

UNIVERSITÉ DU QUÉBEC

MÉMOIRE PRÉSENTÉ À
L'UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

COMME EXIGENCE PARTIELLE
DE LA MAÎTRISE EN BIOLOGIE CELLULAIRE ET MOLÉCULAIRE

PAR
MAXIME VEILLETTE

TRIM5 α : SPÉCIFICITÉ D'ACTION, RÉSISTANCE VIRALE ET
IMPLICATIONS POUR LA THÉRAPIE GÉNIQUE

AOÛT 2013

Université du Québec à Trois-Rivières

Service de la bibliothèque

Avertissement

L'auteur de ce mémoire ou de cette thèse a autorisé l'Université du Québec à Trois-Rivières à diffuser, à des fins non lucratives, une copie de son mémoire ou de sa thèse.

Cette diffusion n'entraîne pas une renonciation de la part de l'auteur à ses droits de propriété intellectuelle, incluant le droit d'auteur, sur ce mémoire ou cette thèse. Notamment, la reproduction ou la publication de la totalité ou d'une partie importante de ce mémoire ou de cette thèse requiert son autorisation.

REMERCIEMENTS

Les travaux réalisés et l'expérience acquise au cours de cette maîtrise forment pour moi les fondations de ma future carrière scientifique. En ce sens, je tiens spécialement à remercier le Dr Lionel Berthoux de m'avoir accueilli dans son équipe de recherche et de m'avoir initié à ce vaste monde qu'est la recherche académique. Son enthousiasme et ses précieux conseils ont fait du temps passé au sein de ce laboratoire un succès qui m'aura permis de me développer grandement.

Je tiens également à remercier Paulina Pawlica et Marie-Édith Nepveu-Traversy, étudiantes au doctorat, pour leur aide et conseils mais, surtout, pour leur amitié. Je garde en mémoire tous ces bons moments passés ensemble au labo et en dehors.

Un grand merci également aux assistantes de recherche du laboratoire, Mélodie B. Plourde et Kathleen Riopel qui ont su m'aider et m'assister dans ces projets tout en mettant de la bonne humeur au sein du laboratoire.

Un merci également aux stagiaires qui m'ont aidé dans la réalisation de ces travaux, Sophie Hamel et Alexandre Paquin.

Bien certainement, je remercie également les Dr Carlos Reyes-Moreno et Céline Van Themsche pour l'assistance technique et le prêt de matériel et d'équipement qui ont été nécessaires dans la réalisation de ces travaux. Un merci va aussi aux organismes m'ayant soutenu, le Fonds de Recherche du Québec – Santé et la fondation C.E.U. de Trois-Rivières.

Et finalement, un grand merci à ma famille et amis pour leur continuel support et encouragements tout au long de ces travaux.

AVANT-PROPOS

Les travaux réalisés au cours de ce mémoire sont présentés sous forme de trois articles scientifiques. Une introduction balisant les notions théoriques pertinentes à la discussion de ces travaux est présentée initialement. Les travaux de recherche sont suivis d'une discussion sur leurs implications et importance dans un cadre théorique plus large que ce qu'il pourrait être présenté indépendamment dans chacun des articles.

Le deuxième chapitre de ce mémoire traite de l'article publié en 2013 dans le journal *Virus Research* dans lequel nous avons identifié un nouveau mutant de la protéine antirétrovirale humaine TRIM5 α . Bien que ma contribution à cette étude ne soit pas la plus importante des trois articles joints à ce mémoire, l'importance des résultats et leur implication dans la suite des travaux justifient son inclusion à ce mémoire. J'ai réalisé environ 15 % des expériences de l'étude en plus d'avoir réalisé le modèle de structure protéique. L'écriture du manuscrit s'est faite en collaboration avec le Dr Lionel Berthoux. Les Drs Alberto Brandariz-Nunez et Felipe Diaz-Griffero ont réalisé l'expérience de liaison aux tubes de capsid virale recombinante dans leur laboratoire au Albert Einstein College of Medicine, É-U. Tous les autres co-auteurs sont responsables de la réalisation des autres expériences dans le laboratoire du Dr Berthoux.

Le troisième chapitre porte sur un article publié en 2013 dans le journal *Retrovirology*. Dans cette étude, nous avons étudié la résistance du VIH-1 à la restriction par des mutants de TRIM5 α humain et le mécanisme qui y est sous-jacent. J'ai contribué d'une manière très importante à cet article en réalisant environ 75 % des expériences et en rédigeant le manuscrit en collaboration avec le Dr Lionel Berthoux. Katsiaryna Bichel, Stefan MV Freund et le Dr Leo C James ont contribué à l'étude en réalisant l'expérience de résonance magnétique nucléaire dans leur laboratoire au Medical Research Council Laboratory of Molecular Biology, Royaume-Uni. Les autres co-auteurs sont responsables de la réalisation ou d'avoir contribué aux expériences dans le laboratoire du Dr Berthoux.

Le quatrième chapitre présente un manuscrit actuellement en préparation pour soumission prochainement au journal Human Gene Therapy. Nous avons étudié la restriction médiée par des mutants de TRIM5 α humain d'un vaste éventail de vecteurs VIH-1 dérivés d'isolats cliniques afin de vérifier l'efficacité de ces transgènes dans un contexte plus clinique. Ma contribution à ce manuscrit consiste en la réalisation de plus de 80 % des expériences et en la rédaction et préparation du manuscrit en collaboration avec le Dr Lionel Berthoux. Sophie Hamel, qui était alors stagiaire sous ma supervision directe, a contribué en réalisant environ 20 % des expériences de ce manuscrit.

RÉSUMÉ

Le VIH/SIDA demeure à ce jour un enjeu médical de taille. Alors que la plupart des approches thérapeutiques actuelles ne parviennent qu'à contrôler le virus et non à l'éradiquer, le développement de stratégies novatrices est essentiel. L'une d'entre elles vise à utiliser la thérapie génique afin d'introduire un transgène efficace contre le VIH chez des patients. Un candidat pour cette thérapie génique est le facteur de restriction TRIM5 α .

TRIM5 α est une protéine de l'immunité innée chez certains mammifères qui empêche l'infection par certains virus. Ce facteur de restriction reconnaît et interagit avec l'assemblage de capsid virale du virus entrant dans la cellule. Cette interaction a plusieurs conséquences néfastes sur le virus, notamment elle provoque une décapsidation accélérée, la dégradation par le protéasome et le blocage du transport de l'ADN viral vers le noyau cellulaire. Des facteurs de restriction de la famille TRIM sont exprimés chez plusieurs espèces différentes, mais tous n'ont pas une efficacité égale contre le VIH. Il faut savoir que chez l'humain, TRIM5 α ne possède pas une activité efficace contre le VIH.

Dans le but de mieux définir des candidats pour une approche par thérapie génique, nous avons premièrement réalisé un crible par mutagenèse aléatoire qui nous a permis d'identifier une nouvelle mutation conférant une activité anti-VIH à la protéine TRIM5 α humaine. À l'instar des mutations identifiées préalablement par d'autres laboratoires et le nôtre, l'ajout d'une charge positive dans le domaine de reconnaissance de la capsid virale de TRIM5 α était essentiel à l'augmentation de l'activité anti-VIH.

La résistance virale aux traitements thérapeutiques est également un enjeu majeur. Afin de mieux cerner les mécanismes moléculaires permettant la résistance aux transgènes de TRIM5 α , nous avons étudié l'effet d'une mutation de résistance préalablement décrite dans la capsid virale (V86M). Nos résultats suggèrent que V86M permet d'échapper à la restriction en modifiant spécifiquement l'interaction de la capsid virale avec un cofacteur de l'hôte, la cyclophiline A, rétablissant ainsi le transport au noyau de l'ADN viral.

Afin de vérifier l'efficacité de nos transgènes contre un vaste éventail de souches virales différentes tel qu'il est nécessaire cliniquement, nous avons analysé la restriction de vecteurs viraux dérivés de plusieurs isolats cliniques. Nous avons ainsi démontré que l'activité de nos transgènes n'était pas limitée aux vecteurs viraux adaptés au laboratoire. Somme toute, les travaux de cet ouvrage permettent de mieux comprendre les transgènes de TRIM5 α et leur potentiel d'être utilisés dans une thérapie génique contre le VIH/SIDA.

Mots-clés : VIH, TRIM5 α , thérapie génique, résistance virale, cyclophiline A, mutagenèse aléatoire.

TABLE DES MATIÈRES

REMERCIEMENTS	ii
AVANT-PROPOS	iii
RÉSUMÉ.....	v
LISTE DES FIGURES ET TABLEAUX.....	x
LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES	xii
CHAPITRE I	
INTRODUCTION.....	1
1.1 Problématique.....	1
1.1.1 L'épidémie du VIH/SIDA	1
1.1.2 Un traitement alternatif au VIH/SIDA : une réelle nécessité	1
1.1.3 Le cas du « patient de Berlin »	3
1.1.4 La thérapie génique.....	3
1.2 Les rétrovirus.....	5
1.2.1 Définition.....	5
1.2.2 Structure.....	5
1.2.3 Génome.....	6
1.2.4 Le cycle viral du VIH-1	7
1.2.5 La pandémie de VIH : pandémie divisée en groupes et sous-groupes ...	9
1.3 Le facteur de restriction TRIM5 α	9
1.3.1 Historique.....	10
1.3.2 Structure.....	10
1.3.3 Spécificité d'action	11
1.3.4 Mécanisme d'action.....	12
1.3.5 Régulation de l'expression de TRIM5 α et autres facteurs de restriction	14
1.3.6 La cyclophiline A.....	15
1.3.7 Des mutants de la protéine TRIM5 α humaine actifs contre le VIH-1	15
1.3.8 TRIM5 α : transgène potentiel pour thérapie génique?	16

CHAPITRE II	
A NOVEL AMINOACID DETERMINANT OF HIV-1 RESTRICTION IN THE TRIM5A VARIABLE 1 REGION ISOLATED IN A RANDOM MUTAGENIC SCREEN	18
Résumé.....	18
Abstract.....	19
Introduction.....	20
Material and Methods	22
Plasmid DNAs	22
Cells and retroviral vectors production	22
Library construction and screening	22
Site-directed mutagenesis	24
Viral challenges	25
HIV-1 CA-NC expression and purification.....	26
<i>In vitro</i> assembly of HIV-1 CA-NC complexes and binding to TRIM5 α variants.....	26
Structural models of the PRYSPRY domain.....	27
Results.....	27
A v1-focused mutagenic screen.....	27
Effect of alternative mutations at Gly330.....	30
Combining G330E with mutations at Arg332 or Arg335	31
<i>In vitro</i> binding analysis	32
Restriction in other viral and cellular environments.....	33
Restriction of a propagating infection	36
Modelling the effect of v1 mutations on the TRIM5 α_{Hu} PRYSPRY domain	37
Discussion	38
Conclusion	39
Acknowledgements.....	40
References.....	41

CHAPITRE III	
THE V86M MUTATION IN HIV-1 CAPSID CONFERS RESISTANCE TO TRIM5A BY ABROGATION OF CYCLOPHILIN A-DEPENDENT RESTRICTION AND ENHANCEMENT OF VIRAL NUCLEAR IMPORT ...	48
Résumé.....	48
Abstract.....	49
Background.....	50
Results.....	52
CA-V86M HIV-1 is partially resistant to restriction by TRIM5 α_{Hu} mutant R332G-R335G.....	52
Restriction of V86M CA HIV-1 by R332G-R335G TRIM5 α_{Hu} is independent of cyclophilin A.....	54
CsA concentration-dependent assays.....	55
The sensitivity of HIV-1 restriction by TRIM5 α_{Hu} mutants to the V86M mutation correlates with its sensitivity to CsA treatment.....	57
Restriction of V86M CA HIV-1 by R332G-R335G TRIM5 α_{Hu} in cat cells is insensitive to CsA.....	58
V86M CA interactions with CypA studied by NMR.....	59
Effects of V86M on early stages of viral replication.....	61
Effect of CypA and V86M on a spreading HIV-1 infection.....	63
Discussion.....	65
Acknowledgements.....	67
Methods.....	68
Plasmid DNAs.....	68
Cells.....	68
Retroviral vectors production.....	69
RNA interference.....	69
Viral challenges.....	70
Quantitative real-time PCR of HIV-1 DNA.....	70
Statistical analyses.....	71
2D 1H - ^{15}N Heteronuclear (ZZ) Exchange Spectroscopy.....	71
References.....	73
Supplementary data.....	80

CHAPITRE IV	
RESTRICTION OF A DIVERSE PANEL OF HIV-1 CLINICAL ISOLATES BY A MUTATED HUMAN TRIM5α	83
Résumé	83
Abstract	84
Introduction	84
Results	86
Discussion	91
Materials and Methods	93
Plasmids DNAs	93
Cell lines	93
Retroviral vectors production	94
Viral challenges and measurement of sensitivity to TRIM5 α	94
Phylogenetic tree analysis	94
Protein sequence alignment	95
Conclusions	95
Acknowledgements	95
References	96
CHAPITRE V	
DISCUSSION ET PERSPECTIVES	103
5.1 L'identification de transgènes aux propriétés antivirales	104
5.1.1 Transgènes de TRIM5 α	104
5.1.2 Transgènes d'autres facteurs de restriction	106
5.1.3 D'autres candidats se cachent dans le génome	106
5.2 La résistance virale et le cas de CA-V86M	107
5.2.1 Transduction, virus répliatif et TRIM5 α : ce que nous apprend V86M	107
5.2.2 La résistance virale dans un contexte clinique	108
5.3 Thérapie génique : implications des résultats obtenus et perspectives	109
5.3.1 Transgènes de TRIM5 α : leur potentiel clinique	109
5.4 Perspectives générales	110
CONCLUSION	112
RÉFÉRENCES BIBLIOGRAPHIQUES	113

LISTE DES FIGURES ET TABLEAUX

Figure		Page
1.1	Tendance de l'incidence mondiale du VIH-1	1
1.2	Nombre de décès associés au VIH/SIDA selon les régions du monde.....	2
1.3	Schéma de la structure du VIH-1	6
1.4	Schéma du génome commun des rétrovirus	6
1.5	Cycle de réplication du VIH-1	8
1.6	Les différents niveaux de classification du VIH.....	9
1.7	Schéma général de la protéine TRIM5 α	11
1.8	Structure du domaine PRYSPRY de la protéine TRIM5 α_{Rh} indiquant la position des quatre régions variables (v1-v4)	12
1.9	Schéma de l'assemblage hexamérique de TRIM5 α sur un cœur viral de CA	13
1.10	Schéma présentant les différents effets de TRIM5 α sur le virus	14
1.11	Schéma général de TRIM5 α_{Hu} identifiant les mutations conférant une activité contre le VIH-1	16
1.12	Position de l'acide aminé V86 dans la CA virale	17
2.1	Isolation of amino acid 330 of TRIM5 α_{Hu} as a determinant of HIV-1 restriction	29
2.2	Restriction of HIV-1 by TRIM5 α_{Hu} mutants of Gly330.....	31
2.3	Antiretroviral effect conferred by G330E when combined with R332G or R335G.....	32
2.4	In vitro TRIM5 α binding to HIV-1 CA	33
2.5	Restriction of HIV-1 replication in human lymphocytic cells and in nonhuman cells	35
2.6	HIV-1 propagation in human lymphocytic cells expressing G330E TRIM5 α_{Hu}	37

2.7	Model representation of the TRIM5 α_{Hu} PRYSPRY domain	38
3.1	V86M mutation in HIV-1 capsid confers partial resistance against R332G-R335G TRIM5 α_{Hu}	53
3.2	Restriction of CA-V86M HIV-1 by R332G-R335G TRIM5 α_{Hu} is independent of cyclophilin A.....	55
3.3	CsA dose-dependent analysis	56
3.4	Direct correlation between the effect of mutation V86M and the effect of CsA treatment	58
3.5	V86M HIV-1 restriction by R332G-R335G TRIM5 α_{Hu} in cat cells is not counteracted by CsA treatment.....	59
3.6	V86M CA interactions with CypA studied by NMR.....	60
3.7	V86M enhances nuclear transport of HIV-1 DNA in restrictive conditions .	62
3.8	Restriction of a spreading CA-V86M HIV-1 infection	65
3.9S	CA-V86M HIV-1 is inhibited by CsA in human lymphocytes and macrophages	80
3.10S	Western blotting analysis of CypA knockdown	81
3.11S	Restriction of WT and CA-V86M HIV-1 by a panel of TRIM5 α_{Hu} mutants.....	82
4.1	Phylogenetic relationships and sequence alignment of the virus strains used in this study.....	88
4.2	Infectivity of different HIV-1 strains in cells expressing a TRIM5 α_{Hu} mutant	90
4.3	Sensitivity of CTL escape mutants to mutated TRIM5 α_{Hu}	91

Tableau**Page**

1.1	Résumé des virus restreints par divers orthologues de TRIM5 α	12
4.1	Summary information on viral strains used.....	87

LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

AML	Leucémie aiguë myéloblastique (<i>Acute Myeloid Leukemia</i>)
CA	Capside virale
CA-NTD	Domaine N-terminal de la capside virale
CCR5-Δ32	Délétion de 32pb dans le gène CCR5
CRF	Formes recombinantes circulante du VIH (<i>Circulating Recombinant Forms</i>)
CsA	Cyclosporine A
CTL	Lymphocytes T cytotoxiques (<i>Cytotoxic T Lymphocytes</i>)
CypA	Cyclophiline A
EIAV	Virus de l'anémie infectieuse équine (<i>Equine Infectious Anemia Virus</i>)
FACS	<i>Fluorescence-activated cell sorting</i>
FIV	Virus de l'immunodéficience féline (<i>Feline Immunodeficiency Virus</i>)
Fv1	<i>Friend Virus Susceptibility 1</i> , facteur de restriction
gp120	glycoprotéine 120 du VIH
gp41	glycoprotéine 41 du VIH
HF	<i>High-Fidelity</i> , se dit des tampons et polymérase à haute fidélité
HIV	<i>Human Immunodeficiency Virus</i>
HSC	Cellules souches hématopoïétiques (<i>Hematopoietic stem cells</i>)
IFN-I	Interférons de type 1
IL2-RG	Récepteur gamma de l'interleukine 2
IN	Intégrase virale
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LTR	<i>Long Terminal Repeat</i>

MA	Matrice virale
MLV	Virus de la leucémie murine (<i>Murine Leukemia Virus</i>)
NC	Nucléocapside virale
NCBI	National Center for Biotechnology Information
NF- κ B	<i>Nuclear factor kappa-light-chain-enhancer of activated B cells</i>
NMR	Résonance magnétique nucléaire (<i>Nuclear Magnetic Resonance</i>)
OMS	Organisation mondiale de la santé
PIC	Complexe viral de pré-intégration (<i>Pre-Integration Complex</i>)
PR	Protéase virale
qPCR	PCR quantitative
RT	Transcriptase inverse virale (<i>Reverse Transcriptase</i>)
SCID-X	<i>Severe Combined Immunodeficiency</i> liée au chromosome X
SIDA	Syndrome d'immunodéficience acquise
SIV	Virus de l'immunodéficience simienne (<i>Simian Immunodeficiency Virus</i>)
TRIM5 α_{Hu}	Orthologue humain de TRIM5 α
TRIM5 α_{Rh}	Orthologue du singe rhésus de TRIM5 α
vDNA	ADN virale
VIH	Virus de l'immunodéficience humaine
VSV	Virus de la stomatite vésiculaire (<i>Vesicular Stomatitis Virus</i>)
WT	Type sauvage (<i>wild type</i>)
ZFN	Nucléases à doigt de zinc (<i>Zinc Finger Nucleases</i>)

CHAPITRE I

INTRODUCTION

1.1 Problématique

1.1.1 L'épidémie du VIH/SIDA

Voilà déjà plus de 25 ans, l'Agence de la santé publique du Canada rapportait les premiers cas d'infection au virus de l'immunodéficience humaine (VIH) au Canada. Malgré tous les efforts déployés par les diverses agences gouvernementales, des milliers de Canadiens sont nouvellement infectés à chaque année. À l'échelle mondiale, les données de l'Organisation mondiale de la santé (OMS) montrent que malgré une tendance à la baisse au cours de la dernière décennie, il y avait en 2009 plus de 2,5 millions de personnes nouvellement infectées par le VIH/SIDA (Figure 1.1).

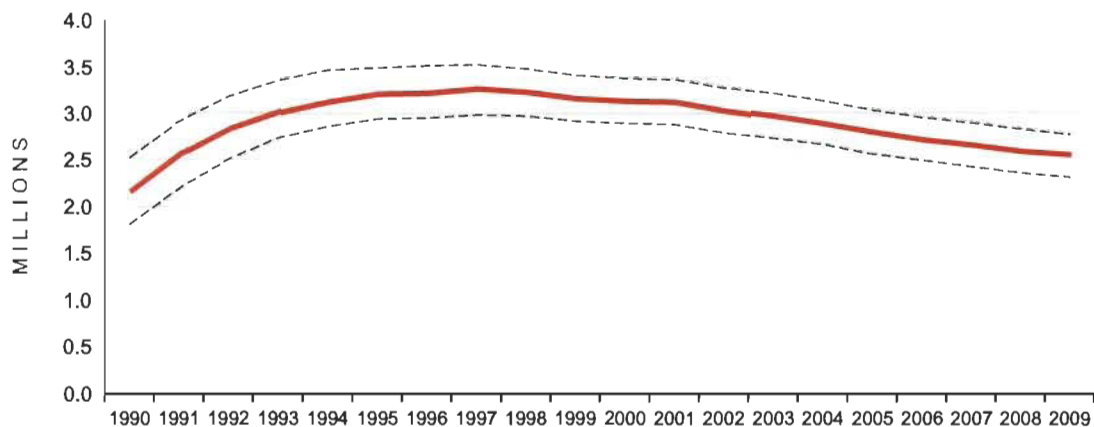


Figure 1.1 Tendence de l'incidence mondiale du VIH-1 [1].

1.1.2 Un traitement alternatif au VIH/SIDA : une réelle nécessité

Les données récentes sur l'incidence du VIH/SIDA démontrent bien que malgré une efficacité grandissante des agents antirétroviraux et de leurs combinaisons, un

nombre important de nouvelles infections est toujours présent au niveau mondial. De plus, bien que le nombre de décès liés au VIH/SIDA présente une tendance à la baisse dans certaines régions du monde (en Amérique du Nord, entre autres), une importante hausse de la mortalité est observée dans d'autres régions, incluant l'Europe de l'Est et l'Asie (Figure 1.2).

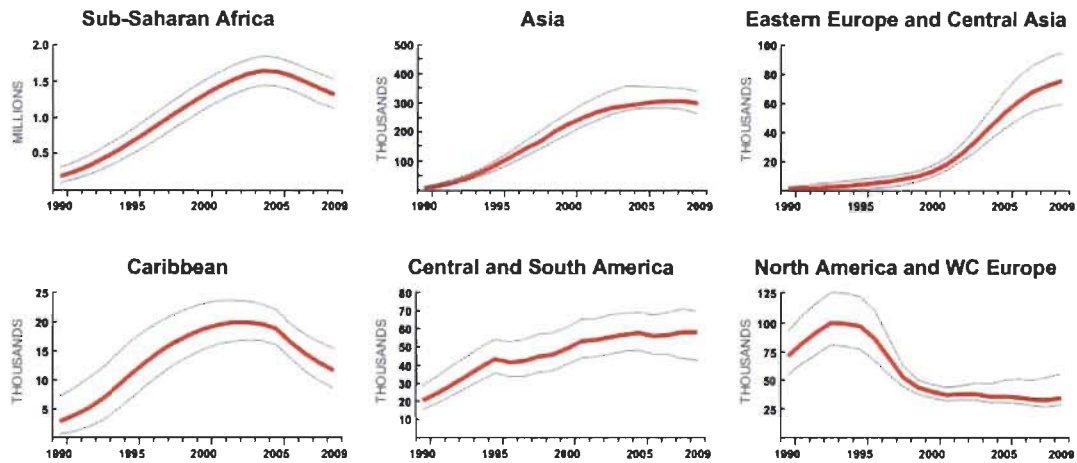


Figure 1.2 Nombre de décès associés au VIH/SIDA selon les régions du monde [1].

De surcroît, aucun traitement actuellement répandu contre le VIH/SIDA ne permet son éradication chez les personnes atteintes. Elles doivent donc continuellement adhérer au traitement, ce qui entraîne une lourde charge économique et augmente la fréquence des interruptions de traitement, ce qui favorise l'émergence de virus résistants aux traitements, en plus de faciliter une éventuelle transmission du virus par l'augmentation de la charge virale [2-4]. La fréquence d'émergence de tels virus est difficile à estimer, compte tenu de la grande quantité de facteurs l'influençant (géographie, contexte socioéconomique, génétiques de l'individu et des différentes souches virales). Néanmoins, des études ont signalé la présence de mutations conférant une résistance à un agent antiviral chez 20 à 50 % des participants à leurs cohortes [5-7]. De plus, la prévalence de la transmission de souches virales portant une mutation de résistance à un agent antiviral augmente au fil du temps, et a récemment été évaluée entre 4 et 12 % selon la localisation géographique [8]. L'élaboration et la mise en place de nouvelles stratégies thérapeutiques sont donc d'une importance capitale dans la lutte contre le VIH/SIDA.

1.1.3 Le cas du « patient de Berlin »

Le cas du « patient de Berlin » était le seul cas jusqu'à plus tôt cette année où un patient a officiellement été guéri du VIH/SIDA. Il s'agit en fait d'un citoyen américain vivant en Allemagne et qui était infecté depuis plus de 10 ans par le VIH-1. Or, ce même patient était atteint d'une leucémie aigüe myéloblastique (AML). L'un des traitements utilisés pour traiter une AML consiste en l'éradication des cellules lymphatiques cancéreuses par radiothérapie suivi d'une repopulation des cellules lymphatiques par greffe de moelle osseuse d'un donneur sain. Or, le docteur traitant ce patient a décidé de trouver un donneur présentant une certaine particularité génétique : le donneur était homozygote pour une délétion de 32 pb dans le gène CCR5 (CCR5- Δ 32). CCR5 est un corécepteur essentiel à l'infection de la plupart des souches transmises du VIH-1, sa délétion rendant ainsi résistant à l'infection par le VIH-1 [9-11]. La repopulation des cellules lymphatiques n'exprimant pas CCR5 chez le patient infecté par le VIH-1 a entraîné sa rémission complète et jusqu'à ce jour aucune trace du virus n'a été détectée dans les divers tissus analysés [12]. Suite à cette réussite médicale, plusieurs équipes de recherche consacrent maintenant beaucoup de temps et d'énergie à développer des approches thérapeutiques qui visent à réintroduire des cellules résistantes au VIH-1 chez les patients infectés.

1.1.4 La thérapie génique

En général, la thérapie génique est une stratégie visant à introduire des modifications ou voire même de nouveaux gènes dans des cellules humaines qui demeureront chez le patient et qui pourront apporter un bénéfice thérapeutique. Cette approche se veut complexe alors que les chercheurs doivent composer avec les risques inhérents et l'efficacité parfois faible des stratégies [13].

Le premier cas de thérapie génique « réussie » est sans aucun doute le traitement des « enfants bulles », jeunes enfants atteints du SCID-X (*Severe Combined Immunodeficiency* liée au chromosome X). Une mutation dans le gène codant pour le récepteur gamma de l'interleukine 2 (IL2-RG) induit une perte de fonction de cette

protéine, rendant ainsi impossible la maturation correcte des lymphocytes T [14]. Sans ces lymphocytes T, les autres cellules du système immunitaire ne peuvent jouer leur rôle, induisant ainsi un état de susceptibilité aux infections opportunistes : l'immunodéficience sévère. Vers la fin des années 1990, le Dr Alain Fischer utilisa un vecteur rétroviral basé sur le virus de la leucémie murine (MLV) qu'il modifia pour y insérer une version fonctionnelle du gène IL2-RG. L'introduction de ce vecteur dans les cellules souches hématopoïétiques des patients a permis de rétablir la maturation et la fonction normale des lymphocytes T chez huit des neuf jeunes enfants traités [15]. Or, les vecteurs rétroviraux s'insèrent d'une manière (quasi) aléatoire dans le génome de cellules infectées, laissant ainsi un risque de dérégler l'équilibre pro-oncogène/suppresseur de tumeurs qui différencie les cellules saines de cellules cancéreuses. C'est ce qui se produisit chez quatre des enfants traités, ces derniers ont développé une leucémie des suites de leur traitement [16]. Malheureusement, l'un d'entre eux en est décédé. Suite à des traitements de chimiothérapie, les trois autres se sont rétablis. Des huit enfants ayant survécu au traitement, sept présentent maintenant un système immunitaire quasi-normal, leur permettant ainsi de se développer normalement dans le monde extérieur. Malgré les graves effets secondaires observés, cette thérapie est néanmoins vue comme un succès pour la thérapie génique.

Plusieurs années se sont écoulées depuis ce premier succès et de nouveaux vecteurs viraux plus sécuritaires sont maintenant disponibles [17, 18]. De plus, de récentes avancées dans le domaine de la modification du génome permettent aujourd'hui de modifier les gènes d'une cellule sans avoir à y introduire de manière permanente des vecteurs viraux exogènes. Ces nouveaux outils, jumelés avec le besoin criant de développer de nouvelles approches thérapeutiques contre le VIH/SIDA, réaffirment la nécessité d'identifier des transgènes antiviraux qui pourront être utilisés dans ce type d'application.

1.2 Les rétrovirus

1.2.1 Définition

Les rétrovirus sont une famille de virus infectant les vertébrés. Ils se distinguent des autres virus entre autres par leur mode de réplication particulier. Ce sont des virus à ARN qui possèdent deux copies de leur génome. Ils codent pour une ADN polymérase ARN dépendante (la transcriptase inverse, RT) qui rétrotranscrit les brins d'ARN en ADN viral qui pourra ainsi être intégré de façon définitive dans le génome de la cellule cible [19]. La famille des rétrovirus se divise en deux sous-familles, soit les orthoretrovirus et les spumaretrovirus. Le virus de l'immunodéficience humaine appartient au genre des *lentivirus* de la sous-famille des orthoretrovirus. Il existe deux types de VIH, soit le VIH-1 et le VIH-2. Le VIH-1 est responsable de la pandémie mondiale alors que le VIH-2, qui est moins facilement transmissible, se limite plutôt à l'Afrique de l'Ouest.

1.2.2 Structure

Ce sont des virus enveloppés d'une bicouche phospholipidique recouverte de protéines de surface (la glycoprotéine 120, gp120) et transmembranaire (glycoprotéine 41, gp41). Cette enveloppe est acquise de la cellule productrice lors du bourgeonnement du virus et sa surface interne est recouverte d'une protéine structurale : la matrice (MA). Au sein de cette enveloppe se trouve le cœur viral, formé de la multimérisation de la protéine de la capsid (CA) virale. Cet assemblage hexamérique de la CA en cœurs viraux contient diverses enzymes associées : la RT, l'intégrase (IN) et la protéase (PR), ainsi que le génome viral entouré de protéines de nucléocapside (NC, Figure 1.3).

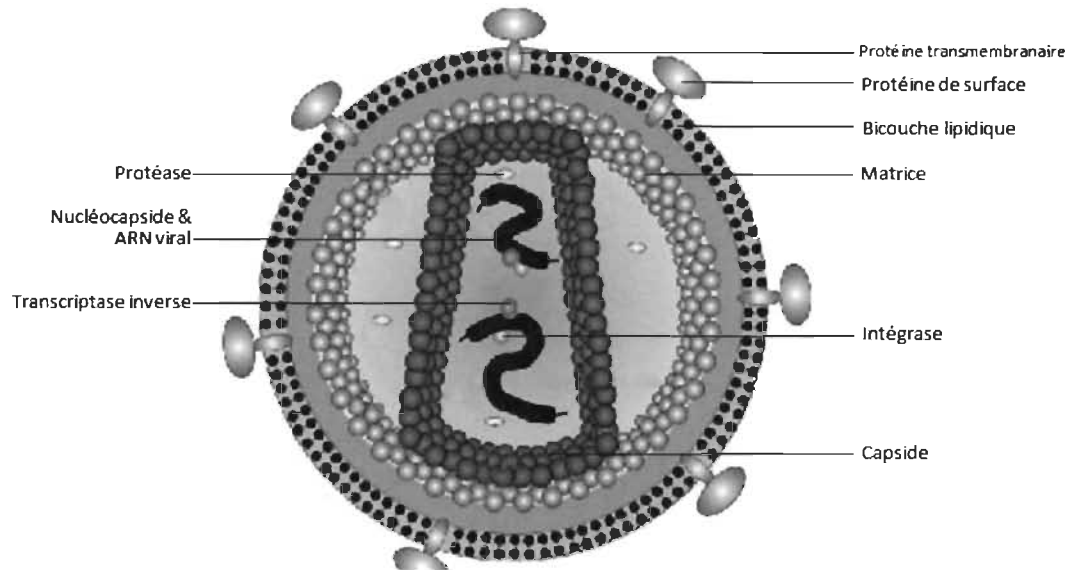


Figure 1.3 Schéma de la structure du VIH-1 [20].

1.2.3 Génome

Le génome des rétrovirus est encodé en deux copies identiques d'ARN simple brin de polarité positive. On y distingue trois gènes principaux qui codent pour des polyprotéines essentielles à la réplication des rétrovirus (Figure 1.4). Ce sont les gènes *gag* (*group-specific antigen*), *pro-pol* (polymérase) et *env* (glycoprotéines d'enveloppe). Ces trois gènes codent pour des précurseurs dont le clivage enzymatique permettra d'obtenir les protéines virales actives (voir Figure 1.4). Le génome complet est encadré entre deux régions nommées LTR (*Long Terminal Repeat*) qui renferment le promoteur (en 5') et le site de polyadénylation (en 3'). Le génome des lentivirus code pour des protéines supplémentaires dites accessoires (pour le VIH-1 : Vif, Vpr, Vpu, Tet, Rev, Tat et Nef) puisqu'elles permettent la régulation de diverses étapes de la réplication virale et de l'interaction avec les protéines de l'hôte.



Figure 1.4 Schéma du génome commun des rétrovirus [20].

1.2.4 Le cycle viral du VIH-1

Bien que les rétrovirus se répliquent selon le même cycle viral, certaines différences existent entre eux. Cette section est donc écrite en fonction du VIH-1 afin d'explorer les particularités de ce virus, facilitant ainsi la compréhension du reste de l'ouvrage. On distingue habituellement deux phases dans le cycle de réplication viral :

- La phase *précoce* allant de la fusion de la particule virale avec la membrane cellulaire jusqu'à l'intégration de l'ADN viral dans le génome cellulaire;
- La phase *tardive* allant de l'expression des protéines du provirus jusqu'au bourgeonnement des nouveaux virions.

Au début de la phase précoce, l'interaction entre la protéine virale de surface gp120 et le récepteur cellulaire CD4 induit un changement de conformation de gp120, permettant ainsi le recrutement du corécepteur cellulaire CCR5 (ou CXCR4 pour certaines souches virales). Le recrutement du corécepteur cellulaire entraîne d'autres changements conformationnels importants qui mènent à l'attachement de la glycoprotéine transmembranaire de l'enveloppe, gp41, à la membrane cellulaire. Grâce à ses deux régions homologues, la gp41 agit comme un ressort et son repliement sur elle-même mène à la fusion des deux membranes lipidiques, permettant ainsi le relargage du cœur viral dans le cytoplasme.

S'en suivent les étapes de la décapsidation du cœur viral et de la transcription inverse de l'ARN viral. Bien que relativement peu connue, la régulation précise de ces étapes est essentielle à l'infectivité maximale du virus [21]. La transcription inverse mène à la formation d'un double-brin d'ADN viral (vDNA) qui fera partie du complexe de pré-intégration (PIC). Les protéines MA et IN ainsi que la protéine accessoire Vpr sont présentes dans le PIC. Plusieurs composantes de ce complexe présentent un signal de localisation nucléaire, lui permettant ainsi de migrer vers le noyau. Une fois arrivé à destination, ce complexe permet l'intégration du vDNA à un site quasi-aléatoire du génome cellulaire.

L'ADN viral ainsi intégré tire profit de la machinerie de transcription et de synthèse protéique de la cellule infectée. Grâce au LTR 5' jouant le rôle de promoteur et du signal de polyadénylation provenant du LTR 3', l'ADN viral est transcrit en ARN. Les transcrits sont alors épissés ou non, puis exportés vers le cytoplasme où ils seront traduits en protéines structurales ou enzymatiques. Ces nouvelles protéines virales seront assemblées à deux brins d'ARN viral près de la membrane plasmique pour ainsi former de nouveaux virions et procéder au bourgeonnement de ces particules jusque-là non-infectieuses. Après le bourgeonnement hors de la cellule, la PR clive les précurseurs protéiques (Gag et Gag-Pol) : le virus est maintenant mature et infectieux. La Figure 1.5 présente une schématisation du cycle de réplication viral.

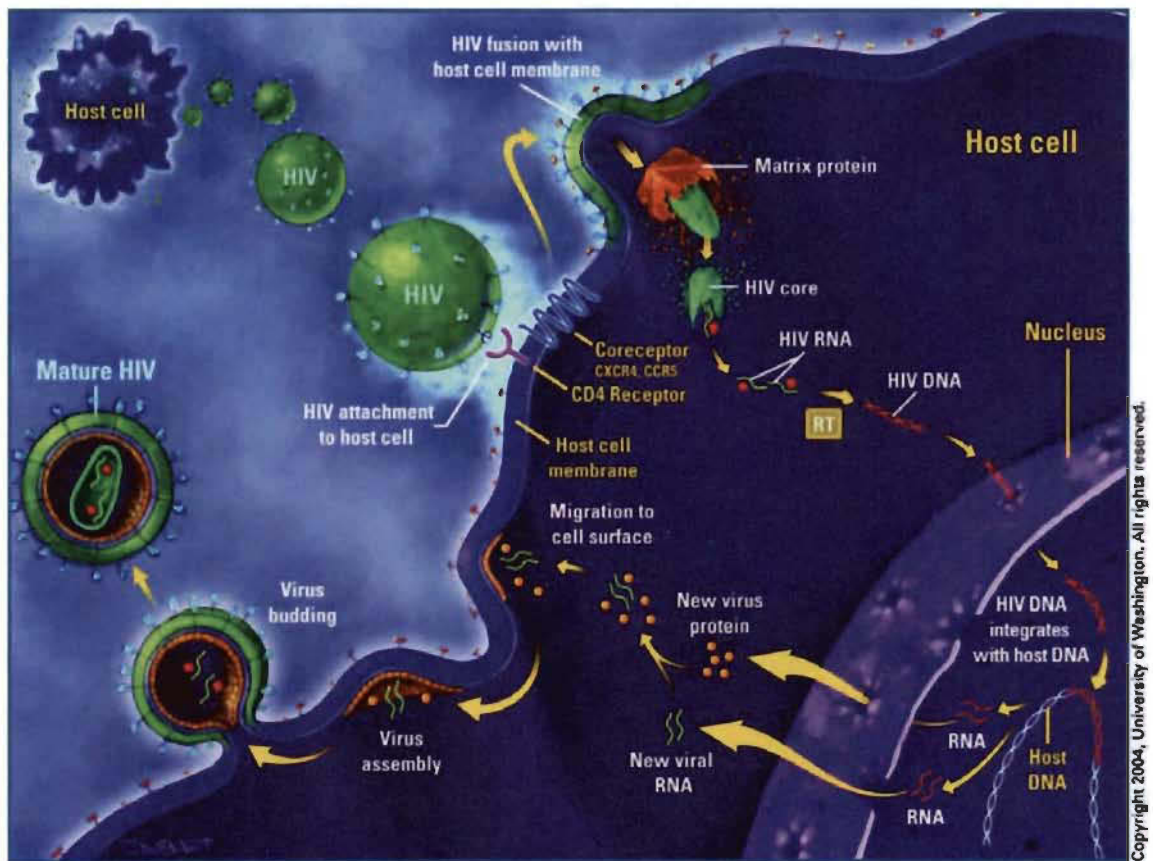


Figure 1.5 Cycle de réplication du VIH-1 [20].

1.2.5 La pandémie de VIH : pandémie divisée en groupes et sous-groupes

Le haut taux de mutations qu'apporte la rétrotranscription de l'ARN en ADN résulte en un très grand nombre de souches virales différentes. Celles-ci sont regroupées en groupes qui partagent des caractéristiques communes de séquences et propriétés phénotypiques. Le groupe M est responsable de la majorité de la pandémie mondiale et on peut y discerner 9 sous-groupes en plus des formes recombinantes circulantes (CRF), qui sont issues de la recombinaison de deux souches de différents sous-groupes au sein d'un même individu. La Figure 1.6 présente un aperçu des différents niveaux de classification utilisés.

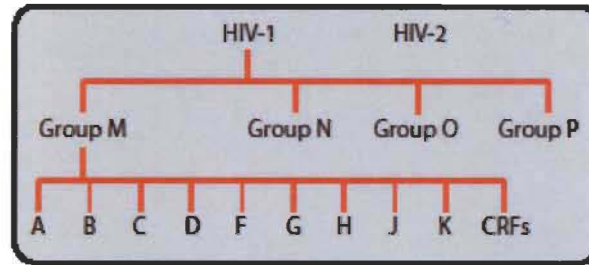


Figure 1.6 Les différents niveaux de classification du VIH.

1.3 Le facteur de restriction TRIM5 α

Des millions d'années d'infection par les rétrovirus ont permis d'exercer une sélection positive sur les mammifères afin qu'ils développent des mécanismes de résistance à ces infections. Ces stratégies sont divisées en deux grandes catégories : l'immunité innée et acquise. Importants acteurs de cette première catégorie, les facteurs de restriction permettent d'empêcher l'infection ou du moins réduire le potentiel infectieux de certains virus. L'un de ces facteurs est un membre de la famille des protéines à motif tripartite, TRIM5 α .

1.3.1 Historique

Vers la fin des années 1960, le croisement de souris Balb/c à des souris NIH-3T3 a permis l'observation d'une activité de restriction présentant un caractère héréditaire. En effet, alors que les souris NIH-3T3 présentaient une résistance face au virus MLV dit B-tropique (B-MLV) et que les Balb/c étaient plutôt résistantes au virus N-tropique (N-MLV), la première génération fille de ce croisement était complètement résistante aux deux souches de virus. Des expériences supplémentaires ont permis de mettre en évidence que le déterminant viral pour cette restriction était la protéine de capsid, plus précisément deux de ses acides aminés (positions 109 et 110) [22]. Toutefois, le gène responsable pour cette restriction n'était toujours pas identifié.

C'est en 1996 qu'il a finalement été cloné; il a été nommé Fv1 pour *Friend Virus Susceptibility*. Les similitudes des caractéristiques de la restriction par Fv1 avec ce qui était observé chez les cellules de primates ont amené les chercheurs à introduire deux autres termes désignant un phénotype de restriction semblable à Fv1 chez l'humain (Ref1) et d'autres primates (Lv1) [23, 24]. Ce n'est cependant pas avant 2004 que TRIM5 isoforme α a été identifié dans des publications démontrant sa capacité de bloquer la réplication du VIH-1 aux étapes de pré-intégration du virus dans les cellules du singe rhésus, identifiant ainsi les phénotypes de Lv1 et Ref1 comme étant bel et bien causés par TRIM5 α [25, 26].

1.3.2 Structure

La famille des protéines à motifs tripartite (*tripartite motif*, TRIM) est caractérisée par la présence des domaines RING-finger, B-Box et Coiled-Coil [27, 28] (voir Figure 1.7). L'épissage du gène TRIM5 donne lieu à l'expression de plusieurs isoformes, dont seul TRIM5 α possède un domaine supplémentaire en C-terminal, nommé PRYSPRY. Cet isoforme est également le seul à avoir une activité de restriction contre le VIH-1 [29].



Figure 1.7 Schéma général de la protéine TRIM5 α .

Le domaine RING confère une activité E3-ubiquitine ligase à la protéine, activité qui est requise pour la restriction des virus [30-32]. Pour sa part, le domaine B-Box peut s'auto-assembler en dimères qui sont essentiels dans l'assemblage des complexes multimériques de TRIM5 α sans quoi la protéine ne peut exercer une restriction maximale sur le virus [33-35].

Le domaine PRYSPRY (aussi appelé B30.2) est responsable de la reconnaissance de la capsid virale [36, 37]. Il est donc le seul élément duquel découle la spécificité d'action de TRIM5 α (voir 1.3.3 ci-dessous) [38]. Le domaine Coiled-Coil permet quant à lui la dimérisation de la protéine, ce qui est essentiel pour que le PRYSPRY puisse convenablement reconnaître la capsid virale [39-41].

1.3.3 Spécificité d'action

Au cours de l'histoire et alors que les différents mammifères étaient infectés par des rétrovirus différents d'une espèce à l'autre, la pression sélective exercée sur les mécanismes de défense a entre autres engendré la divergence de la spécificité d'action des orthologues de TRIM5 α [42]. Bien souvent, les virus restreints ne sont pas des virus qui infectent normalement l'hôte ce qui suggère que TRIM5 α puisse jouer un rôle dans la prévention de la transmission inter-espèces des rétrovirus [43]. Alors que leur structure et leur mécanisme d'action semblent identiques, l'alignement de la séquence des domaines PRYSPRY révèle l'existence de régions variables (Figure 1.8) [42, 44, 45]. C'est au sein de ces régions que l'on retrouve les acides aminés responsables de la spécificité d'action de TRIM5 α , la pression sélective exercée sur ces gènes ayant ainsi favorisé la formation de ces régions variables [46].

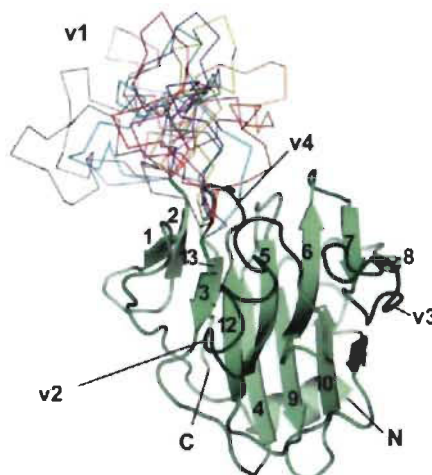


Figure 1.8 Structure du domaine PRYSPRY de la protéine TRIM5 α_{Rh} indiquant la position des quatre régions variables (v1-v4). Les dix conformations de la boucle v1 avec la plus faible énergie sont présentées en différentes couleurs [36].

Tel que l'illustre le Tableau 1.1, le VIH-1 est fortement inhibé par TRIM5 α du singe rhésus (TRIM5 α_{Rh}) alors que la version humaine (TRIM5 α_{Hu}) ne parvient pas à exercer une activité antivirale efficace contre ce virus. Les efforts visant à identifier des mutations de TRIM5 α_{Hu} lui permettant de bloquer efficacement le VIH-1 font l'objet de la section 1.3.7.

Tableau 1.1
Résumé des virus restreints par divers orthologues de TRIM5 α

Orthologue	Espèce	Virus restreints
TRIM5 α_{Hu}	Humain	N-MLV, EIAV, FIV
TRIM5 α_{Rh}	Singe rhésus	VIH-1, FIV
TRIM5 α_{AGM}	Singe vert africain	N-MLV, HIV-1, HIV-2, SIV, EIAV

1.3.4 Mécanisme d'action

La restriction d'un rétrovirus par TRIM5 α s'initie après reconnaissance de la capsid virale par le domaine PRYSPRY. Il a été démontré que la protéine TRIM5 α n'interagit que faiblement avec les monomères de CA [29]. Au contraire, l'assemblage

in vitro de la CA en structures multimériques (tubes ou sphères) qui rappellent la structure des cœurs viraux permet une très forte interaction TRIM5 α -CA [40, 47, 48]. Cette observation suggère que TRIM5 α reconnaît un épitope formé lors de l'assemblage multimérique de CA, ou alors l'avidité de la reconnaissance nécessite plusieurs épitopes. TRIM5 α serait donc un récepteur de reconnaissance de motifs moléculaires de pathogènes. Le fait que la multimérisation de TRIM5 α soit essentielle pour la restriction vient plutôt confirmer ce deuxième modèle. De plus, des études biochimiques et de microscopie électronique ont permis de mettre en évidence que les TRIM5 α restrictifs s'assemblent en structures hexagonales qui sont complémentaires en symétrie et en dimensions avec la CA multimérique (voir Figure 1.9) [35].



Figure 1.9 Schéma de l'assemblage hexamérique de TRIM5 α sur un cœur viral de CA [35].

L'interaction TRIM5 α -CA entraîne un désassemblage accéléré de la particule virale à ses interfaces inter-hexamériques [40, 48-50], ce qui a été démontré comme empêchant l'accumulation des transcrits viraux et donc nuit à l'infectivité [21, 51, 52]. L'interaction du facteur de restriction avec le PIC induit l'activité d'auto-ubiquitination de TRIM5 α , dirigeant ainsi le PIC vers la dégradation par le protéasome [53, 54]. De plus, cette interaction nuit au transport du PIC vers le noyau cellulaire, empêchant ainsi

l'intégration de l'ADN viral dans le génome cellulaire [55] (voir Figure 1.10). Une facette indirecte de l'activité de TRIM5 α a également été récemment identifiée. Jouant le rôle de système de reconnaissance de motifs étrangers (*pattern-recognition*), il activerait la signalisation de l'immunité innée amenant via la protéine NF- κ B, facteur de régulation de la réponse immunitaire [56].

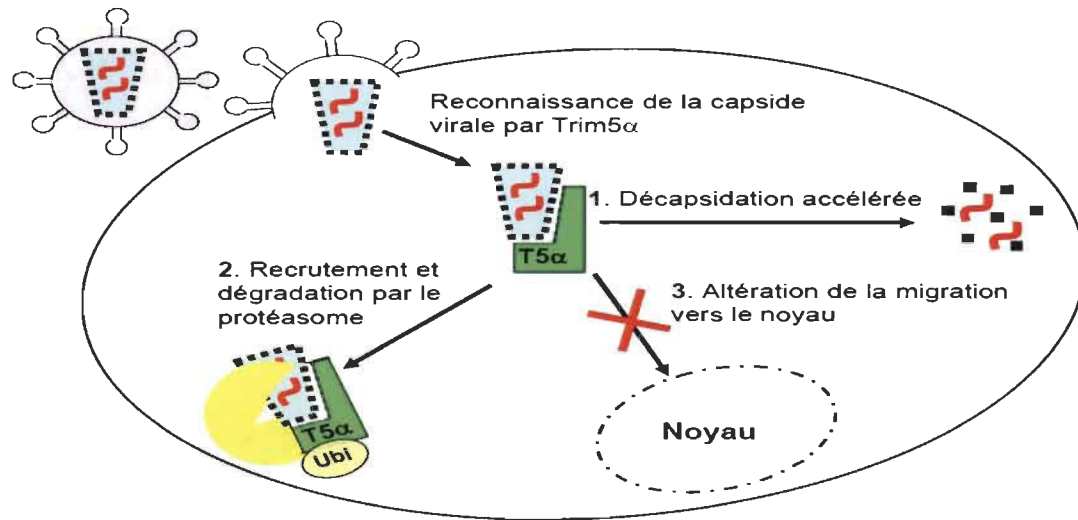


Figure 1.10 Schéma présentant les différents effets de TRIM5 α sur le virus [20].

1.3.5 Régulation de l'expression de TRIM5 α et autres facteurs de restriction

Les interférons de type 1 (IFN-I) comprennent une vaste famille de protéines impliquées dans la signalisation de l'immunité innée. Ils jouent divers rôles dans la réponse antivirale, incluant l'activation des cellules NK et des macrophages [57]. Une autre facette de leur implication dans l'effort antiviral est la régulation qu'ils exercent sur l'expression de différents facteurs de restriction. En effet, le niveau d'expression de TRIM5 α est augmenté en réponse aux IFN-I, ce qui se traduit chez l'humain par une augmentation de la restriction des virus sensibles, notamment N-MLV et quelques isolats du VIH-1 [58-60]. Plusieurs autres facteurs de restriction voient également leur niveau d'expression augmenté en réponse aux IFN-I, notamment APOBEC3G, lequel sera le sujet de la section 5.1.2 [61].

1.3.6 La cyclophiline A

La cyclophiline A (CypA) est une isomérase cis/trans peptidyl-propyl de l'hôte qui est exprimée d'une manière ubiquitaire dans les tissus. Elle peut lier une boucle riche en prolines sur la CA et catalyser l'isomérisation cis/trans du lien peptidique G89-P90 [62, 63]. Le traitement avec l'inhibiteur pharmacologique de CypA, la cyclosporine A (CsA), diminue la restriction par TRIM5 α_{Rh} alors que le même traitement diminue l'infectivité du VIH-1 dans les cellules humaines. CypA est donc, dans un contexte cellulaire-dépendant, nécessaire à la restriction par TRIM5 α ou essentiel à l'infectivité maximale du virus [64-66]. En ce sens, de récentes études suggèrent que CypA pourrait jouer un rôle dans la régulation de la décapsidation du cœur viral [67, 68].

1.3.7 Des mutants de la protéine TRIM5 α humaine actifs contre le VIH-1

Considérant l'ampleur de la problématique qu'implique le VIH/SIDA, il n'est pas étonnant que des chercheurs se soient penchés sur la question d'identifier des déterminants de la restriction du VIH-1 par TRIM5 α . Une équipe a d'abord réalisé des chimères TRIM5 α_{Hu} – TRIM5 α_{Rh} afin de cartographier les différences et déterminants. Cette approche a permis l'identification d'une mutation en position 332 (R332G) de la protéine humaine qui lui confère une activité contre le VIH-1 d'environ 5 fois supérieure à celle de la protéine sauvage [69].

La position 332 fait partie de la région variable 1 (v1) du domaine PRYSPRY. Ceci implique que d'autres positions au sein de v1 ou autres régions variables pourraient jouer un rôle dans la détermination du potentiel restrictif de TRIM5 α_{Hu} sur le VIH-1, bien qu'elles puissent ne pas avoir été identifiées dans l'approche comparative avec TRIM5 α_{Rh} . Notre laboratoire a donc réalisé un crible par gain de fonction basé sur une mutagenèse aléatoire du domaine PRYSPRY de TRIM5 α_{Hu} . Une nouvelle mutation a été identifiée, R335G [70]. Lorsque combinée avec la mutation en position 332 (mutant R332G-R335G, voir Figure 1.11), son efficacité s'est trouvée augmentée (20 fois comparativement à la protéine sauvage). Fait notable, dans les deux cas le changement d'acide aminé venant supprimer une charge positive dans la région de v1 était important

pour l'augmentation de l'activité de restriction. Le chapitre de cet ouvrage présente une deuxième stratégie de notre laboratoire qui a mené à l'identification d'une troisième mutation dans la région v1.

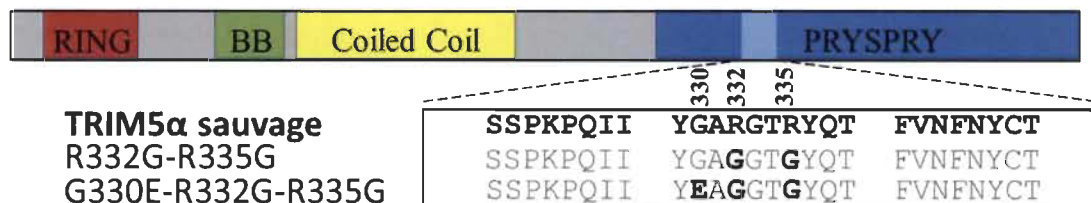


Figure 1.11 Schéma général de TRIM5 α_{Hu} identifiant les mutations conférant une activité contre le VIH-1.

1.3.8 TRIM5 α : transgène potentiel pour thérapie génique?

Alors que les technologies de thérapie génique s'améliorent et que l'identification de nouveaux transgènes antiviraux continue, il est important de comprendre et d'étudier les prérequis pour un traitement efficace et sécuritaire.

Du point de vue sécurité et considérant que la stratégie d'administration du transgène serait idéalement intrinsèquement sécuritaire, le transgène en lui-même ne devrait pas avoir d'effets négatifs sur l'hôte. De ce côté, l'introduction d'une chimère TRIM5 α_{Hu} – TRIM5 α_{Rh} dans des cellules souches hématopoïétiques (HSC) humaines s'est révélée comme ne nuisant pas à la différenciation et l'activation de ces cellules [71]. Cependant, une récente étude a démontré que la surexpression constitutive de TRIM5 α provoque l'activation de la signalisation liée à l'immunité innée, résultant ainsi en une activation constitutive de NF- κ B [56]. La dérégulation de l'activation de NF- κ B peut avoir de lourdes conséquences, allant du cancer aux maladies auto-immunes [72, 73]. L'utilisation de vecteurs rétroviraux pour introduire les transgènes de TRIM5 α chez l'hôte, d'où ils seraient généralement exprimés grâce à de puissants promoteurs tels que CMV-IE, est donc risquée en soi. Une connaissance plus approfondie des mécanismes par lesquels la surexpression de TRIM5 α induit l'activation de NF- κ B pourrait permettre d'identifier un moyen de contrôler cet effet indésirable dans une thérapie génique.

Du point de vue de l'efficacité, plusieurs critères sont à prendre en considération. Tout d'abord, étant donné que les rétrovirus sont de par leur cycle de réplication des virus qui mutent et s'adaptent rapidement à la pression qui est exercée sur eux, l'émergence de virus résistant est un risque réel qui doit être limité. Pour ce faire, une meilleure connaissance des mécanismes permettant au VIH-1 d'échapper à la restriction médiée par TRIM5 α est essentielle. Le chapitre III de cet ouvrage présente notre étude de ces mécanismes de résistance d'un virus résistant aux mutants de TRIM5 α _{Hu}. Plus spécifiquement, nous y avons étudié la mutation virale V86M qui se situe au sein de la boucle de liaison à CypA de CA (voir Figure 1.12).

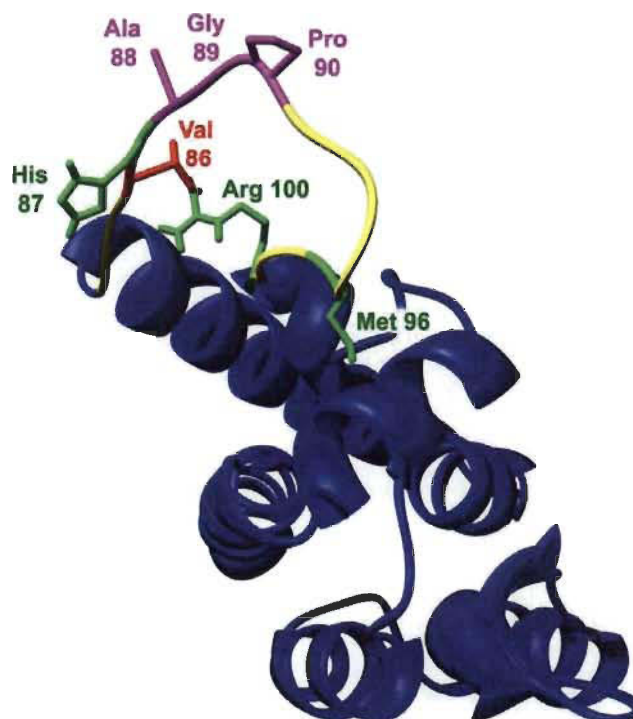


Figure 1.12 Position de l'acide aminé V86 dans la CA virale [74].

Une autre facette de l'efficacité repose sur la variabilité entre les différentes souches de VIH-1 qui infectent les individus à l'échelle mondiale. Les transgènes candidats devraient donc idéalement être capables de bloquer l'infection par plusieurs voire toutes ces différentes variations. Nous avons également étudié cet aspect important de la démarche vers une thérapie génique, étude qui est présentée au chapitre IV.

CHAPITRE II

A NOVEL AMINOACID DETERMINANT OF HIV-1 RESTRICTION IN THE TRIM5 α VARIABLE 1 REGION ISOLATED IN A RANDOM MUTAGENIC SCREEN

QUANG TOAN PHAM¹, MAXIME VEILLETTE¹, ALBERTO BRANDARIZ-NUÑEZ²,
PAULINA PAWLICA, CAROLINE THIBERT-LEFEBVRE, NADIA CHANDONNET,
FELIPE DIAZ-GRIFFERO², LIONEL BERTHOUX

Publié dans *Virus Research*, le 25 janvier 2013.

Résumé

Les gènes antirétroviraux dérivés de l'humain sont d'une haute importance biomédicale et sont activement recherchés. Le VIH-1 est efficacement inhibé aux étapes précoces du cycle de réplication virale par des mutants de la région variable 1 (v1) du facteur de restriction humain TRIM5 α . Dans cette étude, nous avons utilisé un megaprimer muté afin de créer une librairie de mutants de la région v1 de TRIM5 α_{Hu} . Nous avons isolé un variant présentant une mutation à la position 330 (G330E) qui inhibe la transduction d'un vecteur VIH-1 aussi efficacement que les mutations déjà décrites aux positions 332 et 335 (R332G et R335G). À l'instar de ces dernières, l'ajout d'une charge acide locale dans la région v1 était la clef pour la restriction du VIH-1. TRIM5 α_{Hu} G330E inhibait également la propagation d'un virus VIH-1 répliquatif dans une lignée de lymphocytes T humains. Fait intéressant, la combinaison de G330E avec les mutations aux positions 332 et 335 n'a pas augmenté la restriction du VIH-1. Par ailleurs, le triple mutant G330E-R332G-R335G liait les tubes de capsid recombinante purifiée moins efficacement que le double mutant R332G-R335G. Dans un modèle de la

¹ Ces auteurs ont contribué également.

² Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, É.-U.

structure du domaine PRYSPRY de TRIM5 α_{Hu} , l'ajout de la mutation G330E au double mutant R332G-R335G a causé d'importants changements à la surface de liaison à la capsid, ce qui pourrait expliquer pourquoi le triple mutant n'était pas plus restrictif que le double mutant. Par ailleurs, le potentiel des mutants de Gly330 d'inhiber le VIH-1 n'a pas été prédit par l'étude d'orthologues de TRIM5 α qui sont connus pour inhiber fortement le VIH-1. Cette étude souligne donc le potentiel des stratégies de mutagenèse aléatoire à isoler des variants de protéines humaines avec des propriétés antivirales.

Abstract

Human-derived antiretroviral transgenes are of great biomedical interest and are actively pursued. HIV-1 is efficiently inhibited at post-entry, pre-integration replication stages by point mutations in the variable region 1 (v1) of the human restriction factor TRIM5 α . Here we use a mutated megaprimer approach to create a mutant library of TRIM5 α_{Hu} v1 and to isolate a mutation at Gly330 (G330E) that inhibits transduction of an HIV-1 vector as efficiently as the previously described mutants at positions Arg332 and Arg335. As was the case for these other mutations, modification of the local v1 charge toward increased acidity was key to inhibiting HIV-1. G330E TRIM5 α_{Hu} also disrupted replication-competent HIV-1 propagation in a human T cell line. Interestingly, G330E did not enhance restriction of HIV-1 when combined with mutations at Arg332 or Arg335. Accordingly, the triple mutant G330E-R332G-R335G bound purified recombinant HIV-1 capsid tubes less efficiently than the double mutant R332G-R335G did. In a structural model of the TRIM5 α_{Hu} PRYSPRY domain, the addition of G330E to the double mutant R332G-R335G caused extensive changes to the capsid-binding surface, which may explain why the triple mutant was no more restrictive than the double mutant. The HIV-1 inhibitory potential of Gly330 mutants was not predicted by examination of natural TRIM5 α orthologs that are known to strongly inhibit HIV-1. This work underlines the potential of random mutagenesis to isolate novel variants of human proteins with antiviral properties.

Introduction

It has long been recognized that treatment with type I IFN (IFN- α especially) efficiently inhibits HIV-1 propagation *in vitro*, suggesting an important role for innate immunity in the control of this infection (Chakrabarti and Simon, 2010). Several mediators of the anti-HIV-1 activity of type I IFNs in human cells have been discovered in the last 15 years. These proteins, sometimes called “restriction factors”, can inhibit the replication of retroviruses at various stages. All the restriction factors isolated so far are dominant and some are antagonized by HIV-1 gene products (Goffinet et al., 2009; Mangeat et al., 2009; Marin et al., 2003; Mehle et al., 2004). Some restriction factors show a high level of specificity and typically target non-cognate retroviruses more efficiently, implying an important role in the prevention of inter-species transmission (Cullen, 2006; Evans et al., 2010; Mariani et al., 2003).

TRIM5 α was isolated as a retroviral restriction factor in 2004 (Stremlau et al., 2004) and acts within the post-entry, pre-integration window (Owens et al., 2003; Perez-Caballero et al., 2005b). The viral molecular target of TRIM5 α is the correctly matured N-terminal domain of capsid (CA) proteins forming the outer surface of the retroviral core (Cowan et al., 2002; Forshey et al., 2005; Ikeda et al., 2004; Owens et al., 2003; Shi and Aiken, 2006; Stremlau et al., 2006). A direct interaction between the two proteins, each present as high molecular weight multimers, occurs shortly after entry and is required for downstream inhibition of viral replication (Kar et al., 2008; Langelier et al., 2008; Perez-Caballero et al., 2005a; Sebastian and Luban, 2005; Stremlau et al., 2006). The mechanism of TRIM5 α -mediated restriction can be broken down to discrete events, some of them inter-dependent: (i) virus entrapment into TRIM5 α cytoplasmic bodies (Campbell et al., 2008), (ii) decreased stability of the virus core (Black and Aiken, 2010; Perron et al., 2007; Stremlau et al., 2006; Zhao et al., 2011) (iii) targeting to a proteasome-dependent degradation pathway (Anderson et al., 2006; Diaz-Griffero et al., 2006; Lienlaf et al., 2011; Rold and Aiken, 2008); , and (iv) inhibition of nuclear import (Anderson et al., 2006; Berthoux et al., 2004; Wu et al., 2006). The linear structure of TRIM5 α reveals a partition between effector domains at the N-terminus and

a target recognition domain called PRYSPRY (also known as SPRY or B30.2) found at the C-terminus. The PRYSPRY domain contains evolutionarily variable regions that determine binding to CA and restriction potency (Diaz-Griffero et al., 2008; Ohkura et al., 2006; Sawyer et al., 2005; Song et al., 2005).

The human ortholog of TRIM5 α moderately restricts HIV-1 (~2-fold), even when over-expressed (Pham et al., 2010; Stremlau et al., 2004; Uchil et al., 2008), but many natural isolates of HIV-1 are more sensitive to TRIM5 α_{Hu} (Battivelli et al., 2011). Thus, stable transduction of HIV-1-targeting TRIM5 α has been proposed as a way to decrease the permissiveness of human cells to HIV-1 infection in medical applications (Rossi et al., 2007). Rhesus macaque TRIM5 α (TRIM5 α_{Rh}) over-expressed exogenously is dominant over its human endogenous counterpart and efficiently restricts HIV-1, including upon transduction into hematopoietic progenitor cells (Anderson and Akkina, 2005). Human/rhesus chimeric TRIM5 α , in which regions of the PRYSPRY domain originate from the rhesus ortholog, have also been used (Kambal et al., 2011). Other human/rhesus mapping experiments led to the discovery that mutations at Arg332 in the variable 1 (v1) region of PRYSPRY conferred HIV-1 restriction activity (Li et al., 2006; Yap et al., 2005), although not to the level of TRIM5 α_{Rh} (Li et al., 2006; Pham et al., 2010). Based on the dual hypothesis that (i) TRIM5 α_{Rh} and other Old world primate TRIM5 α might be too immunogenic for human gene transfer, (ii) novel discrete mutations in TRIM5 α could increase its affinity for HIV-1 CA, we previously introduced a random mutagenic screen to isolate point mutants of TRIM5 α_{Hu} targeting HIV-1 (Pham et al., 2010). Using this approach, we found that several mutations abrogating the positive charge at position 335 of the PRYSPRY domain restricted HIV-1 in human cell lines. Here we describe a second, different mutagenic screen that led to the identification of additional HIV-1-restrictive mutants.

Material and Methods

Plasmid DNAs

pMIP-TRIM5 α_{Hu} and pMIP-TRIM5 α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before (Berthoux et al., 2005a; Berthoux et al., 2005b; Bérubé et al., 2007; Pham et al., 2010; Sebastian et al., 2006). pNL-GFP, pMD-G, p Δ R8.9, pTRIP_{CMV-GFP} and pCL-Eco have all been described elsewhere (Berthoux et al., 2005a; Berthoux et al., 2004; Berthoux et al., 2005b; Berthoux et al., 2003; Naviaux et al., 1996; Zufferey et al., 1997). The proviral clone pNL4-3 (Adachi et al., 1986) was used for propagation experiments.

Cells and retroviral vectors production

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C. Human T lymphoblast Sup-T1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 37 °C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA). VSV G-pseudotyped HIV-1_{NL4-3} HIV-1 and MLV-based vectors were produced through transient transfection of 293T cells and collected as previously described (Bérubé et al., 2007; Pham et al., 2010). To produce HIV-1_{TRIP-CMV-GFP}, cells were co-transfected with pTRIP_{CMV-GFP}, p Δ R8.9 and pMD-G. To produce MIP or MIP-TRIM5 α vectors, cells were co-transfected with the relevant pMIP vector, pCl-Eco and pMD-G.

Library construction and screening

The following oligodeoxynucleotide (ODN) named "38-doping" was ordered from Sigma-Genosys: 5'-aga caa gtg agc tct CCg AAa CCa CAg ATa ATa TAt GGg GCa CGa GGg ACa AGa TAc CAg ACa TTt GTg AAt ttc aat tat tgt act. This ODN was designed to introduce random mutations in the coding region for TRIM5 α_{Hu} (NCBI

accession # NM_033034) residues Pro323 to Asn341. The manufacturer was instructed to introduce random mutations at nucleotides shown in capital letters in the sequence above. At each of these positions, 95% of the ODNs were requested to have the correct residue, while 5% would have any of the 3 other ones. Mutations were confined to the first and second residues of each codon in order to minimize the occurrence of silent mutations. Because 38 residues were targeted for mutagenesis, and assuming a 5% chance of being mutated for each, the expected ODNs synthesized should bear 1.9 mutations on average. However, we found that TRIM5 α_{Hu} generated by this procedure had 3.2 mutations on average.

Sewing PCR was used to transfer the mutant library to a retroviral vector expressing TRIM5 α_{Hu} . For this, TRIM5 α_{Hu} upstream of the mutated region was amplified with primers TRIM5fwd (5'-GTTCCCTCGAGATGGCTTCTGGAATCCTGGTTAAT) and TRIM5₉₆₃₋₉₄₀ (GCTCACTTGTCTCTTATCTTCAGA). The rest of TRIM5 α_{Hu} was amplified using the 38-doping ODN and TRIM5rev (5'-TCCTGAATTCTTACTTATCGTCGTCATCCTTGTAATC). Each PCR reaction was done in 1× High Fidelity (HF) buffer, contained 170 ng of matrix DNA, 20 units/ml of high fidelity Phusion enzyme from New England Biolabs (Pickering, ON), 0.2 mM of dNTP, 0.2 μ M of each primer and proceeded for 25 cycles (98 °C, 10 sec; 56 °C, 30 sec; 72 °C, 45 sec). PCR products were agarose gel-purified using the QIAquick gel extraction kit. About 1/10 of the purified product from each PCR were combined and used to perform a second PCR reaction with ODNs TRIM5fwd and TRIM5rev. DNA was amplified for 25 cycles (98 °C, 20 sec; 56 °C, 20 sec; 72 °C, 1 min) using the Phusion enzyme. The PCR product was purified using the QIAGEN QIAquick PCR purification kit, then digested with PshA1 and EcoR1. Digested DNA was again gel-purified and then ligated overnight to pMIP-TRIM5 α_{Hu} cut with the same enzymes. The ligation product was divided in 6 aliquots and then entirely transformed in *E. coli* DH5 α bacteria by electroporation, yielding a 21,000-clone library.

MLV-based vectors carrying the mutant library were produced by transfection of 293T cells in a 10-cm plate with 10 μ g of mutated pMIP-TRIM5 α_{Hu} , 10 μ g of pCL-Eco

and 5 μ g of pMD-G. 100,000 TE671 cells in 6-well plates were infected with various amounts of the MLV-TRIM5 α_{Hu} , in triplicate. 2 days later, cells were treated with 2 μ g per ml of puromycin for 2 to 3 days, at which point we visually examined the cultures and discarded the ones for which transduction efficiency was higher than 15%. The remaining cells were pooled. Puromycin-resistant cells were subsequently challenged with a preparation of the GFP-expressing HIV-1 vector HIV-1_{TRIP-CMV-GFP} at a high multiplicity of infection, exactly as before (Pham et al., 2010). GFP-negative cells were then sorted out by FACS, and 0.18% of intact cells were recovered. These cells were individually distributed into 2 x 96-wells plates and the surviving clones were challenged again with HIV-1_{TRIP-CMV-GFP}. Cell clones that expressed mutated versions of TRIM5 α_{Hu} and that were confirmed as being more than 2-times resistant to transduction by HIV-1_{TRIP-CMV-GFP} were analyzed further.

Site-directed mutagenesis

The R332G, R335G and R332G-R335G mutations were described before (Pham et al., 2010). Other mutations were introduced in MIP-TRIM5 α_{Hu} by using the following primers through the same “sewing PCR” procedure as before (the modified codons are underlined):

K324Q_{fwd}, GAGCTCTCCGCAACCCACAGATAATA;
 K324Q_{rev}, ATCTGTGGTTGCGGAGAGCTCACTT;
 I328S_{fwd}, ACCACAGATATCATATGGGGCACGAG;
 I328S_{rev}, TGCCCCATATGATATCTGTGGTTTCG;
 Y329C_{fwd}, CAGATAATATTGTGGGGCACGAGGGA;
 Y329C_{rev}, CCTCGTGCCCCACATATTATCTGT;
 G330E_{fwd}, ATAATATATGAGGCACGAGGGACAA;
 G330E_{rev}, CCCTCGTGCCTCATATATTATCTGT;
 G333V_{fwd}, GGGGCACGAGTGACAAAGATACCAGA;
 G333V_{rev}, GGTATCTTGTCACTCGTGCCCCAT;
 Y336C_{fwd}, GGGACAAGATTGCCCAGACATTTGTGA;
 Y336C_{rev}, AAATGTCTGGCATCTTGTCCCTCGT;

Q337P_{fwd}, GGACAAGATACCCGACATTTGTGAA;
 Q337P_{rev}, TTCACAAATGTCGGGTATCTTGTCC;
 T338P_{fwd}, CAAGATACCAGCCATTTGTGAATTTC;
 T338P_{rev}, TTCACAAATGGCTGGTATCTTGTCC;
 G330E-R332G_{fwd}, CAGATAATATATGAGGCAGGAGGGACAAGATACC;
 G330E-R332G_{rev}, GGTATCTTGTCCCTCCTGCCTCATATATTA;
 G330E-R335G_{fwd}, ATAATATATGAGGCACGAGGGACAGGATACCAGAC;
 G330E-R335G_{rev}, GTCTGGTATCCTGTCCCTCGTGCCTCATATATTA;
 G330E-R332G-R335G_{fwd},
 ATAATATATGAGGCAGGAGGGACAGGATACCAGAC;
 G330E-R332G-R335G_{rev},
 GTCTGGTATCCTGTCCCTCCTGCCTCATATATTA.

TE671, CRFK and Sup-T1 cells stably expressing these TRIM5 α _{Hu} variants were generated by transduction with the MLV-based vector MIP-TRIM5 α _{Hu} followed by puromycin selection, exactly as described previously (Pham et al., 2010).

Viral challenges

Cells expressing various TRIM5 α orthologs or mutants were challenged with single doses of HIV-1_{TRIP-CMV-GFP} or HIV-1_{NL-GFP} as described previously (Pham et al., 2010). The viral dose used was typically set so that approximately 10% of control permissive cells are infected, thus allowing reliable quantification of infection when the magnitude of restriction is up to 100-fold. Two days post-infection, adherent cells were trypsinized and fixed in 2% formaldehyde-phosphate-buffered saline while Sup-T1 cells were PBS-washed and then treated with 2% formaldehyde. All cells were subjected to fluorescence-activated cell sorting analysis on a FC500 MPL instrument (Beckman Coulter, Mississauga, ON, USA) using the MXP/CXP software. On the basis of light scatter profiles, only intact cells were included in the analysis. GFP-positive cells were gated and counted as a percentage of total intact cells following analysis of 10,000 to 25,000 cells. For the propagation assay, 2×10^6 Sup-T1 cells were infected with 50 μ l of

supernatant from 293T cells transfected with pNL4-3, containing approximately 10 ng of CAp24 as measured by ELISA. The next day, cells were washed to remove input virus and then kept in culture for 4 weeks. Cell-free supernatants were collected every 2 or 3 days for analysis. p24 (CA) concentrations in the supernatants were determined by a home-made ELISA assay using the 183 CA monoclonal antibody (Bounou et al., 2002).

HIV-1 CA-NC expression and purification

The HIV-1 capsid-nucleocapsid (CA-NC) protein was expressed, purified and assembled as previously described (Ganser-Pomillos et al., 2004; Ganser et al., 1999). Briefly, a pET11a-based vector (Novagen) expressing the CA-NC protein of HIV-1 was used to transform BL-21(DE3) *E. coli*. CA-NC expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when the culture reached an optical density of 0.6 at 600 nm. After 4 hours of induction, cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.5), 1 μ M ZnCl₂, 10 mM 2-mercaptoethanol and protease inhibitors (Roche). Lysis was performed by sonication, and debris were pelleted for 30 minutes at 35,000 x *g*. Nucleic acids were precipitated by adding 0.11 equivalents of 2M (NH₄)₂SO₄ and the same volume of 10% polyethylenimine and then pelleted by centrifugation at 29,500 x *g* for 15 minutes. Proteins were recovered by adding 0.35 equivalents of saturated (NH₄)₂SO₄ and centrifuging at 9,820 x *g* for 15 min. Pellets were resuspended in 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μ M ZnCl₂ and 10 mM 2-mercaptoethanol. Finally the CA-NC protein was dialyzed against 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 μ M ZnCl₂ and 10 mM 2-mercaptoethanol, and stored at -80 °C.

In vitro assembly of HIV-1 CA-NC complexes and binding to TRIM5 α variants

HIV-1 CA-NC particles were assembled *in vitro* by diluting the CA-NC protein to a concentration of 0.3 mM in 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl and 2 mg/ml DNA oligo-(TG)₅₀. The mixture was incubated at 4°C overnight and centrifuged at 8,600 x *g* for 5 minutes. The pellet was resuspended in assembly buffer (50 mM Tris-HCl

(pH 8.0), 0.5 M NaCl) at a final protein concentration of 0.15 mM and stored at 4 °C until needed. 293T cells were transfected with plasmids expressing wild-type or mutant TRIM5 α proteins. 48 h after transfection, cell lysates were prepared as follows. Cells were PBS-washed and resuspended in capsid-binding buffer (10 mM Tris, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT). Cells were disrupted by freeze-thawing, and incubated on ice for 10 minutes. Lysates were centrifuged in a refrigerated microcentrifuge (~ 14,000 x g) for 5 minutes. Supernatants were supplemented with 1/10 volume of 10X PBS and then used in the binding assay. In some cases, samples containing the TRIM5 α variants were diluted with extracts prepared in parallel from untransfected cells. To assay binding, 5 μ l of CA-NC particles assembled *in vitro* were incubated with 200 μ l of cell lysate at room temperature for 1 hour. A portion of this mixture, referred to as “input” was stored. The mixture was spun through a 70% sucrose cushion (70% sucrose, 1X PBS and 0.5 mM DTT) at 100,000 x g in an SW55 rotor (Beckman) for 1 hour at 4 °C. After centrifugation, the supernatant was carefully removed and the pellet was resuspended in 1X SDS-PAGE loading buffer. Levels of TRIM5 α proteins were determined by western blotting with an anti-FLAG antibody as described above. Levels of HIV-1 CA-NC protein in the pellet were assessed by western blotting with an anti-p24 capsid antibody.

Structural models of the PRYSPRY domain

Prediction structures were generated using the web-based I-TASSER system (Roy et al., 2010; Zhang, 2008) with default parameters. Molecular graphics and analyses were performed with the UCSF Chimera 1.6.2 package (Pettersen et al., 2004).

Results

A v1-focused mutagenic screen

Previously published data pointed to v1 as the major region determining retroviral restriction specificity (Ohkura et al., 2006; Sawyer et al., 2005; Stremlau et al., 2005).

We thus developed a novel mutagenic screen specifically targeting this domain. A megaprimer was used to introduce mutations at residues 323 to 341 of TRIM5 α_{Hu} (Figure 2.1A). A library of more than 20,000 clones was generated, each carrying about 3 mutations on average. The library was retrovirally transduced in human TE671 cells and we applied the same functional screen that was used previously by us (Pham et al., 2010) and others (Stremlau et al., 2004) to isolate cells with decreased permissiveness to transduction by the GFP-encoding, VSV G-pseudotyped HIV-1 vector TRIP-CMV-GFP. Following two rounds of functional selection to retain only the clones that restricted HIV-1 by 2-fold or more compared to the control transduced with the “empty” vector, we recovered 5 cell lines in which GFP transduction by HIV-1_{TRIP-CMV-GFP} was inhibited about 5-fold (Figure 2.1B). Sequencing of the TRIM5 α_{Hu} expressed from the retroviral vector showed that 3 of them contained mutations at position 332 (Figure 2.1B). Two of those were the R332G mutation that has been well-characterized by others (Li et al., 2006). These three clones were not analyzed any further as their restriction potential was entirely explained by Arg332 mutations.

Each of the 7 substitutions found in clones B3 and G4 was introduced in the parental retroviral vector expressing TRIM5 α_{Hu} by site-directed mutagenesis and then transduced into TE671 cells. Permissiveness to GFP transduction by HIV-1_{TRIP-CMV-GFP} was then analyzed for each of the mutants. Only the mutation G330E of G4 conferred a strong level of HIV-1 resistance, close to 10-fold (Figure 2.1C). The fact that G330E was more restrictive than the parental clone G4 was probably due to the presence of mitigating mutations in clone G4, such as Y329C. This mutation alone abolishes the slight level of restriction (2-fold) conferred by over-expression of the wild-type (WT) TRIM5 α_{Hu} , and thus it is likely that it would also decrease restriction when combined with G330E, although we did not test this. Thus, HIV-1 restriction in TE671-B3 cells was due to a combination effect involving 2 or 3 of the substitutions found in this clone, while restriction in TE671-G4 cells was explained by mutation G330E. In this preliminary analysis, G330E was found to be as efficient at inhibiting HIV-1 as previously described mutation R332G. However, it was approximately 2-times less restrictive than the double mutation R332G-R335G and 10-times less restrictive than the Rhesus macaque ortholog (Figure 2.1C).

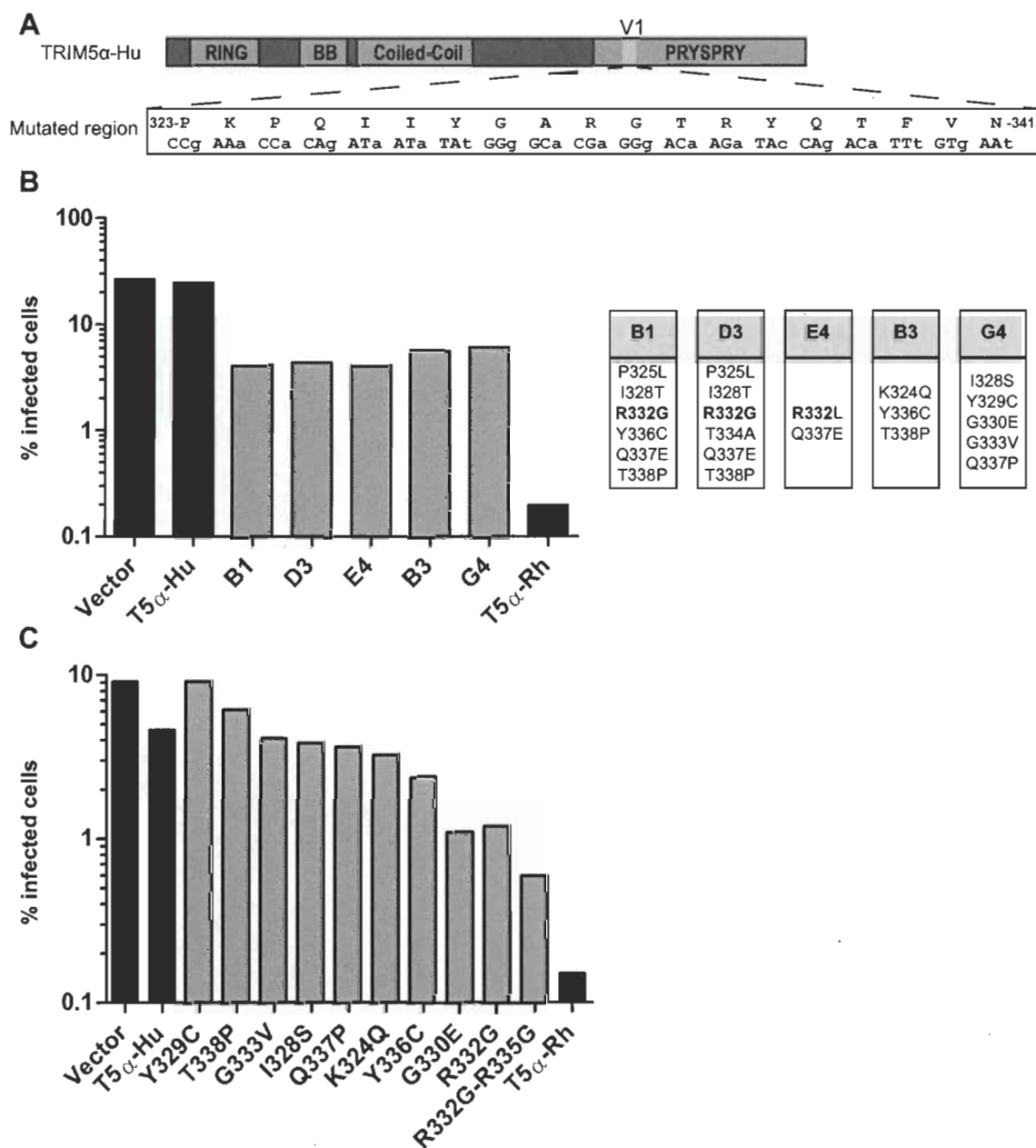


Figure 2.1 Isolation of amino acid 330 of TRIM5 α _{Hu} as a determinant of HIV-1 restriction.

(A) Megaprimer approach to introduce mutations in the v1 region of TRIM5 α _{Hu}. The nucleotide sequence of the region targeted by the primer is shown in the box under the corresponding amino acid sequence. Partly degenerated primer synthesis was used to introduce random mutations at the 38 nucleotide positions shown in capital letters, and the mix of mutated megaprimers were cloned into the retroviral vector construct pMIP-TRIM5 α _{Hu} by sewing PCR, and then transduced into TE671 cells. (B) HIV-1 restriction potential of 5 TE671 clones isolated in a functional screen. Presumed HIV-1-resistant cell clones were grown and tested for HIV-1 permissiveness. The left panel shows percentages of infected

(GFP-positive) cells two days after challenging the 5 clones with a single dose of HIVTRIP-CMV-GFP. Control cells transduced with the empty MIP vector or cells transduced with the weakly restrictive wild-type TRIM5 α_{Hu} or with restrictive TRIM5 α_{Rh} were included. The right panel lists mutations found in the TRIM5 α_{Hu} variants expressed by each of the TE671 cell clones isolated. (C) HIV-1 restriction potential of individual point mutations found in cell clones B3 and G4. Mutations were introduced into pMIP-TRIM5 α_{Hu} and TE671 cells transduced with the constructed vectors were challenged with HIVTRIP-CMV-GFP as above. Previously characterized restrictive TRIM5 α_{Hu} mutants R332G and R332G-R335G were included in the assay.

Effect of alternative mutations at Gly330

Previous mutations modulating the capacity of TRIM5 α_{Hu} to inhibit restriction of HIV-1 affected the charge of the protein. Removal of the positive charge conferred by Arg332 or Arg335 was, indeed, key to HIV-1 restriction (Li et al., 2006; Pham et al., 2010). The negatively charged glutamic acid brought by the G330E mutation likewise affects the protein charge. In order to test whether restriction was primarily due to the removal of the glycine or to the introduction of negatively charged residue, we tested the restriction potential of other substitutions at position Gly330. We found that introduction of an aspartic acid significantly (3- to 4-fold) inhibited HIV-1 replication, while G330A or G330S had little or no effect (Figure 2.2). These data suggest that introducing a negative charge at that position was important to inhibit HIV-1. However, G330D was not as restrictive as G330E, and only the latter mutant was retained for further investigation.

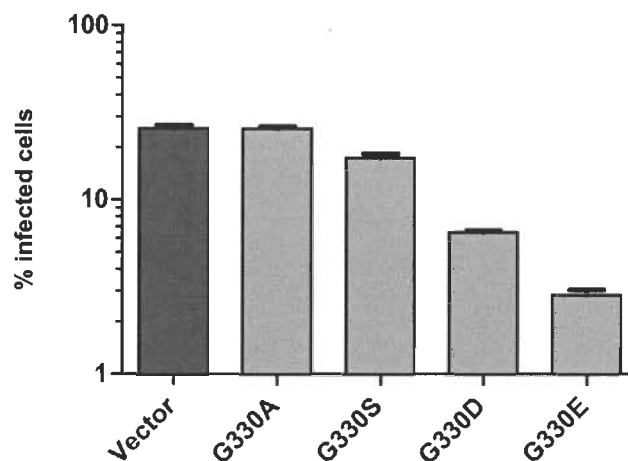


Figure 2.2 Restriction of HIV-1 by TRIM5 α_{Hu} mutants of Gly330. TE671 cells were transduced to express the indicated TRIM5 α and then challenged with a single dose of HIV_{TRIP-CMV-GFP} exactly as in Figure 2.1. The percentages of infected cells were determined by FACS 2 days later. Shown are average values from triplicate infections with standard deviations.

Combining G330E with mutations at Arg332 or Arg335

Previously, we found that R332G and R335G had additive effects on HIV-1 replication (Pham et al., 2010). Thus, we aimed at analyzing whether the inhibition of HIV-1 conferred by G330E was, similarly, additive to that of R332G, R335G, or the double mutant R332G-R335G. As shown in Figure 2.3, however, any combination including G330E was no more restrictive, and even slightly less restrictive, than the relevant controls lacking it. Therefore, G330E has a mitigating effect on HIV-1 restriction stemming from removing positive charges in the v1 region.

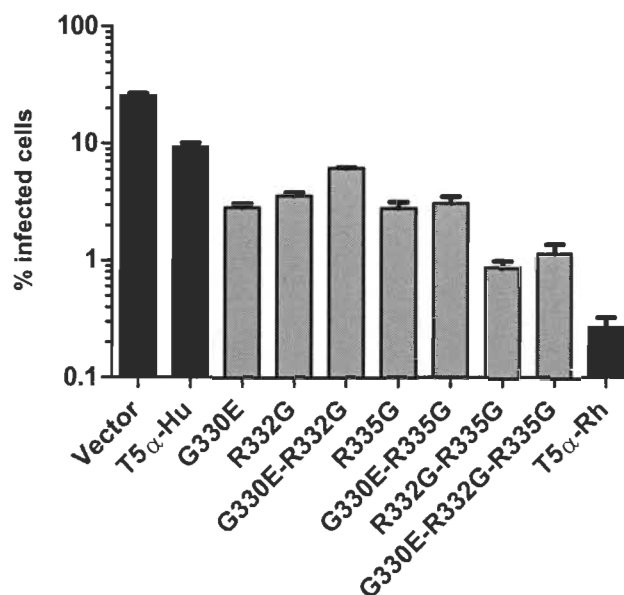


Figure 2.3 Antiretroviral effect conferred by G330E when combined with R332G or R335G.

The indicated single, double or triple TRIM5 α_{Hu} mutants were constructed and retrovirally transduced into TE671 cells. The cell lines created were then challenged with HIV_{TRIP-CMV-GFP} as before. Bars show averages of percent infected cells after single-dose infections in triplicates, with standard deviations.

In vitro binding analysis

Mutations at Arg332 of TRIM5 α_{Hu} have been demonstrated to promote HIV-1 restriction through increased binding of TRIM5 α_{Hu} to its molecular target, the CA protein of HIV-1 (Li et al., 2006). Presumably, mutations at positions 330 and 335 would also increase CA recognition, but this had not been demonstrated for Arg335 mutations. We analyzed the potential of TRIM5 α_{Hu} mutants to bind *in vitro* assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes, as described before (Diaz-Griffero et al., 2009). TRIM5 α_{Rh} and both R332G-R335G and G330E-R332G-R335G mutants of TRIM5 α_{Hu} clearly bound *in vitro* assembled CA-NC complexes, and the strength of the interaction roughly correlated with the magnitude of restriction (Figure 2.4). G330E-R332G-R335G was less efficient than R332G-R335G at binding *in vitro* assembled HIV-1 CA-NC complexes, supporting the finding that the G330E mutation does not increase restriction when it is combined with Arg332 and Arg335 mutations.

Single mutants G330E and R335G were not observed to significantly bind *in vitro* assembled HIV-1 CA-NC complexes in these experimental conditions, but this may be simply due to a lack of sensitivity of our assay.

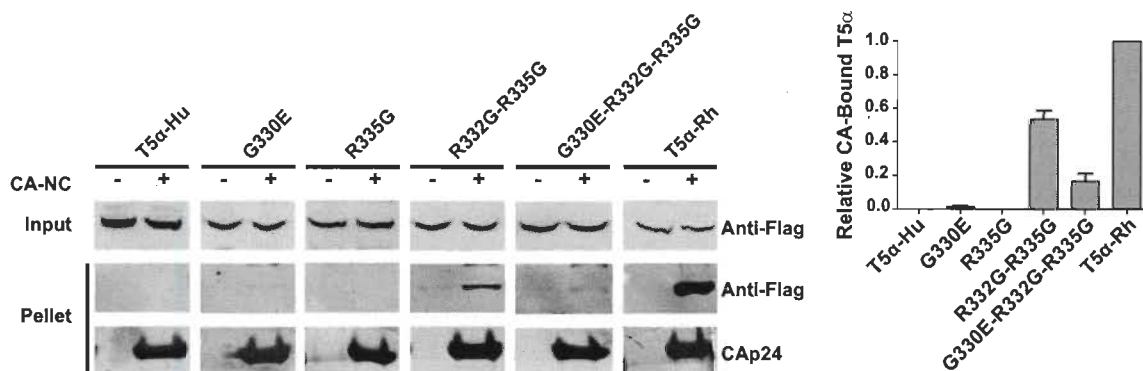


Figure 2.4 *In vitro* TRIM5 α binding to HIV-1 CA.

CA-NC complexes were assembled *in vitro* and mixed with lysates from 293T cells transfected with the relevant TRIM5 α -FLAG-expressing plasmid constructs. CA-NC complexes were separated from soluble proteins by ultracentrifugation through a sucrose cushion and analyzed by Western blotting using CA and FLAG antibodies (pellet). A fraction of the pre-centrifugation mix was analyzed by Western blot for CA content (input). The graph on the right shows relative CA-bound TRIM5 α as determined by the ratio between pellet-associated and total (input) TRIM5 α , relative to that obtained for TRIM5 α_{Rh} . Bars represent the average of 3 experiments with standard deviations.

Restriction in other viral and cellular environments

T lymphocytes are the main host cells in natural HIV-1 infection. To analyze whether G330E and other mutants would inhibit HIV-1 when stably expressed in lymphocytes, Sup-T1 cells were transduced with the MIP-TRIM5 α vectors. Like before, untransduced cells were eliminated by antibiotic treatment and cells were then challenged with a single dose of HIV-1_{NL-GFP}. This HIV-1 vector is similar to HIV-1_{TRIP-CMV-GFP} in that it is derived from a subtype B HIV-1 strain and it does not encode a viral envelope. However, HIV-1_{NL-GFP} resembles wild-type HIV-1 since all viral proteins except for Nef and Env are expressed following infection. As shown in Figure 2.5A, the infectivity data obtained closely mirrored those generated with HIV-1_{TRIP-CMV-GFP} in TE671 cells. Although the overall level of restriction was smaller

than in TE671 cells, as observed before (Pham et al., 2010), it is worth noting that expression of either R332G-R335G or G330E-R332G-R335G in Sup-T1 cells inhibited HIV-1 almost as efficiently as TRIM5 α_{RH} . Like before, G330E inhibited HIV-1 as efficiently as mutations in Arg332 or Arg335, but G330E-R332G-R335G was not more efficient than R332G-R335G.

Human cells express low levels of endogenous TRIM5 α that do not significantly affect HIV-1 replication, and over-expressed transgenic TRIM5 α is known to be dominant over its endogenous counterpart (Berthoux et al., 2005a). However, it was still possible that some of the mutants constructed might behave differently in the absence of endogenous TRIM5 α . To test this directly, we transduced feline cells, which do not express any TRIM5, with the same TRIM5 α orthologs and mutants that had been tested in human cells. These cells were then challenged with HIV-1_{NL-GFP} and the percentages of infected cells were determined. We found that the restriction pattern in feline cells (Figure 2.5B) closely resembled what had been obtained in human TE671 and Sup-T1 cells (Figure 2.5A). Specifically, G330E restricts HIV-1 in these cells about as efficiently as R332G and R335G do (about 10-fold compared with the empty vector control), and G330E does not potentiate HIV-1 restriction by the R332G-R335G double mutant.

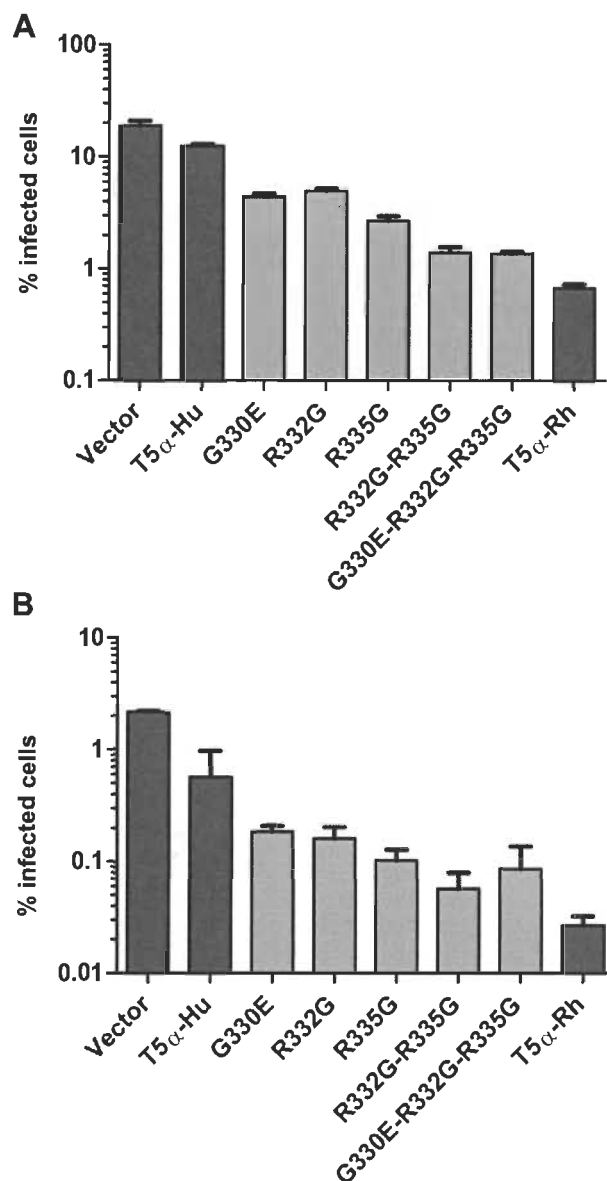


Figure 2.5 Restriction of HIV-1 replication in human lymphocytic cells and in nonhuman cells.

Human Sup-T1 cells (A) and feline fibroblast CRFK cells (B) were retrovirally transduced with the “empty” MIP vector or with MIP expressing the indicated TRIM5 α . The cells were then challenged in triplicates with a single dose of HIV-1_{NL-GFP}. Percentages of infected (GFP-positive) cells were determined 2 days later by FACS, as described in Materials and Methods.

Restriction of a propagating infection

Experiments using VSV G-pseudotyped HIV-1 vectors may not always faithfully recapitulate TRIM5 α -mediated restriction of WT viruses. Indeed, TRIM5 α may also interfere with late (assembly) steps of the retroviral cycle (Sakuma et al., 2007) although this conclusion has been rebutted by others (Zhang et al., 2008). In addition, HIV-1 propagation occurs mostly through so-called viral synapses forming between cells, rather than by transmission of cell-free viruses (Groot et al., 2008; Haller and Fackler, 2008; Rudnicka et al., 2009). It is unclear whether TRIM5 α restricts HIV-1 originating from viral synapses the same way it would restrict infection by cell-free viruses, but there are some indications that it does not (Richardson et al., 2008). In order to test whether G330E TRIM5 α_{Hu} would efficiently disrupt the spread of HIV-1 in susceptible cells, we infected Sup-T1 cells expressing WT, G330 or R332G-R335G TRIM5 α_{Hu} with HIV-1_{NL4-3} (Figure 2.6A). Over-expression of WT TRIM5 α_{Hu} slightly delayed the peak of replication from ~5 to ~7 days post infection, an effect seen before (Pham et al., 2010). Spreading of HIV-1 in cells expressing G330E or R332G-R335G TRIM5 α_{Hu} was apparent but no clear peak was visible during the course of the experiment. Indeed, while the p24 concentration in the supernatants of these two cultures was between 10 and 100 pg/ml from day 5 to day 16 of the propagation assay, these concentrations were more than 10,000-times smaller than those measured at the peak of replication in control cells and in cells over-expressing WT TRIM5 α_{Hu} . Yet, viral replication did occur in these cells, as evidenced by the presence of syncytia (Figure 2.6B) followed by widespread cell death (not shown). Therefore, transduction of G330E or R332G-R335G TRIM5 α_{Hu} is able to efficiently attenuate the spread of HIV-1 in susceptible cells, without completely disrupting it. That HIV-1 was roughly equally inhibited in G330E and R332G-R335G cells, despite the fact that the latter is more efficient at inhibiting HIV-1 single-cycle vector transduction, indicates that restriction of vector transduction may not always be a faithful model for the restriction of HIV-1 propagation.

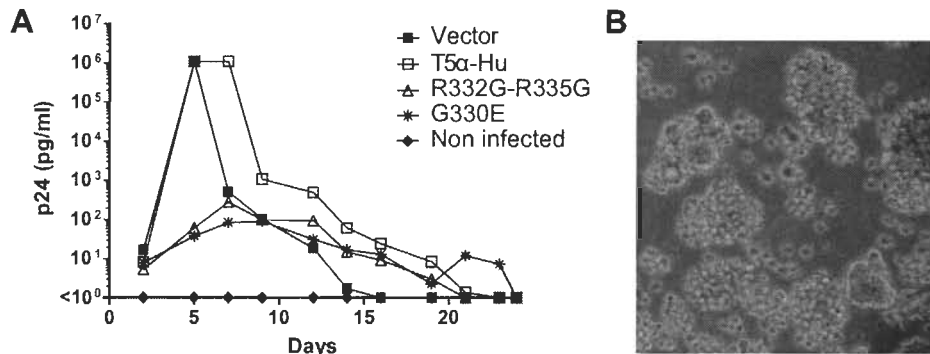


Figure 2.6 HIV-1 propagation in human lymphocytic cells expressing G330E TRIM5 α_{Hu} .

Sup-T1 cells transduced with WT or mutated TRIM5 α_{Hu} or untransduced (vector) were then infected with the replication-competent HIV-1 clone NL4-3. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 24 days. (A) CA (p24) content in the supernatant of infected cells and of uninfected cells as a control were determined periodically by ELISA. (B) Micrograph of R332G-R335G TRIM5 α_{Hu} -expressing cells 14 days post-infection, showing the presence of syncytia.

Modelling the effect of v1 mutations on the TRIM5 α_{Hu} PRYSPRY domain

The 4 hyper-variable regions of TRIM5 α are present at the surface of the protein and constitute the binding interface for interactions with retroviral capsids, as has been confirmed recently (Biris et al., 2012). We used an *in silico* assay to predict the impact of mutations at positions 330, 332 and 335 on this CA-binding interface (Figure 2.7). The I-TASSER program integrated more than 10 published structures of domains similar to the WT TRIM5 α_{Hu} PRYSPRY domain. Among those was the recently published structure of the Rhesus macaque TRIM5 α PRYSPRY (Biris et al., 2012). According to this prediction tool, introduction of the R332G and R335G mutations caused significant changes in all 3 main variable regions (v1, v2, v3). This was most apparent in the surface representation (Figure 2.7B). Interestingly, the triple mutant (G330E-R332G-R335G) had a surface structure that was very different from that of the R332G-R335G mutant and, in fact, more closely resembled that of the WT protein. These observations help explain why G330E-R332G-R335G was slightly less restrictive than the 332-335 double mutant.

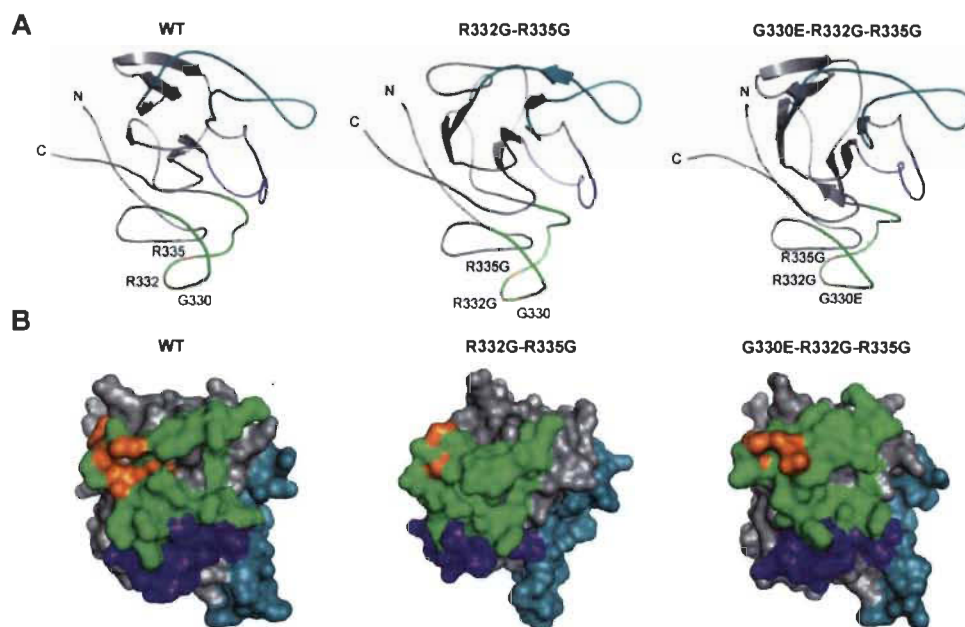


Figure 2.7 Model representation of the TRIM5 α_{Hu} PRYSPRY domain. The predicted impact of v1 mutants on the structure of the PRYSPRY domain was modeled *in silico* based on the published structures of similar domains. (A) Ribbon model and (B) surface representation of WT, R332G-R335G and G330E-R332G-R335G TRIM5 α_{Hu} . The v1, v2 and v3 regions are shown in green, blue and cyan, respectively. The aminoacids at positions 330, 332 and 335 are shown in orange.

Discussion

Our data reveal that a novel mutation in the v1 region of the human TRIM5 α results in a 10-fold decrease in permissiveness to HIV-1 vector transduction, similar to what was previously achieved by other point mutations in this region (Li et al., 2006; Pham et al., 2010; Yap et al., 2005). This mutation, G330E, was isolated using a degenerated oligonucleotide, a technique which to our knowledge had never been applied to generate anti-HIV-1 restriction factors. The change in charge associated with the substitution is crucial to G330E inhibitory activity, as was previously found to be the case for other mutants in this region (Li et al., 2006; Pham et al., 2010). Interestingly, the inhibitory effect of G330E is not additive to that of R332G or R335G, while R332G and R335G do have additive effects. In fact, G330E slightly decreased restriction stemming from Arg332 and/or Arg335 mutations and seemed to decrease binding of

R332G-R335G to *in vitro* assembled HIV-1 CA-NC complexes. The molecular interactions between TRIM5 α and its molecular target, the N-terminal region of CA, have not been elucidated at the structural level despite some recent advances (Biris et al., 2012; Ganser-Pornillos et al., 2011). Future progress in that field is a prerequisite to the full understanding of phenotypes associated with mutations in the v1 domain of TRIM5 α_{Hu} . However, our structure prediction analysis suggests that discrete mutations in v1 can have long-range effects on the whole CA-interaction surface of the PRYSPRY domain. This explains how single mutations in TRIM5 α_{Hu} v1 can modulate the affinity of TRIM5 binding to CA even though this binding depends on multiple weak interactions involving all the variable regions (Biris et al., 2012; Ohkura et al., 2006).

Neither E330 nor D330 are found in TRIM5 α of primates that do or do not restrict HIV-1 (Song et al., 2005); therefore, the mutation found in our screen could not have been predicted from the available literature, an indication of the usefulness of random mutagenesis approaches. However, TRIM5 α from Rhesus macaques and from African green monkeys, two orthologs that strongly restrict HIV-1, bear the following residues at the corresponding positions: Q330, P332, L335. Because this particular combination is different from what we have been testing, it would be valuable to test all the possible combinations (Q330-G332-G335, E330-P332-L335, etc.) in order to isolate (i) the most potent anti-HIV-1 TRIM5 α_{Hu} variants and (ii) the least likely to allow HIV-1 escape. In addition, our spreading infection experiment suggests that assays with fully replicative viruses cannot be circumvented in the search for the most efficient HIV-1 restriction factors. HIV-1 vector transduction assays are convenient and informative but do not provide definitive predictions of antiviral activity.

Conclusion

An innovative random mutagenesis screen has led to the isolation of a novel determinant of HIV-1 restriction by TRIM5 α_{Hu} with potential applications in gene therapy and genome editing.

Acknowledgements

We thank Michel J. Tremblay and Réjean Cantin for sharing their BSL3 facility and the p24 ELISA assay. This work was supported by the Canadian Institutes for Health Research and the Canada Research Chairs program (L.B.). M.V. was supported by a FRQS Master's Training Award. Work in F.D.-G.'s laboratory was supported by NIH R01 AI087390 to F.D.-G, and a K99/R00 Pathway to Independence Award from the National Institutes of Health.

References

- Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A. and Martin, M.A. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* 59(2), 284-91.
- Anderson, J. and Akkina, R. (2005) TRIM5alpha(rh) expression restricts HIV-1 infection in lentiviral vector-transduced CD34(+)-cell-derived macrophages. *Mol Ther* 12(4), 687-96.
- Anderson, J.L., Campbell, E.M., Wu, X., Vandegraaff, N., Engelman, A. and Hope, T.J. (2006) Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *J Virol* 80(19), 9754-60.
- Battivelli, E., Migraine, J., Lecossier, D., Yeni, P., Clavel, F. and Hance, A.J. (2011) Gag cytotoxic T lymphocyte escape mutations can increase sensitivity of HIV-1 to human TRIM5alpha, linking intrinsic and acquired immunity. *J Virol* 85(22), 11846-54.
- Berthoux, L., Sebastian, S., Sayah, D.M. and Luban, J. (2005a) Disruption of human TRIM5alpha antiviral activity by nonhuman primate orthologs. *J Virol* 79(12), 7883-8.
- Berthoux, L., Sebastian, S., Sokolskaja, E. and Luban, J. (2004) Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *J Virol* 78(21), 11739-50.
- Berthoux, L., Sebastian, S., Sokolskaja, E. and Luban, J. (2005b) Cyclophilin A is required for TRIM5 α -mediated resistance to HIV-1 in Old World monkey cells. *Proc Natl Acad Sci U S A* 102(41), 14849-53.
- Berthoux, L., Towers, G.J., Gurer, C., Salomoni, P., Pandolfi, P.P. and Luban, J. (2003) As(2)O(3) enhances retroviral reverse transcription and counteracts Ref1 antiviral activity. *J Virol* 77(5), 3167-80.
- Bérubé, J., Bouchard, A. and Berthoux, L. (2007) Both TRIM5alpha and TRIMCyp have only weak antiviral activity in canine D17 cells. *Retrovirology* 4, 68.

- Biris, N., Yang, Y., Taylor, A.B., Tomashevski, A., Guo, M., Hart, P.J., Diaz-Griffero, F. and Ivanov, D.N. (2012) Structure of the rhesus monkey TRIM5alpha PRYSPRY domain, the HIV capsid recognition module. *Proc Natl Acad Sci U S A* 109(33), 13278-83.
- Black, L.R. and Aiken, C. (2010) TRIM5alpha disrupts the structure of assembled HIV-1 capsid complexes *in vitro*. *J Virol* 84(13), 6564-9.
- Bounou, S., Leclerc, J.E. and Tremblay, M.J. (2002) Presence of host ICAM-1 in laboratory and clinical strains of human immunodeficiency virus type 1 increases virus infectivity and CD4(+)-T-cell depletion in human lymphoid tissue, a major site of replication *in vivo*. *J Virol* 76(3), 1004-14.
- Campbell, E.M., Perez, O., Anderson, J.L. and Hope, T.J. (2008) Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha. *J Cell Biol* 180(3), 549-61.
- Chakrabarti, L.A. and Simon, V. (2010) Immune mechanisms of HIV control. *Curr Opin Immunol* 22(4), 488-96.
- Cowan, S., Hatzioannou, T., Cunningham, T., Muesing, M.A., Gottlinger, H.G. and Bieniasz, P.D. (2002) Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc Natl Acad Sci U S A* 99(18), 11914-9.
- Cullen, B.R. (2006) Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J Virol* 80(3), 1067-76.
- Diaz-Griffero, F., Li, X., Javanbakht, H., Song, B., Welikala, S., Stremlau, M. and Sodroski, J. (2006) Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology* 349(2), 300-15.
- Diaz-Griffero, F., Perron, M., McGee-Estrada, K., Hanna, R., Maillard, P.V., Trono, D. and Sodroski, J. (2008) A human TRIM5alpha B30.2/SPRY domain mutant gains the ability to restrict and prematurely uncoat B-tropic murine leukemia virus. *Virology* 378(2), 233-42.
- Diaz-Griffero, F., Qin, X.R., Hayashi, F., Kigawa, T., Finzi, A., Sarnak, Z., Lienlaf, M., Yokoyama, S. and Sodroski, J. (2009) A B-box 2 surface patch important for TRIM5alpha self-association, capsid binding avidity, and retrovirus restriction. *J Virol* 83(20), 10737-51.

- Evans, D.T., Serra-Moreno, R., Singh, R.K. and Guatelli, J.C. (2010) BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends Microbiol* 18(9), 388-96.
- Forshey, B.M., Shi, J. and Aiken, C. (2005) Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells. *J Virol* 79(2), 869-75.
- Ganser-Pornillos, B.K., Chandrasekaran, V., Pornillos, O., Sodroski, J.G., Sundquist, W.I. and Yeager, M. (2011) Hexagonal assembly of a restricting TRIM5alpha protein. *Proc Natl Acad Sci U S A* 108(2), 534-9.
- Ganser-Pornillos, B.K., von Schwedler, U.K., Stray, K.M., Aiken, C. and Sundquist, W.I. (2004) Assembly properties of the human immunodeficiency virus type 1 CA protein. *J Virol* 78(5), 2545-52.
- Ganser, B.K., Li, S., Klishko, V.Y., Finch, J.T. and Sundquist, W.I. (1999) Assembly and analysis of conical models for the HIV-1 core. *Science* 283(5398), 80-3.
- Goffinet, C., Allespach, I., Homann, S., Tervo, H.M., Habermann, A., Rupp, D., Oberbremer, L., Kern, C., Tibroni, N., Welsch, S., Krijnse-Locker, J., Banting, G., Krausslich, H.G., Fackler, O.T. and Keppler, O.T. (2009) HIV-1 antagonism of CD317 is species specific and involves Vpu-mediated proteasomal degradation of the restriction factor. *Cell Host Microbe* 5(3), 285-97.
- Groot, F., Welsch, S. and Sattentau, Q.J. (2008) Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. *Blood* 111(9), 4660-3.
- Haller, C. and Fackler, O.T. (2008) HIV-1 at the immunological and T-lymphocytic virological synapse. *Biol Chem* 389(10), 1253-60.
- Ikeda, Y., Ylinen, L.M., Kahar-Bador, M. and Towers, G.J. (2004) Influence of gag on human immunodeficiency virus type 1 species-specific tropism. *J Virol* 78(21), 11816-22.
- Kambal, A., Mitchell, G., Cary, W., Gruenloh, W., Jung, Y., Kalomoiris, S., Nacey, C., McGee, J., Lindsey, M., Fury, B., Bauer, G., Nolte, J.A. and Anderson, J.S. (2011) Generation of HIV-1 resistant and functional macrophages from hematopoietic stem cell-derived induced pluripotent stem cells. *Mol Ther* 19(3), 584-93.
- Kar, A.K., Diaz-Griffero, F., Li, Y., Li, X. and Sodroski, J. (2008) Biochemical and biophysical characterization of a chimeric TRIM21-TRIM5alpha protein. *J Virol* 82(23), 11669-81.

- Langelier, C.R., Sandrin, V., Eckert, D.M., Christensen, D.E., Chandrasekaran, V., Alam, S.L., Aiken, C., Olsen, J.C., Kar, A.K., Sodroski, J.G. and Sundquist, W.I. (2008) Biochemical characterization of a recombinant TRIM5alpha protein that restricts human immunodeficiency virus type 1 replication. *J Virol* 82(23), 11682-94.
- Li, Y., Li, X., Stremlau, M., Lee, M. and Sodroski, J. (2006) Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *J Virol* 80(14), 6738-44.
- Lienlaf, M., Hayashi, F., Di Nunzio, F., Tochio, N., Kigawa, T., Yokoyama, S. and Diaz-Griffero, F. (2011) Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha. *J Virol* 85(17), 8725-37.
- Mangeat, B., Gers-Huber, G., Lehmann, M., Zufferey, M., Luban, J. and Piguet, V. (2009) HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathog* 5(9), e1000574.
- Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H. and Landau, N.R. (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114(1), 21-31.
- Marin, M., Rose, K.M., Kozak, S.L. and Kabat, D. (2003) HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* 9(11), 1398-403.
- Mehle, A., Strack, B., Ancuta, P., Zhang, C., McPike, M. and Gabuzda, D. (2004) Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J Biol Chem* 279(9), 7792-8.
- Naviaux, R.K., Costanzi, E., Haas, M. and Verma, I.M. (1996) The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* 70(8), 5701-5.
- Ohkura, S., Yap, M.W., Sheldon, T. and Stoye, J.P. (2006) All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction. *J Virol* 80(17), 8554-65.
- Owens, C.M., Yang, P.C., Gottlinger, H. and Sodroski, J. (2003) Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol* 77(1), 726-31.

- Perez-Caballero, D., Hatzioannou, T., Yang, A., Cowan, S. and Bieniasz, P.D. (2005a) Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. *J Virol* 79(14), 8969-78.
- Perez-Caballero, D., Hatzioannou, T., Zhang, F., Cowan, S. and Bieniasz, P.D. (2005b) Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *J Virol* 79(24), 15567-72.
- Perron, M.J., Stremlau, M., Lee, M., Javanbakht, H., Song, B. and Sodroski, J. (2007) The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. *J Virol* 81(5), 2138-48.
- Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. and Ferrin, T.E. (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* 25(13), 1605-12.
- Pham, Q.T., Bouchard, A., Grutter, M.G. and Berthoux, L. (2010) Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. *Gene Ther* 17(7), 859-71.
- Richardson, M.W., Carroll, R.G., Stremlau, M., Korokhov, N., Humeau, L.M., Silvestri, G., Sodroski, J. and Riley, J.L. (2008) Mode of transmission affects the sensitivity of human immunodeficiency virus type 1 to restriction by rhesus TRIM5alpha. *J Virol* 82(22), 11117-28.
- Rold, C.J. and Aiken, C. (2008) Proteasomal degradation of TRIM5alpha during retrovirus restriction. *PLoS Pathog* 4(5), e1000074.
- Rossi, J.J., June, C.H. and Kohn, D.B. (2007) Genetic therapies against HIV. *Nat Biotechnol* 25(12), 1444-54.
- Roy, A., Kucukural, A. and Zhang, Y. (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5(4), 725-38.
- Rudnicka, D., Feldmann, J., Porrot, F., Wietgreffe, S., Guadagnini, S., Prevost, M.C., Estaquier, J., Haase, A.T., Sol-Foulon, N. and Schwartz, O. (2009) Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses. *J Virol* 83(12), 6234-46.
- Sakuma, R., Noser, J.A., Ohmine, S. and Ikeda, Y. (2007) Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. *Nat Med* 13(5), 631-5.

- Sawyer, S.L., Wu, L.I., Emerman, M. and Malik, H.S. (2005) Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8), 2832-7.
- Sebastian, S. and Luban, J. (2005) TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* 2, 40.
- Sebastian, S., Sokolskaja, E. and Luban, J. (2006) Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologs in a cell type-dependent manner. *J Virol* 80(4), 2051-4.
- Shi, J. and Aiken, C. (2006) Saturation of TRIM5 alpha-mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid. *Virology* 350(2), 493-500.
- Song, B., Gold, B., O'Huigin, C., Javanbakht, H., Li, X., Stremlau, M., Winkler, C., Dean, M. and Sodroski, J. (2005) The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates. *J Virol* 79(10), 6111-21.
- Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P. and Sodroski, J. (2004) The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977), 848-53.
- Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I. and Sodroski, J. (2006) Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* 103(14), 5514-9.
- Stremlau, M., Perron, M., Welikala, S. and Sodroski, J. (2005) Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *J Virol* 79(5), 3139-45.
- Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M. and Mothes, W. (2008) TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *PLoS Pathog* 4(2), e16.
- Wu, X., Anderson, J.L., Campbell, E.M., Joseph, A.M. and Hope, T.J. (2006) Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proc Natl Acad Sci U S A* 103(19), 7465-70.
- Yap, M.W., Nisole, S. and Stoye, J.P. (2005) A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol* 15(1), 73-8.

- Zhang, F., Perez-Caballero, D., Hatzioannou, T. and Bieniasz, P.D. (2008) No effect of endogenous TRIM5alpha on HIV-1 production. *Nat Med* 14(3), 235-6; author reply 236-8.
- Zhang, Y. (2008) I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9, 40.
- Zhao, G., Ke, D., Vu, T., Ahn, J., Shah, V.B., Yang, R., Aiken, C., Charlton, L.M., Gronenborn, A.M. and Zhang, P. (2011) Rhesus TRIM5alpha disrupts the HIV-1 capsid at the inter-hexamer interfaces. *PLoS Pathog* 7(3), e1002009.
- Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L. and Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat Biotechnol* 15(9), 871-5.

CHAPITRE III

THE V86M MUTATION IN HIV-1 CAPSID CONFERS RESISTANCE TO TRIM5 α BY ABROGATION OF CYCLOPHILIN A-DEPENDENT RESTRICTION AND ENHANCEMENT OF VIRAL NUCLEAR IMPORT

MAXIME VEILLETTE, KATSIARYNA BICHEL¹, PAULINA PAWLICA,
STEFAN M.V. FREUND¹, MÉLODIE B. PLOURDE, QUANG TOAN PHAM,
CARLOS REYES-MORENO, LEO C. JAMES¹, LIONEL BERTHOUX

Publié dans *Retrovirology*, le 28 février 2013.

Résumé

Le VIH-1 est inhibé rapidement après l'entrée du virus dans les cellules qui expriment certains orthologues simiens de TRIM5 α . Des mutants de l'orthologue humain (TRIM5 α_{Hu}) peuvent également induire une protection contre le VIH-1. Une protéine de l'hôte, la cyclophiline A (CypA), se lie aux protéines de la capsid (CA) virale et favorise les étapes précoces de la réplication du VIH-1 par des mécanismes encore inconnus. D'un autre côté, l'interaction CA-CypA augmente la susceptibilité du VIH-1 à la restriction par TRIM5 α . Préalablement, la mutation V86M dans la boucle de liaison à CypA de la CA du VIH-1 a été sélectionnée après des passages en série du VIH-1 dans des cellules exprimant TRIM5 α du singe rhésus (TRIM5 α_{Rh}). Les objectifs de cette étude étaient de (i) analyser si V86M permet au VIH-1 d'échapper aux mutants de TRIM5 α_{Hu} , et (ii) de caractériser le rôle de CypA dans la résistance à TRIM5 α par V86M.

¹ Medical Research Council Laboratory of Molecular Biology, Division of Protein and Nucleic Acid Chemistry, Hills Road, Cambridge, Angleterre.

Nos résultats montrent que dans des expériences de transduction de vecteurs VIH-1 non-répliquatifs, V86M confère une résistance partielle contre TRIM5 α_{Hu} R332G-R335G et d'autres mutants de la région variable 1 qui ont été isolés précédemment. Par contre, V86M ne semble pas être résistant à TRIM5 α_{Hu} R332G-R335G dans un contexte de propagation virale. De manière surprenante, la restriction des vecteurs VIH-1 V86M par les mutants de TRIM5 α_{Hu} est surtout insensible à la présence de CypA dans les cellules infectées. Des expériences de RMN montrent que V86M modifie l'interaction CypA-CA ainsi que l'isomérisation de CA par CypA. D'un autre côté, V86M n'affecte pas l'augmentation de la réplication du VIH-1 médiée par CypA dans les cellules humaines. Finalement, des expériences de qPCR montrent que V86M augmente le transport de l'ADN viral au noyau des cellules exprimant un TRIM5 α restrictif.

Somme toute, notre étude montre que V86M sépare les deux fonctions normalement associées avec l'interaction CA-CypA. V86M favorise les étapes précoces de la réplication du VIH-1 dans des cellules restrictives en améliorant le transport nucléaire. Nos résultats suggèrent donc que V86M échappe à la restriction médiée par TRIM5 α en empêchant sélectivement l'augmentation CypA-dépendante du blocage de l'importation nucléaire médié par TRIM5 α .

Abstract

Background: HIV-1 is inhibited early after entry into cells expressing some simian orthologues of the tripartite motif protein family member TRIM5 α . Mutants of the human orthologue (TRIM5 α_{hu}) can also provide protection against HIV-1. The host protein cyclophilin A (CypA) binds incoming HIV-1 capsid (CA) proteins and enhances early stages of HIV-1 replication by unknown mechanisms. On the other hand, the CA-CypA interaction is known to increase HIV-1 susceptibility to restriction by TRIM5 α . Previously, the mutation V86M in the CypA-binding loop of HIV-1 CA was found to be selected upon serial passaging of HIV-1 in cells expressing Rhesus macaque TRIM5 α (TRIM5 α_{rb}). The objectives of this study were (i) to analyze whether

V86M CA allows HIV-1 to escape mutants of TRIM5 α_{hu} , and (ii) to characterize the role of CypA in the resistance to TRIM5 α conferred by V86M.

Results: We find that in single-cycle HIV-1 vector transduction experiments, V86M confers partial resistance against R332G-R335G TRIM5 α_{hu} and other TRIM5 α_{hu} variable 1 region mutants previously isolated in mutagenic screens. However, V86M HIV-1 does not seem to be resistant to R332G-R335G TRIM5 α_{hu} in a spreading infection context. Strikingly, restriction of V86M HIV-1 vectors by TRIM5 α_{hu} mutants is mostly insensitive to the presence of CypA in infected cells. NMR experiments reveal that V86M alters CypA interactions with, and isomerisation of CA. On the other hand, V86M does not affect the CypA-mediated enhancement of HIV-1 replication in permissive human cells. Finally, qPCR experiments show that V86M increases HIV-1 transport to the nucleus of cells expressing restrictive TRIM5 α .

Conclusions: Our study shows that V86M de-couples the two functions associated with CA-CypA binding, i.e. the enhancement of restriction by TRIM5 α and the enhancement of HIV-1 replication in permissive human cells. V86M enhances the early stages of HIV-1 replication in restrictive cells by improving nuclear import. In summary, our data suggest that HIV-1 escapes restriction by TRIM5 α through the selective disruption of CypA-dependent, TRIM5 α -mediated inhibition of nuclear import. However, V86M does not seem to relieve restriction of a spreading HIV-1 infection by TRIM5 α_{hu} mutants, underscoring context-specific restriction mechanisms.

Background

TRIM5 α was isolated as a retroviral restriction factor in 2004 [1] and acts within the post-entry, pre-integration window [2, 3]. The viral molecular target of TRIM5 α is the correctly matured N-terminal domain of capsid (CA) proteins forming the outer surface of the retroviral core [2, 4-8]. A direct interaction between the two proteins, each present as high molecular weight multimers, occurs shortly after entry and is required

for downstream inhibition of viral replication [8-12]. The mechanism of TRIM5 α -mediated restriction can be broken down to discrete events, some of them interdependent: (i) virus entrapment into TRIM5 α cytoplasmic bodies [13], (ii) decreased stability of the virus core [8, 14-16], (iii) targeting to a proteasome-dependent degradation pathway [17-20], and (iv) inhibition of nuclear transport [17, 21, 22].

CypA, a host peptidyl-prolyl *cis/trans* isomerase that is ubiquitously expressed in tissues, is known to play roles in both HIV-1 infection of human cells and in HIV-1 restriction by TRIM5 α in monkey cells. In dividing permissive human cells, CypA enhances HIV-1 infectivity by regulating the disassembly of its core [23-25] independently of TRIM5 α [26, 27]. On the other hand, restriction of HIV-1 by simian TRIM5 α orthologues is enhanced by CypA, and inhibition of CypA expression or of its activity partially rescues infectivity in restrictive conditions [21, 26, 28-30]. CypA binds to an exposed proline-rich loop on the viral CA [31, 32] and catalyzes the isomerisation of the peptide bond G89-P90 [33, 34]. The mutation V86M in the CypA-binding loop of HIV-1 CA has been identified as conferring partial resistance to TRIM5 α_{Rh} [35]. The mechanism of HIV-1 resistance to TRIM5 α_{Rh} conferred by V86M CA was not addressed in this study. However, it was established that this mutation in the CypA-binding loop did not disrupt CA-CypA interactions *in vitro* or in cell cultures [35].

We and others have proposed that point mutations in the variable region 1 (v1) of TRIM5 α_{Hu} could confer HIV-1 restriction capability [36-41]. These mutations were discovered by mapping of HIV-1 restriction determinants in non-human TRIM5 α orthologues [36, 39-41] or through the use of random mutagenesis-based screens [38, 42]. Such antiviral genes are promising candidates for gene therapy applications, owing to a few key characteristics: (i) They block replication early after virus entry and before integration can occur; (ii) They are human-like and thus probably nonimmunogenic; (iii) They inhibit HIV-1 by a well-established, natural mechanism with little side effect expected. However, it is currently unknown whether HIV-1 can acquire resistance to TRIM5 α_{Hu} mutants. Here we investigate the extent and mechanism of resistance of the HIV-1 CA mutant V86M to R332G-R335G TRIM5 α_{Hu} and other mutants of the

v1 domain. Our data show that this mutation affects physical and functional interactions with CypA in order to decrease HIV-1 sensitivity to TRIM5 α while retaining replication-enhancement functions also conferred by CypA binding.

Results

CA-V86M HIV-1 is partially resistant to restriction by TRIM5 α_{Hu} mutant R332G-R335G

We analyzed whether V86M could protect HIV-1 against restriction by TRIM5 α_{Hu} mutant R332G-R335G in human TE671, human Sup-T1 and feline CRFK cells. The choice of human cells as an experimental model was justified by the need to gather data in cells representative of the HIV-1 natural host, while the analysis in cat cells was prompted by the absence of an endogenous TRIM5 α protein in this species, thus allowing us to analyse restriction in the absence of potentially interfering effects from the endogenous gene. All the cell lines created were challenged by wild-type (WT) or V86M HIV-1 NL43-based vectors (Figure 3.1). The two vectors had almost identical titers in non-restrictive human or cat cells. In addition, when those vector preparations were normalized according to reverse transcriptase activity or titer in CRFK cells, they were found to have similar titers in activated human lymphocytes or in human M0, M1 and M2 macrophages (Figure 3.9S). However, V86M HIV-1_{NL-GFP} was about 4-times more infectious than its WT counterpart in cells expressing R332G-R335G TRIM5 α_{Hu} , and this was true in all the three cell lines tested (Figure 3.1). In other words, restriction of V86M HIV-1_{NL-GFP} was 4-times less efficient than restriction of WT HIV-1_{NL-GFP}.

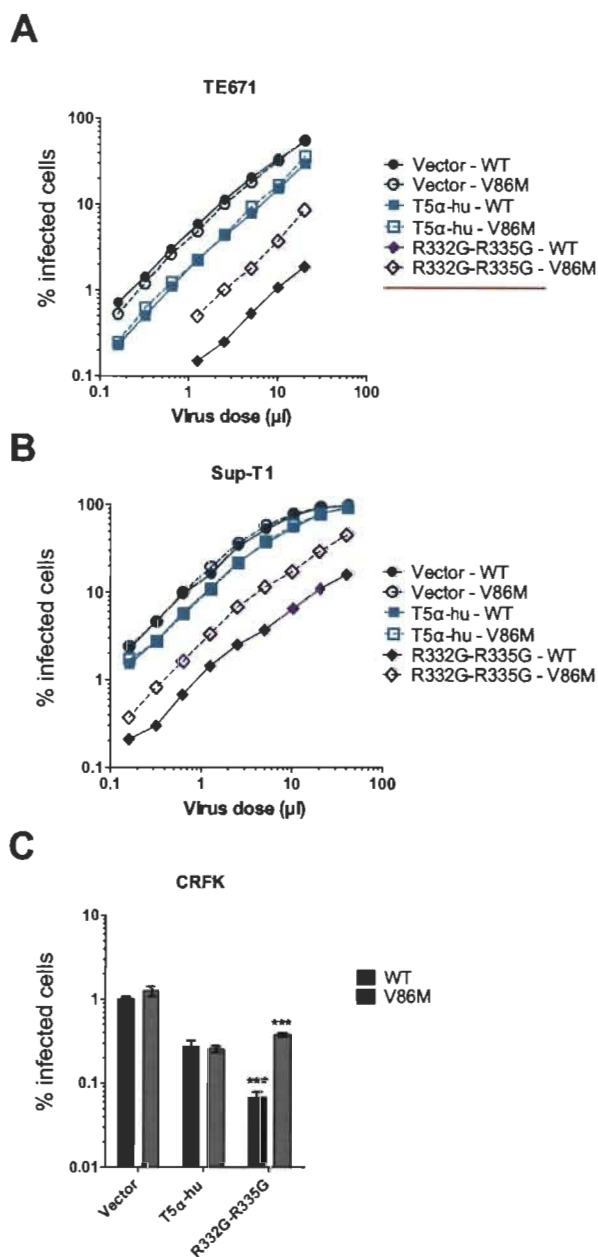


Figure 3.1 V86M mutation in HIV-1 capsid confers partial resistance against R332G-R335G TRIM5 α _{Hu}.

TE671 (A), Sup-T1 (B) and CRFK (C) cells were transduced with either WT TRIM5 α _{Hu}, R332G-R335G TRIM5 α _{Hu}, or with the “empty” vector as a control. Following antibiotic selection, cells were infected with multiple doses (A, B) or with a single dose (C) of WT or V86M HIV-1NL-GFP. The two viral preparations had similar virion content as determined by RT assay. The percentage of infected (GFP-positive) cells was determined two days later by flow cytometry. In (C), infections were done at a multiplicity of infection of ~ 0.1 as determined for the WT virus in parental cells. Bars show the average data from 3 infections with standard deviations (***, P-value < 0.0001).

Restriction of V86M CA HIV-1 by R332G-R335G TRIM5 α_{Hu} is independent of cyclophilin A

Although V86 is located in the CypA-binding loop of HIV-1 CA, this mutant was previously shown to retain CA binding to CypA [35]. Accordingly, transduction of lymphocytes and macrophages by V86M HIV-1 was significantly affected by treatment by 5 μ M of cyclosporine A (CsA), a drug that competes with CA for binding to CypA (Figure 3.9S). We nonetheless hypothesized that the mutation might affect functional interactions between CypA, TRIM5 α and CA. To directly analyze the role of CypA in V86M-mediated resistance, we knocked down its expression in TE671 and in Sup-T1 cells that had been transduced with WT or R332G-R335G TRIM5 α_{Hu} or with the empty vector. The knockdown was efficient for all the TRIM5 variants and in both cell contexts (Figure 3.10S). Non-transduced cells were eliminated by antibiotic treatment, and cells were then challenged with either WT or V86M NL4-3 vectors in the presence or absence of 2 μ M CsA (Figure 3.2). In control permissive cells, replication of both WT and V86M HIV-1_{NL-GFP} was decreased (25 to 35%) either by expression of the CypA shRNA or by CsA treatment [25, 43] (Figure 3.2, left panels). As expected, combining CsA treatment and CypA knockdown had no additional inhibitory effect on HIV-1 replication in the two cell lines, confirming that CsA inhibits incoming HIV-1 in human cells by interfering with its interactions with CypA [25, 27, 43]. In TE671 and Sup-T1 cells expressing R332G-R335G TRIM5 α_{Hu} , replication of WT HIV-1 was strongly reduced compared with the control “vector” cells (~50-fold and 20-fold, respectively) and like before, V86M partly rescued infection of HIV-1 in these cells (5-fold in TE671 cells, 2.5-fold in Sup-T1 cells). CypA knockdown enhanced infectivity of WT HIV-1_{NL-GFP} by 5-fold in TE671 cells and by 3-fold in Sup-T1 cells, but had no significant effect on the replication of the V86M mutant. Likewise, CsA treatment strongly increased replication of WT HIV-1_{NL-GFP} in R332G-R335G TRIM5 α_{Hu} cells (about 10-times in TE671, 5-times in Sup-T1 cells) but had a much smaller effect on V86M HIV-1_{NL-GFP} (1.5- to 2-fold in both cell lines). Therefore, replication of V86M CA HIV-1 in cells expressing R332G-R335G TRIM5 α_{Hu} was not efficiently rescued by depletion or inhibition of CypA.

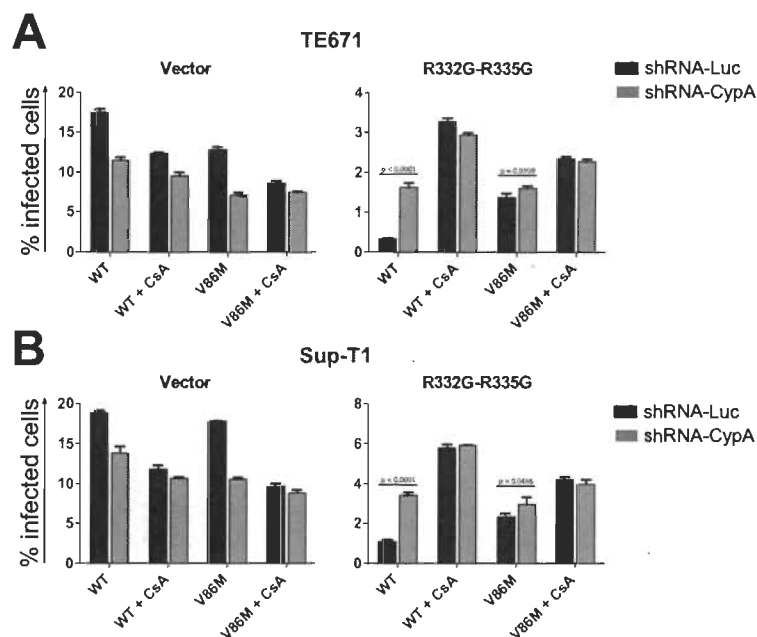


Figure 3.2 Restriction of CA-V86M HIV-1 by R332G-R335G TRIM5 α_{Hu} is independent of cyclophilin A.

TE671 cells (A) and Sup-T1 cells (B) transduced with either WT TRIM5 α_{Hu} , R332G-R335G TRIM5 α_{Hu} or with the “empty” vector were also transduced with shRNAs targeting CypA or the non-relevant luciferase mRNAs. Following antibiotic selection, cells were challenged with a single dose of WT or V86M HIV-1_{NL-GFP}, in the presence or absence of 2 μ M CsA. A multiplicity of infection of \sim 0.2 was used, as determined for the WT virus in parental cells and in the absence of drug. WT and V86M virus preparations were adjusted by RT assay. Cells were analyzed by FACS two days after infection, and the bars show the average % infected cells from 3 infections with standard deviations. Note the difference in scale between the y-axis of different graphs.

CsA concentration-dependent assays

In this experimental setting, instead of using a single volume of HIV-1_{NL-GFP} for all infections as in Figure 3.2, we adjusted the amount of virus used for each virus/cell combination so that approximately 1% of cells were infected in the absence of drug, and we then repeated the infection in presence of increasing CsA concentrations (Figure 3.3). Like before, CsA decreased replication of both WT and V86M HIV-1_{NL-GFP} in the permissive control “vector” cells by 30% to 50% in both TE671 cells (Figure 3.3A) and Sup-T1 cells (Figure 3.3B). CsA rescued replication of WT HIV-1_{NL-GFP} in cells expressing R332G-R335G TRIM5 α_{Hu} , by up to 6-fold in TE671 cells and

8-fold in Sup-T1 cells. In contrast, the impact of the drug on the restriction of the V86M mutant by this variant of TRIM5 α_{Hu} was much smaller: up to 1.5-fold and 2-fold in TE671 and Sup-T1 cells, respectively.

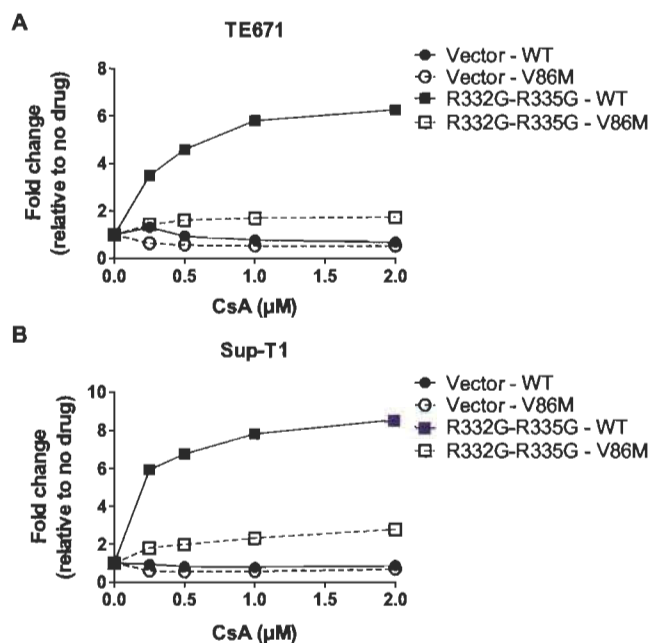


Figure 3.3 CsA dose-dependent analysis. TE671 cells (A) and Sup-T1 cells (B) expressing R332G-R335G TRIM5 α_{Hu} or transduced with the “empty” vector were challenged with WT or V86M HIV-1_{NL-GFP} in the presence of increasing concentrations of CsA. Virus doses were adjusted so that approximately 1% of cells were infected in the absence of CsA. The percentages of infected cells were determined two days later as before and results are shown as –fold increases relative to the relevant no-drug controls.

Altogether, data in Figure 3.2 and Figure 3.3 show that restriction of V86M HIV-1 replication by R332G-R335G TRIM5 α_{Hu} is poorly sensitive to the presence of CypA compared to restriction of the WT control. However, replication of V86M HIV-1 in permissive human cells is as much sensitive as WT HIV-1 to the presence of CypA.

The sensitivity of HIV-1 restriction by TRIM5 α_{Hu} mutants to the V86M mutation correlates with its sensitivity to CsA treatment

Data shown in Figure 3.1 and Figure 3.2 revealed that restriction of HIV-1 by R332G-R335G TRIM5 α_{Hu} was counteracted either by the V86M mutation or by CsA treatment, and suggested that the two interventions had redundant effects. In order to better characterize the role of CypA in the TRIM5 α resistance conferred by V86M, we analyzed the effect of this mutation on other mutants of TRIM5 α_{Hu} . We also analyzed the effect of CsA treatment on restriction by these same mutants. G330E, R332G, R335G, and G330E-R332G-R335G TRIM5 α_{Hu} all restricted HIV-1 when expressed in TE671 cells, albeit with various efficacies (Figure 3.11SA), and as shown before [38, 42]. Replication of HIV-1_{NL-GFP} in these cells was enhanced either by introducing the V86M mutation (Figure 3.11SA) or by CsA treatment (Figure 3.11SB). When we plotted the increase in HIV-1_{NL-GFP} infectivity resulting from V86M against the increase in infectivity resulting from CsA treatment in TE671 expressing these various TRIM5 α_{Hu} , we observed a highly significant correlation (Figure 3.4). Also, the level of rescue of HIV-1_{NL-GFP} infectivity by either V86M mutation or CsA treatment was grossly proportional to the level of restriction. This analysis supports our findings that V86M and CsA treatment rescue HIV-1 replication in restrictive conditions by similar mechanisms. However, the slope of the linear curve (1.89) also shows that CsA is more efficient than V86M at rescuing HIV-1 replication.

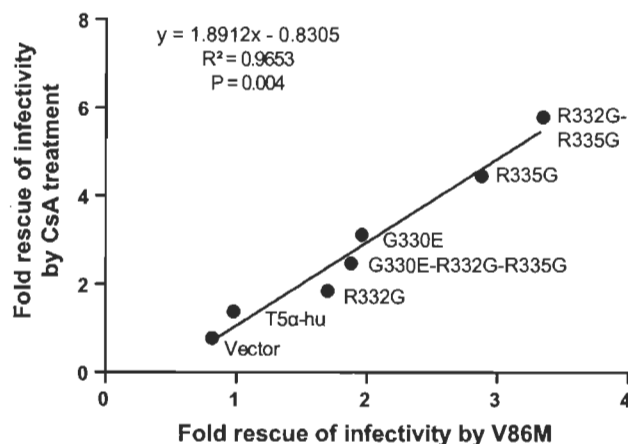


Figure 3.4 Direct correlation between the effect of mutation V86M and the effect of CsA treatment.

The increase in HIV-1 infectivity in TE671 cells expressing the indicated TRIM5 α_{Hu} in presence of 2 μ M CsA was plotted against the increase in infectivity resulting from the V86M mutation. All values are -fold increases relative to the untreated WT control virus. Data were gathered at a multiplicity of infection yielding close to 1% infected cells for the untreated WT HIV-1 in the cell lines expressing each the various TRIM5 α . The correlation is significant, as shown by the R^2 obtained and the Spearman's P-value. The linear regression equation is shown.

Restriction of V86M CA HIV-1 by R332G-R335G TRIM5 α_{Hu} in cat cells is insensitive to CsA

In human cells expressing an HIV-1-restrictive TRIM5 α , CsA treatment might cause dual, opposite effects on HIV-1: enhancement by counteracting TRIM5 α , and inhibition by a yet unclear, CypA-dependent mechanism. In addition, although endogenous TRIM5 α_{Hu} is thought to be recessive against the over-expressed TRIM5 α in our experimental system, there was still a slight possibility of interference effects. To address these issues, we analyzed the rescue of HIV-1 replication by V86M mutation and/or by CsA treatment under restriction by R332G-R335G TRIM5 α_{Hu} in CRFK cat fibroblasts. These cells do not express any TRIM5 α , and CsA treatment does not inhibit WT HIV-1 infection in cat cells [26, 44]. We found that R332G-R335G TRIM5 α_{Hu} strongly restricted HIV-1 $_{NL-GFP}$, and the mutation V86M in CA decreased restriction by about 3-fold (Figure 3.5). WT HIV-1 $_{NL-GFP}$ replication was enhanced 10-times by CsA treatment while the effect of the drug on the replication of V86M HIV-1 $_{NL-GFP}$ was much smaller (1.5-fold), thus mirroring the data obtained in human cells.

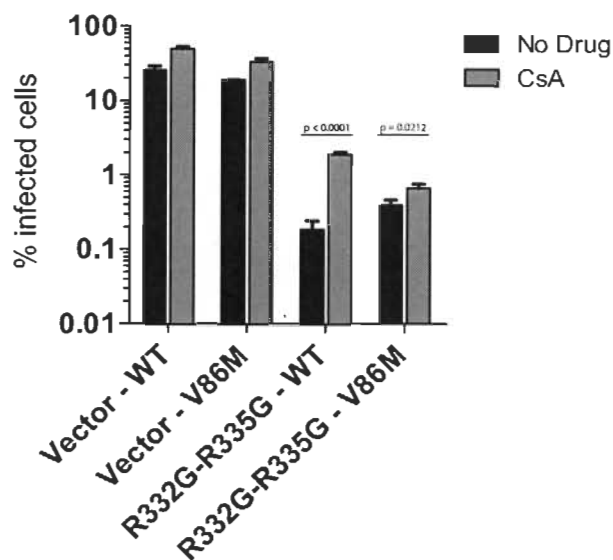


Figure 3.5 V86M HIV-1 restriction by R332G-R335G TRIM5 α_{Hu} in cat cells is not counteracted by CsA treatment.

CRFK cells were transduced with R332G-R335G TRIM5 α_{Hu} or with the empty vector as control and subsequently challenged with a single dose of WT or V86M HIV-1_{NL-GFP} in presence or absence of 2 μ M CsA. Infections were done at a multiplicity of infection of \sim 0.2 as determined in parental cells infected with the WT virus, and the amounts of viruses used were normalized by RT assay. The percentages of infected cells were determined two days later by FACS.

V86M CA interactions with CypA studied by NMR

Data in Figures 3.1-3.5 showed that V86M made HIV-1 resistant to CypA-dependent, TRIM5 α -mediated restriction mechanisms. Thus, we hypothesized that V86M could modify the interactions between HIV-1 CA and CypA, or the isomerisation of the former by the latter [33, 45]. The interaction of CA with CypA was studied by 2D 1H , ^{15}N ZZ-exchange NMR spectroscopy (Figure 3.6). In the absence of CypA, we observed two G89 N-H correlation peaks in the V86M capsid spectra, indicating that, as in WT, the GP bond exists in both *cis* and *trans* states. However, the peak positions for both states in V86M were chemically shifted compared to WT, suggesting that the mutation influences the conformations adopted by the cyclophilin-binding loop (Figure 3.6A). Furthermore, there is evidence of a second minor G89_{*cis*} peak in the V86M spectra that is indicative of discrete CypA-binding loop isomers.

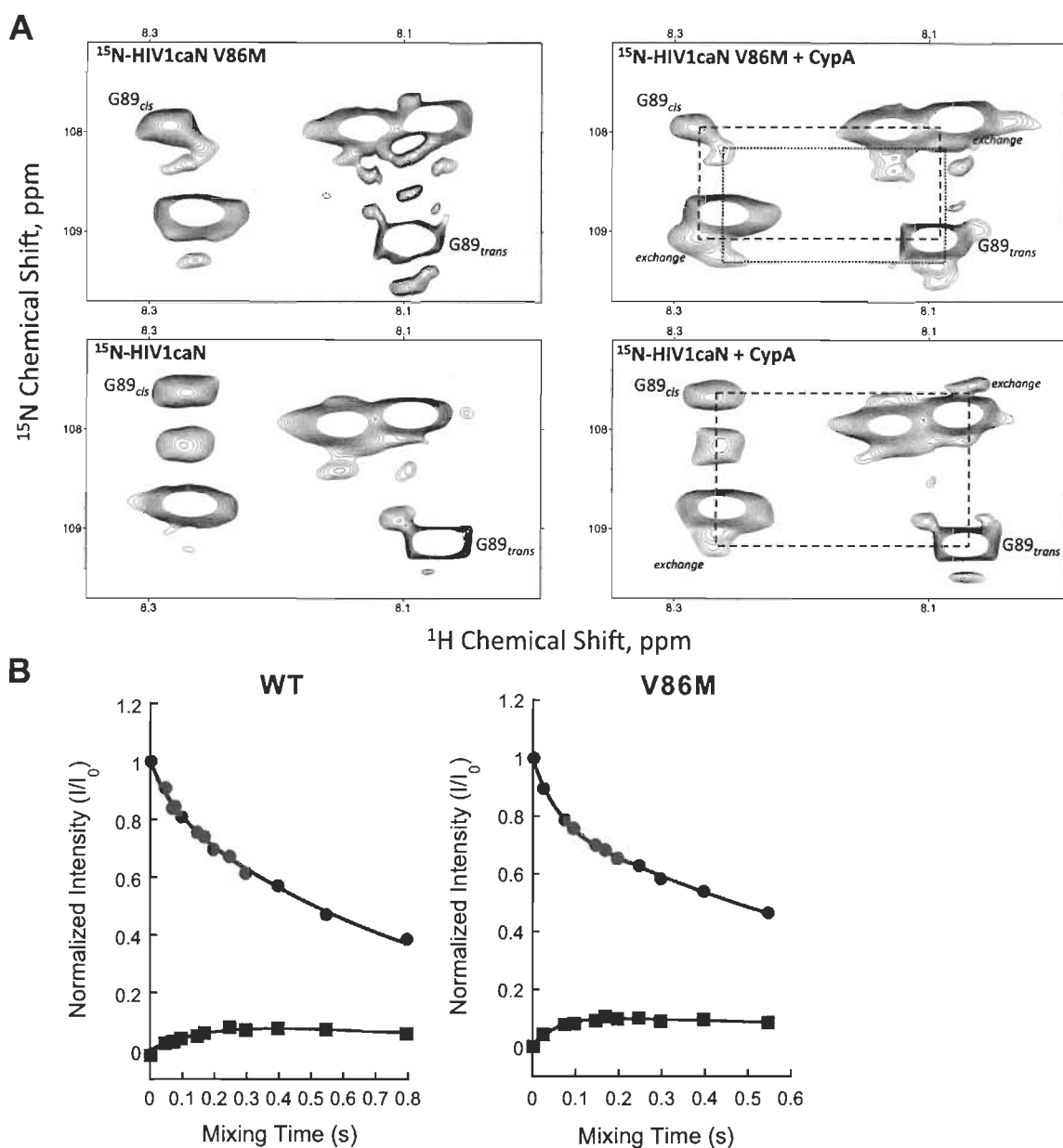


Figure 3.6 V86M CA interactions with CypA studied by NMR. (A) Expansions of 2D ^1H - ^{15}N ZZ-exchange spectra illustrating CypA catalysed *cis-trans* proline isomerisation of P90 in HIV1caN. The four panels represent backbone amide ^1H , ^{15}N correlations for the preceding G89 residue in HIV1caN and in HIV1caN V86M in the presence and absence of CypA. In the absence of CypA, the isomerisation reaction is slow and only distinct G89 *cis*- and *trans*- correlation peaks are observed. The presence of CypA leads to an increase in the isomerisation rate and the accumulation of *cis* and *trans* exchange peaks. The dotted box denotes the location of the exchange peaks and indicates their symmetrical distribution with regard to G89_{*cis*} and G89_{*trans*}. Note that in the case of HIV1caN V86M a broad G89_{*cis*} peak and an additional *cis*-exchange peak indicates a second conformer. All spectra were acquired at the same

mixing time (69 ms) and processed with the same contour levels. (B) Evaluation of exchange rates for CypA catalyzed isomerisation of G89-P90 in HIV1caN and HIV1caN V86M. Normalised peak intensities of *trans* auto peaks and the corresponding exchange peaks for HIV1caN/CypA and HIV1caN V86M/CypA were plotted as a function of mixing time (s). Exchange rates (k_{ex}) in the order of 5 s^{-1} for HIV1caN/CypA and 20 s^{-1} for HIV1caN V86M/CypA were extracted by fitting auto peaks and exchange peaks as described by Bosco et al (2002).

Upon addition of CypA, symmetrical exchange peaks were observed for WT and V86M indicating that both capsids are a substrate for CypA and that increased *cis-trans* exchange occurs during the ZZ-exchange experiment. However, in the V86M mutant, at least two *cis* exchange peaks were observed compared to a single peak in the WT. Furthermore, the intensities and line shapes of these additional peaks were directly affected by addition of CypA and dependent upon the mixing time of the experiment (Figure 3.6B). This supports the hypothesis that the V86M mutation results in distinct populations of conformers for the G89-P90 bond.

Next, we determined the catalysed isomerisation rate of the G89-P90 bond in both WT and V86M capsids in the presence of CypA (Figure 3.6B). For WT capsid we observed a similar rate as previously published, of $\sim 5\text{ s}^{-1}$. For the V86M capsid, the exchange rate for the major G89 peak was $\sim 20\text{ s}^{-1}$. This suggests that the V86M mutation has both altered the conformations adopted by the CypA-binding loop and increased the rate at which it is isomerised. The presence of an additional G89_{*cis*} peak in V86M in the absence of CypA suggests that the mutant capsid may also undergo faster uncatalysed isomerisation, however we were not able to quantify this under the conditions tested.

Effects of V86M on early stages of viral replication

qPCR assays were performed on DNA extracted from TE671 cells expressing either WT TRIM5 α_{Hu} , R332G-R335G TRIM5 α_{Hu} or, as a positive control, TRIM5 α_{Rb} , and then infected with WT or V86M HIV-1_{NL-GFP} (Figure 3.7). We quantified GFP DNA as a marker for late reverse transcription products and 2-LTR circles as a marker for nuclear viral DNA. As expected, accumulation of HIV-1 DNA was reduced in cells

expressing TRIM5 α_{Rh} and R332G-R335G TRIM5 α_{Hu} , and levels of nuclear HIV-1 DNA were even more strongly decreased. This is consistent with HIV-1 replication being impeded by TRIM5 α at both the reverse transcription and nuclear transport stages [17, 22]. The amounts of viral DNAs were decreased further in TRIM5 α_{Rh} cells than in R332G-R335G TRIM5 α_{Hu} cells, consistent with the greater magnitude of restriction conferred by TRIM5 α_{Rh} . V86M had no effect on total DNA levels but significantly increased relative amounts of 2-LTR nuclear DNA species. However, V86M 2-LTR DNA levels in restrictive cells were still reduced 5- to 10-times compared with levels of WT 2-LTR DNA in non-restrictive conditions, as expected from the observation that V86M only partially rescues HIV-1 in restrictive conditions. In summary, escape from restriction by this mutant is associated with increased HIV-1 transport to the nucleus.

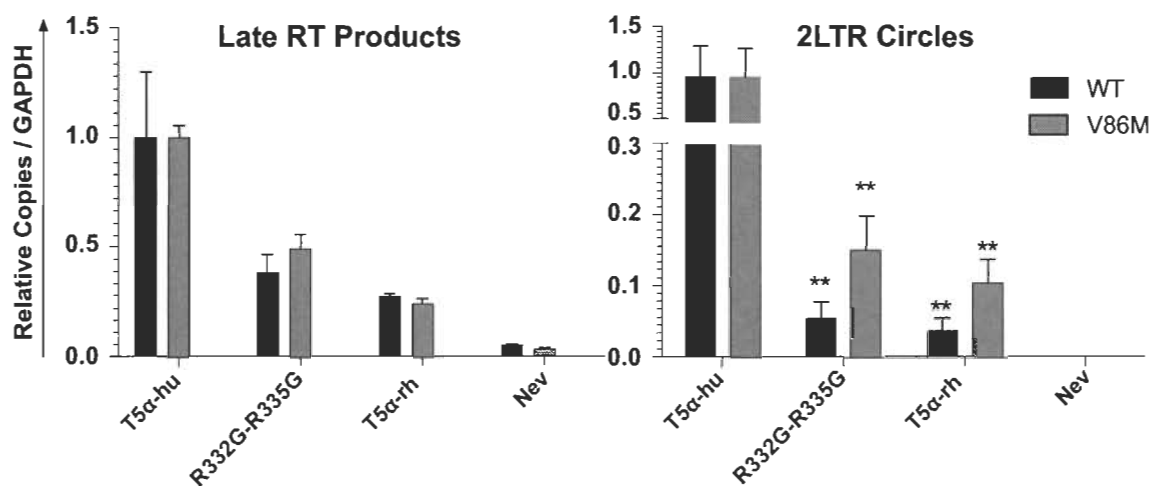


Figure 3.7 V86M enhances nuclear transport of HIV-1 DNA in restrictive conditions.

TE671 cells expressing the indicated TRIM5 α were challenged with WT or V86M HIV-1_{NL-GFP}. An RT inhibitor (nevirapine) control was included to show the absence of contaminating DNA. The levels of HIV-1 late reverse transcription products and 2-LTR circles were determined by real-time quantitative PCR using primers to detect GFP or 2-LTR junctions, respectively. DNA amounts were calculated using serially diluted, linearized DNAs as standards. Data are presented as mean ratio of late RT or 2-LTR DNA copies over the number of GAPDH DNA copies, normalized to the permissive control TRIM5 α_{Hu} . Shown are values with standard deviations of 3 or 5 independent infections (late RT products and 2-LTR, respectively). Student's t-test was used to test for significance of data, specifically WT vs V86M viruses in cells expressing R332G-R335G or TRIM5 α ($p = 0.0020$ and $p = 0.0015$, respectively).

Effect of CypA and V86M on a spreading HIV-1 infection

Single-cycle experiments using HIV-1 vectors are useful to investigate restriction events taking place between entry and integration. However, restriction of fully replicating HIV-1 may be more complex than what is reflected in these assays. Indeed, TRIM5 α may also interfere with late stages of the retroviral cycle [46] although these conclusions have been rebutted [47]. Perhaps even more significantly, HIV-1 propagation occurs mainly through so-called virological synapses forming between cells [48-50]. It is still unclear whether TRIM5 α restricts HIV-1 originating from these viral synapses in the same way that it targets incoming capsids from cell-free viruses, but there are some indications that it does not [51]. In order to investigate the role of CypA and the effect of V86M on HIV-1 restriction in a spreading infection context, we infected Sup-T1 cells with the NL4-3 strain of HIV-1 (Figure 3.8). The cell lines included in the experiment expressed R332G or R332G-R335G TRIM5 α_{Hu} and were co-transduced with the shRNA against CypA or the control shRNA targeting luciferase. The V86M mutation in CA prevented the use of a sensitive CAP24 assay to monitor replication. Instead, we performed a reverse transcriptase (RT) assay [38]. Replication of WT NL4-3 in the control (vector) shRNA-Luc cells peaked at about 18 days post-infection, while replication of the V86M mutant in the same cells peaked at about 15 days. Thus, V86M did not affect the fitness of HIV-1 in Sup-T1 cells. Replication of either WT or V86M NL4-3 was not detectable in the shRNA-Luc cells also expressing R332G-R335G TRIM5 α_{Hu} (Figure 3.8, top panel). Therefore, if the V86M mutation conferred some level of resistance to R332G-R335G TRIM5 α_{Hu} in a spreading infection context, it did not do so at levels high enough to allow detection of replication. In cells expressing R332G TRIM5 α_{Hu} , active replication was detected but only at a single time-point (day 35 post-infection). Replication of V86M NL4-3 was not detected in the same cells, which suggests that V86M does not provide protection against this particular mutant of TRIM5 α_{Hu} in a spreading infection assay. In cells transduced with the shRNA targeting CypA, viral replication was delayed compared to the control shRNA-Luc cells, as expected. This was more obvious for the V86M virus which peaked at about 18 days, confirming that CA-V86M HIV-1 depends on CypA for optimal replication in human cells. Interestingly, replication of WT and V86M NL4-3 in shRNA-CypA/R332G

TRIM5 α_{Hu} -expressing cells was highly increased compared with replication in the shRNA-Luc cells. Specifically, replication of WT NL4-3 peaked at 28 days post-infection, compared to 35 days post-infection in the control cells, and the amount of viruses in the supernatants was also much higher. V86M NL4-3 peaked at about 35 days post-infection in the shRNA-CypA/R332G TRIM5 α_{Hu} -expressing cells while its replication was not detected in the shRNA-Luc/R332G cells. This suggests that CypA knockdown can rescue replication of these two viruses in TRIM5-restrictive conditions and also that V86M NL4-3 is more sensitive to restriction by R332G TRIM5 α_{Hu} , in contradiction with the data obtained in vector transduction experiments. In shRNA-CypA/R332G-R335G TRIM5 α_{Hu} -expressing cells, replication of both WT and V86M NL4-3 was detected, although at low levels. Replication of V86M NL4-3 was no more efficient than that of its WT counterpart in these cells. Altogether, this experiment shows that restriction of a spreading HIV-1 infection by mutant TRIM5 α_{Hu} in Sup-T1 cells is enhanced by CypA, and suggests that the V86M mutation does not rescue HIV-1 replication in this context.

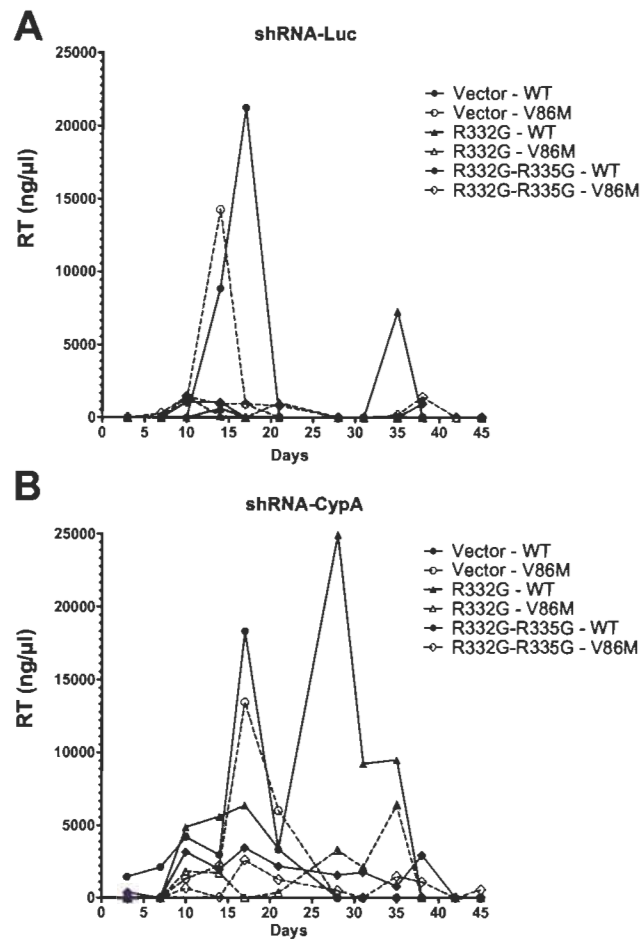


Figure 3.8 Restriction of a spreading CA-V86M HIV-1 infection.

Sup-T1 cells transduced with a control shRNA against luciferase (A) or with an shRNA targeting CypA (B) and expressing the indicated TRIM5 α proteins were infected with an identical dose of WT or V86M HIV-1_{NL4-3} normalized by RT activity. Unbound virus was eliminated 16 h later and infection was allowed to proceed for 45 days. Reverse transcriptase activity in the supernatant of infected cells and of uninfected cells as a control were determined periodically. Background noise as determined from the uninfected cells was subtracted from the other samples at each time-point.

Discussion

HIV-1 resistance to treatment is a hallmark of pharmacological interventions against this virus. Invariably, mutations appear in the coding sequences of the proteins targeted by antiretroviral drugs, including protease, reverse transcriptase and integrase [52]. It is expectable that genetic interventions will similarly lead to the occurrence of

viral resistance. Indeed, restriction of HIV-1 by CPSF6-358, a truncated form of the RNA processing factor, cleavage and polyadenylation specific factor 6 (CPSF6), is counteracted by the mutation N74D in CA [53]. This mutation, isolated by serial passages of HIV-1 in cells expressing CPSF6-358, abrogates direct binding of CA to CPSF6-358 [54]. Several groups, including ours, have produced mutants of TRIM5 α_{Hu} to be used as antiviral transgenes [36, 38-41]. In order to predict potential HIV-1 escape from inhibition mediated by these TRIM5 α_{Hu} variants, it is important to isolate those escape mutants *in vitro*, and to understand by which mechanism they decrease sensitivity to restriction. Our initial efforts had not led us to isolate R332G-R335G TRIM5 α_{Hu} -resistant HIV-1 by serial passaging. We then decided to test whether V86M, an HIV-1 CA mutant isolated from cells expressing TRIM5 α_{Rh} by the Sodroski group and shown to confer some level of protection against this orthologue, would also make HIV-1 resistant to TRIM5 α_{Hu} mutants.

Our data show that V86M indeed confers some level of protection against various mutants of TRIM5 α_{Hu} , at least in the context of single-cycle infections with HIV-1 vectors. We saw no protection in the context of HIV-1 spreading infections, although we tested only one HIV-1 strain (NL4-3) in one cell line (Sup-T1). Sodroski and collaborators [35] have isolated the V86M mutant in HeLa-CD4 cells, which we have not tested here. They observed modest levels (2-fold) of protection against TRIM5 α_{Rh} in these HeLa-derived cells when infectivity was measured in single-cycle assays, and they saw an even more modest effect in canine Cf2Th cells [35]. Altogether, V86M can confer HIV-1 protection against restriction by various TRIM5 α proteins in specific replication settings. However, even in situations in which protection takes place, restriction still occurs, albeit weakened. On the basis of our data, we do not expect V86M to be highly significant in an *in vivo* context, although this is of course rather hazardous to predict.

In order to investigate the mechanism of CA-V86M HIV-1 resistance to R332G-R335G TRIM5 α_{Hu} , we analyzed the role of CypA in the restriction. Altogether, our results show that restriction of V86M CA HIV-1 is largely insensitive to the

presence of functional CypA, while the same virus is still inhibited in human cells devoid of CypA. In other words, HIV-1 is able to subtly alter its interactions with CypA in order to downregulate a mechanism of restriction while preserving other benefits associated with this interaction. Accordingly, our NMR data showed that the molecular interactions between CA and CypA were altered by the V86M mutation, as was the isomerisation reaction catalysed by CypA.

Finally, our qPCR data correlate modifications in CypA-CA interactions with effects on nuclear transport in restrictive conditions. Interestingly, experiments pre-dating the discovery of TRIM5 α restriction had demonstrated that CsA treatment of Old World monkey cells increased nuclear transport of HIV-1 in these cells [21]. More recently, Lin and Emerman similarly observed that CsA treatment of the sMAGI Rhesus macaque cell line increases HIV-1 nuclear transport more than it does enhance reverse transcription [55]. Other recently published data also link nuclear transport and CA-CypA interactions in permissive human cells. Specifically, CypA knockdown and CsA treatment reduce HIV-1 dependency toward nucleopore components Nup153 and Nup358 for its nuclear transport [56, 57]. It should be informative to analyze whether V86M modifies the interactions between HIV-1 CA and these nucleoporins.

Acknowledgements

This work was funded by the Canadian Institutes for Health Research and the Canada Research Chairs program (LB). LCJ and KB were supported by the Medical Research Council [U105181010] and the Darwin Trust of Edinburgh, respectively. MV received a FRQS Master's Training Award.

Methods

Plasmid DNAs

pMIP-TRIM5 α_{Hu} and pMIP-TRIM5 α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before [28, 38, 44, 58, 59]. pMIP-TRIM5 α_{Hu} with the mutation R332G-R335G has been described [38], and additional mutants have been described as well [42]. pSRBI-CypA expresses a short hairpin RNA (shRNA) targeting the human CypA mRNA. Shortly, it is the previously described pSRP-CypA plasmid [27] in which the puromycin resistance cassette has been changed to one conferring resistance to blasticidin. The control plasmid, pSRBI-Luc, encodes a shRNA targeting luciferase [60]. pNL-GFP, pMD-G and pCL-Eco have all been described elsewhere [21, 28, 58, 61-63]. The fully replication-competent HIV-1 clone pNL4-3 [64] was used for propagation experiments.

Cells

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C. Human T lymphoblast Sup-T1 cells and monocytic cells THP-1 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 37°C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA). Human PBMCs were isolated from diluted whole blood of a healthy donor by Ficoll density gradient centrifugation as previously described [65]. CD4⁺ T cells were then isolated using a negative isolation kit (Dynabeads Untouched Human CD4 T Cells, Invitrogen) according to manufacturer specifications. The isolated cells were cultivated in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. Activation and proliferation of CD4⁺ T cells were performed by supplementing the culture medium with 60 U/ml IL-2 (Peprotech, Canada) and incubating the cells with anti-CD3/CD28 coated beads (Dynabeads Human T-Activator CD3/CD28, Invitrogen) according to the manufacturer instructions. Infections of these cells were performed in the absence of

IL-2 and activating beads. THP-1 monocytic cells (7.5×10^5) were differentiated into M0 macrophages by incubation in serum-free medium supplemented with 50 ng/ml PMA and 10 ng/ml GM-CSF for 48 h. Polarization into pro-inflammatory M1 macrophages was induced by addition of 100 ng/ml LPS and 25 U/ml IFN- γ . Anti-inflammatory M2 macrophages were induced by addition of 20 ng/ml IL-4 and 20 ng/ml IL-10 (all reagents were from Peprotech). In all cases, non-adherent cells were washed away before infection.

Retroviral vectors production

HIV-1 and MLV-based vectors were produced through transient transfection of 293T cells and collected as previously described [38, 44]. To produce HIV-1_{NL-GFP}, cells were co-transfected with pNL-GFP and pMD-G. To produce the MLV-based vectors SRBI and MIP, cells were transfected with the relevant pMIP or pSRBI plasmid and co-transfected with pCL-Eco and pMD-G. To produce HIV-1 VLPs, cells were co-transfected with p Δ R8.9 and pMD-G. When necessary, amounts of viruses were normalized using the nonradioactive EnzChek RT Assay Kit (Invitrogen, Burlington, ON), or based on their titer in permissive human cells.

RNA interference

pSRBI-based retroviral vectors encoding an shRNA targeting either CypA or Luc mRNAs were produced in 293T cells as described [28, 38]. TE671 and Sup-T1 cells were plated at 250,000 cells per well in 12-well plates and exposed to 1 mL of either SRBL-CypA or -Luc vector preparation. Two days post-transduction, cells were placed in medium containing 10 μ g/ml of blasticidin (Sigma-Aldrich). Selection was allowed to proceed for 1 week. Efficiency of the knockdown was verified by assaying CypA expression levels in the transduced cells by western blotting, using antibodies directed against CypA (rabbit polyclonal IgG; Sigma-Aldrich, StLouis, MI) and actin (mouse monoclonal; Chemicon International).

Viral challenges

Cells were plated in 24-well plates at 50,000 cells /well (TE671 and CRFK), 100,000 cells/well (Sup-T1), 200,000 cells/well (primary CD4 T cells) or 750,000 cells/well (differentiated THP-1) and infected with HIV-1 vectors (either HIV-1_{TRIP-CMV-GFP} or HIV-1_{NL-GFP}) with or without CsA treatment (2 or 5 μ M). Drugs were added 15 min prior to infections. In CsA dose-dependent experiments, virus doses were adjusted for each virus-cell combination so that approximately 1% of the cells would be infected in the absence of CsA. Two days post-infection, cells were trypsinised when necessary, fixed in 1 to 2% formaldehyde in a PBS solution. The % of GFP-positive cells were then determined by analyzing 10,000 to 20,000 cells with a FC500 MPL cytometer (Beckman Coulter) using the CXP software (Beckman Coulter). For spreading infections, quantification of RT activity in the supernatants of infected cells was done exactly as before [38].

Quantitative real-time PCR of HIV-1 DNA

Cells were plated in 12-well plates at 3×10^5 cells/well and infected with HIV-1_{NL-GFP} WT or V86M vectors. Viruses were passed through 0.45 μ m filters and pretreated for 1 hour at 37°C with 20U/ml DNase I (New England Biolabs) to prevent contamination by carry-over plasmid DNA. In addition, control infections were performed in presence of 80 μ M nevirapine to ascertain the absence of such contaminating plasmid DNA. Total cellular DNA was prepared after 6 hours of infection (late RT products) or 6 hours of infection followed by 18 hours of incubation in virus-free medium (2LTR-circles) and DNA preparation was done using the DNeasy Blood and Tissue Kit (Qiagen, California).

The primer sets to detect each DNA species were as follows: GFP forward, GACGACGGCAACTACAAGAC; GFP reverse, TCGTCCATGCCGAGAGTGAT; 2LTR-circles forward (MH535), AACTAGGGAACCCACTGCTTAAG [66]; 2LTR-circles reverse (MH536), TCCACAGATCAAGGATATCTTGTC; GAPDH forward, GTCAGTGGTGGACCTGACCT; GAPDH reverse, TGAGCTTGACAAAGTGGTCG.

In each experiment, a standard curve of the amplicon being measured was run in duplicate ranging from 30 to 3×10^5 copies plus a no-template control. Reactions contained $1 \times$ SensiFAST SYBR Lo-Rox kit (Bioline, UK), 300nM forward and reverse primers, and 5 μ l template DNA (100-300ng) in 20 μ l-sized reactions. After initial incubation step of 3 min at 95°C, 40 cycles of amplification were carried out at 10s at 95°C, 10s at 62°C (GFP) or 65°C (2LTR, GAPDH) and 10s (GFP) or 15s (2LTR, GAPDH) at 72°C on a MX3000P qPCR system (Agilent Technologies, California). Results were analyzed with the MxPro software (Agilent Technologies).

Statistical analyses

Statistical data were calculated using GraphPad Prism version 5.03. Student's unpaired t-tests were used for tests of difference between means.

2D ^1H - ^{15}N Heteronuclear (ZZ) Exchange Spectroscopy

Uniformly ^{15}N -labeled CA^N domain of HIV1 (^{15}N HIV1caN) was expressed in DE3 bacteria in K-MOPS buffer supplemented with 20 mM $^{15}\text{NH}_4\text{Cl}$ as a sole source of nitrogen, 4 mM pH 8.0 KPOi, 0.1 ‰ sodium ampicillin salt, vitamins and 0.4% glucose. Bacterial culture was grown until 0.500-0.700 OD₆₀₀ at 37°C, induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG), and the protein was expressed at 20°C overnight followed by purification as previously described [67] except that the final step of purification, i.e. gel filtration, was run in 50 mM potassium phosphate, pH 6.5, 100 mM NaCl and 1 mM DTT. Uniformly ^{15}N -labeled CA^N domain of HIV1 V86M (^{15}N HIV1caN V86M) was expressed and purified as per ^{15}N HIV1caN. Non-isotopically labeled N-terminally his₆-tagged human CypA was expressed in DE3 bacteria in 2xTY medium supplemented with 0.6% glucose and 0.1 ‰ sodium ampicillin salt, induced at 0.6-0.8 OD₆₀₀ at 37°C with 1 mM IPTG, expressed at 20°C and purified using Ni-NTA beads (Qiagen) and gel-filtration chromatography in 75 mM Tris pH8.0, 50 mM NaCl and 1 mM DTT buffer. For all NMR experiments, proteins were dialysed against the same 50 mM KPOi, pH 6.5 buffer and supplemented with

1 mM DTT. The capsids were used at 12-fold excess concentration over CypA as previously described [33, 68]: 430 μM CA^N/35 μM Cyp A or 430 μM CA^N only. All NMR samples contained 5% D₂O.

2D ¹H-¹⁵N heteronuclear (ZZ) exchange experiment is described in detail in [69] and was previously applied for a similar model [33, 68]. The experiments were performed on a Bruker 800 MHz spectrometer at 298 K using in-house written pulse program with the following mixing times in a randomised order: 3 ms, 25 ms, 47 ms, 69 ms, 75 ms, 97 ms, 147 ms, 169 ms, 197 ms, 247 ms, 297 ms, 397 ms, 547 ms and 796 ms. The first time-point was acquired twice to assess the error. G89 assignment for ¹⁵N HIV1caN was assumed from ppm chemical shifts referred to in [33, 68]. It was also assumed that chemical shifts for G89 in ¹⁵N HIV1caN V86M did not change dramatically. The data was processed in TopSpin 2.0 (Bruker, Karlsruhe) and after analysis in Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

References

1. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J: **The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys.** *Nature* 2004, **427**:848-853.
2. Owens CM, Yang PC, Gottlinger H, Sodroski J: **Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells.** *J Virol* 2003, **77**:726-731.
3. Perez-Caballero D, Hatzioannou T, Zhang F, Cowan S, Bieniasz PD: **Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity.** *J Virol* 2005, **79**:15567-15572.
4. Cowan S, Hatzioannou T, Cunningham T, Muesing MA, Gottlinger HG, Bieniasz PD: **Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism.** *Proc Natl Acad Sci U S A* 2002, **99**:11914-11919.
5. Forshey BM, Shi J, Aiken C: **Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells.** *J Virol* 2005, **79**:869-875.
6. Ikeda Y, Ylinen LM, Kahar-Bador M, Towers GJ: **Influence of gag on human immunodeficiency virus type 1 species-specific tropism.** *J Virol* 2004, **78**:11816-11822.
7. Shi J, Aiken C: **Saturation of TRIM5 α -mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid.** *Virology* 2006, **350**:493-500.
8. Stremlau M, Perron M, Lee M, Li Y, Song B, Javanbakht H, Diaz-Griffero F, Anderson DJ, Sundquist WI, Sodroski J: **Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5 α restriction factor.** *Proc Natl Acad Sci U S A* 2006, **103**:5514-5519.
9. Langelier CR, Sandrin V, Eckert DM, Christensen DE, Chandrasekaran V, Alam SL, Aiken C, Olsen JC, Kar AK, Sodroski JG, Sundquist WI: **Biochemical characterization of a recombinant TRIM5 α protein that restricts human immunodeficiency virus type 1 replication.** *J Virol* 2008, **82**:11682-11694.

10. Sebastian S, Luban J: **TRIM5alpha selectively binds a restriction-sensitive retroviral capsid.** *Retrovirology* 2005, **2**:40.
11. Perez-Caballero D, Hatzioannou T, Yang A, Cowan S, Bieniasz PD: **Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity.** *J Virol* 2005, **79**:8969-8978.
12. Kar AK, Diaz-Griffero F, Li Y, Li X, Sodroski J: **Biochemical and biophysical characterization of a chimeric TRIM21-TRIM5alpha protein.** *J Virol* 2008, **82**:11669-11681.
13. Campbell EM, Perez O, Anderson JL, Hope TJ: **Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha.** *J Cell Biol* 2008, **180**:549-561.
14. Black LR, Aiken C: **TRIM5alpha disrupts the structure of assembled HIV-1 capsid complexes *in vitro*.** *J Virol* 2010, **84**:6564-6569.
15. Perron MJ, Stremlau M, Lee M, Javanbakht H, Song B, Sodroski J: **The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid.** *J Virol* 2007, **81**:2138-2148.
16. Zhao G, Ke D, Vu T, Ahn J, Shah VB, Yang R, Aiken C, Charlton LM, Gronenborn AM, Zhang P: **Rhesus TRIM5alpha disrupts the HIV-1 capsid at the inter-hexamer interfaces.** *PLoS Pathog* 2011, **7**:e1002009.
17. Anderson JL, Campbell EM, Wu X, Vandegraaff N, Engelman A, Hope TJ: **Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins.** *J Virol* 2006, **80**:9754-9760.
18. Diaz-Griffero F, Li X, Javanbakht H, Song B, Welikala S, Stremlau M, Sodroski J: **Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5.** *Virology* 2006, **349**:300-315.
19. Rold CJ, Aiken C: **Proteasomal degradation of TRIM5alpha during retrovirus restriction.** *PLoS Pathog* 2008, **4**:e1000074.
20. Lienlaf M, Hayashi F, Di Nunzio F, Tochio N, Kigawa T, Yokoyama S, Diaz-Griffero F: **Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha.** *J Virol* 2011, **85**:8725-8737.

21. Berthoux L, Sebastian S, Sokolskaja E, Luban J: **Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA.** *J Virol* 2004, **78**:11739-11750.
22. Wu X, Anderson JL, Campbell EM, Joseph AM, Hope TJ: **Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection.** *Proc Natl Acad Sci U S A* 2006, **103**:7465-7470.
23. Hatziioannou T, Perez-Caballero D, Cowan S, Bieniasz PD: **Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells.** *J Virol* 2005, **79**:176-183.
24. Li Y, Kar AK, Sodroski J: **Target Cell Type-Dependent Modulation of Human Immunodeficiency Virus (HIV-1) Capsid Disassembly by Cyclophilin A.** *J Virol* 2009, **83**:10951-10962.
25. Sokolskaja E, Sayah DM, Luban J: **Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity.** *J Virol* 2004, **78**:12800-12808.
26. Keckesova Z, Ylinen LM, Towers GJ: **Cyclophilin A renders human immunodeficiency virus type 1 sensitive to Old World monkey but not human TRIM5 alpha antiviral activity.** *J Virol* 2006, **80**:4683-4690.
27. Sokolskaja E, Berthoux L, Luban J: **Cyclophilin A and TRIM5alpha independently regulate human immunodeficiency virus type 1 infectivity in human cells.** *J Virol* 2006, **80**:2855-2862.
28. Berthoux L, Sebastian S, Sokolskaja E, Luban J: **Cyclophilin A is required for TRIM5 α -mediated resistance to HIV-1 in Old World monkey cells.** *Proc Natl Acad Sci U S A* 2005, **102**:14849-14853.
29. Stremlau M, Song B, Javanbakht H, Perron M, Sodroski J: **Cyclophilin A: an auxiliary but not necessary cofactor for TRIM5alpha restriction of HIV-1.** *Virology* 2006, **351**:112-120.
30. Kootstra NA, Munk C, Tonnu N, Landau NR, Verma IM: **Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells.** *Proc Natl Acad Sci U S A* 2003, **100**:1298-1303.
31. Franke EK, Yuan HE, Luban J: **Specific incorporation of cyclophilin A into HIV-1 virions.** *Nature* 1994, **372**:359-362.

32. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP: **Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B.** *Cell* 1993, **73**:1067-1078.
33. Bosco DA, Eisenmesser EZ, Pochapsky S, Sundquist WI, Kern D: **Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A.** *Proc Natl Acad Sci U S A* 2002, **99**:5247-5252.
34. Gamble TR, Vajdos FF, Yoo S, Worthylake DK, Houseweart M, Sundquist WI, Hill CP: **Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid.** *Cell* 1996, **87**:1285-1294.
35. Pacheco B, Finzi A, Stremlau M, Sodroski J: **Adaptation of HIV-1 to cells expressing rhesus monkey TRIM5alpha.** *Virology* 2010, **408**:204-212.
36. Li Y, Li X, Stremlau M, Lee M, Sodroski J: **Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection.** *J Virol* 2006, **80**:6738-6744.
37. Maillard PV, Reynard S, Serhan F, Turelli P, Trono D: **Interfering residues narrow the spectrum of MLV restriction by human TRIM5alpha.** *PLoS Pathog* 2007, **3**:e200.
38. Pham QT, Bouchard A, Grutter MG, Berthoux L: **Generation of human TRIM5alpha mutants with high HIV-1 restriction activity.** *Gene Ther* 2010, **17**:859-871.
39. Stremlau M, Perron M, Welikala S, Sodroski J: **Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction.** *J Virol* 2005, **79**:3139-3145.
40. Yap MW, Nisole S, Stoye JP: **A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction.** *Curr Biol* 2005, **15**:73-78.
41. Anderson JS, Javien J, Nolta JA, Bauer G: **Preintegration HIV-1 inhibition by a combination lentiviral vector containing a chimeric TRIM5 alpha protein, a CCR5 shRNA, and a TAR decoy.** *Mol Ther* 2009, **17**:2103-2114.
42. Pham QT, Veillette M, Brandariz-Nunez A, Pawlica P, Thibert-Lefebvre C, Chandonnet N, Diaz-Griffero F, Berthoux L: **A novel aminoacid determinant of HIV-1 restriction in the TRIM5alpha variable 1 region isolated in a random mutagenic screen.** *Virus Res* 2013.

43. Towers GJ, Hatziioannou T, Cowan S, Goff SP, Luban J, Bieniasz PD: **Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors.** *Nat Med* 2003, **9**:1138-1143.
44. Bérubé J, Bouchard A, Berthoux L: **Both TRIM5alpha and TRIMCyp have only weak antiviral activity in canine D17 cells.** *Retrovirology* 2007, **4**:68.
45. Bosco DA, Eisenmesser EZ, Clarkson MW, Wolf-Watz M, Labeikovsky W, Millet O, Kern D: **Dissecting the microscopic steps of the cyclophilin A enzymatic cycle on the biological HIV-1 capsid substrate by NMR.** *J Mol Biol* 2010, **403**:723-738.
46. Sakuma R, Noser JA, Ohmine S, Ikeda Y: **Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins.** *Nat Med* 2007, **13**:631-635.
47. Zhang F, Perez-Caballero D, Hatziioannou T, Bieniasz PD: **No effect of endogenous TRIM5alpha on HIV-1 production.** *Nat Med* 2008, **14**:235-236; author reply 236-238.
48. Groot F, Welsch S, Sattentau QJ: **Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses.** *Blood* 2008, **111**:4660-4663.
49. Haller C, Fackler OT: **HIV-1 at the immunological and T-lymphocytic virological synapse.** *Biol Chem* 2008, **389**:1253-1260.
50. Rudnicka D, Feldmann J, Porrot F, Wietgreffe S, Guadagnini S, Prevost MC, Estaquier J, Haase AT, Sol-Foulon N, Schwartz O: **Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses.** *J Virol* 2009, **83**:6234-6246.
51. Richardson MW, Carroll RG, Stremlau M, Korokhov N, Humeau LM, Silvestri G, Sodroski J, Riley JL: **Mode of transmission affects the sensitivity of human immunodeficiency virus type 1 to restriction by rhesus TRIM5alpha.** *J Virol* 2008, **82**:11117-11128.
52. Kuritzkes DR: **Drug resistance in HIV-1.** *Curr Opin Virol* 2011, **1**:582-589.
53. Lee K, Ambrose Z, Martin TD, Oztop I, Mulky A, Julias JG, Vandegraaff N, Baumann JG, Wang R, Yuen W, et al: **Flexible use of nuclear import pathways by HIV-1.** *Cell Host Microbe* 2010, **7**:221-233.

54. Price AJ, Fletcher AJ, Schaller T, Elliott T, Lee K, Kewalramani VN, Chin JW, Towers GJ, James LC: **CPSF6 Defines a Conserved Capsid Interface that Modulates HIV-1 Replication.** *PLoS Pathog* 2012, **8**:e1002896.
55. Lin TY, Emerman M: **Determinants of cyclophilin A-dependent TRIM5 alpha restriction against HIV-1.** *Virology* 2008, **379**:335-341.
56. Matreyek KA, Engelman A: **The requirement for nucleoporin NUP153 during human immunodeficiency virus type 1 infection is determined by the viral capsid.** *J Virol* 2011, **85**:7818-7827.
57. Schaller T, Ocwieja KE, Rasaiyaah J, Price AJ, Brady TL, Roth SL, Hue S, Fletcher AJ, Lee K, KewalRamani VN, et al: **HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting and replication efficiency.** *PLoS Pathog* 2011, **7**:e1002439.
58. Berthoux L, Sebastian S, Sayah DM, Luban J: **Disruption of human TRIM5alpha antiviral activity by nonhuman primate orthologues.** *J Virol* 2005, **79**:7883-7888.
59. Sebastian S, Sokolskaja E, Luban J: **Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologues in a cell type-dependent manner.** *J Virol* 2006, **80**:2051-2054.
60. Sayah DM, Sokolskaja E, Berthoux L, Luban J: **Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1.** *Nature* 2004, **430**:569-573.
61. Berthoux L, Towers GJ, Gurer C, Salomoni P, Pandolfi PP, Luban J: **As(2)O(3) enhances retroviral reverse transcription and counteracts Ref1 antiviral activity.** *J Virol* 2003, **77**:3167-3180.
62. Naviaux RK, Costanzi E, Haas M, Verma IM: **The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses.** *J Virol* 1996, **70**:5701-5705.
63. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D: **Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*.** *Nat Biotechnol* 1997, **15**:871-875.

64. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA: **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** *J Virol* 1986, **59**:284-291.
65. Dufresne M, Dumas G, Asselin E, Carrier C, Pouliot M, Reyes-Moreno C: **Pro-inflammatory type-1 and anti-inflammatory type-2 macrophages differentially modulate cell survival and invasion of human bladder carcinoma T24 cells.** *Molecular immunology* 2011, **48**:1556-1567.
66. Butler SL, Hansen MS, Bushman FD: **A quantitative assay for HIV DNA integration *in vivo*.** *Nat Med* 2001, **7**:631-634.
67. Price AJ, Marzetta F, Lammers M, Ylinen LM, Schaller T, Wilson SJ, Towers GJ, James LC: **Active site remodeling switches HIV specificity of antiretroviral TRIMCyp.** *Nat Struct Mol Biol* 2009, **16**:1036-1042.
68. Bosco DA, Kern D: **Catalysis and binding of cyclophilin A with different HIV-1 capsid constructs.** *Biochemistry* 2004, **43**:6110-6119.
69. Farrow NA, Zhang O, Forman-Kay JD, Kay LE: **A heteronuclear correlation experiment for simultaneous determination of ¹⁵N longitudinal decay and chemical exchange rates of systems in slow equilibrium.** *J Biomol NMR* 1994, **4**:727-734.

Supplementary data

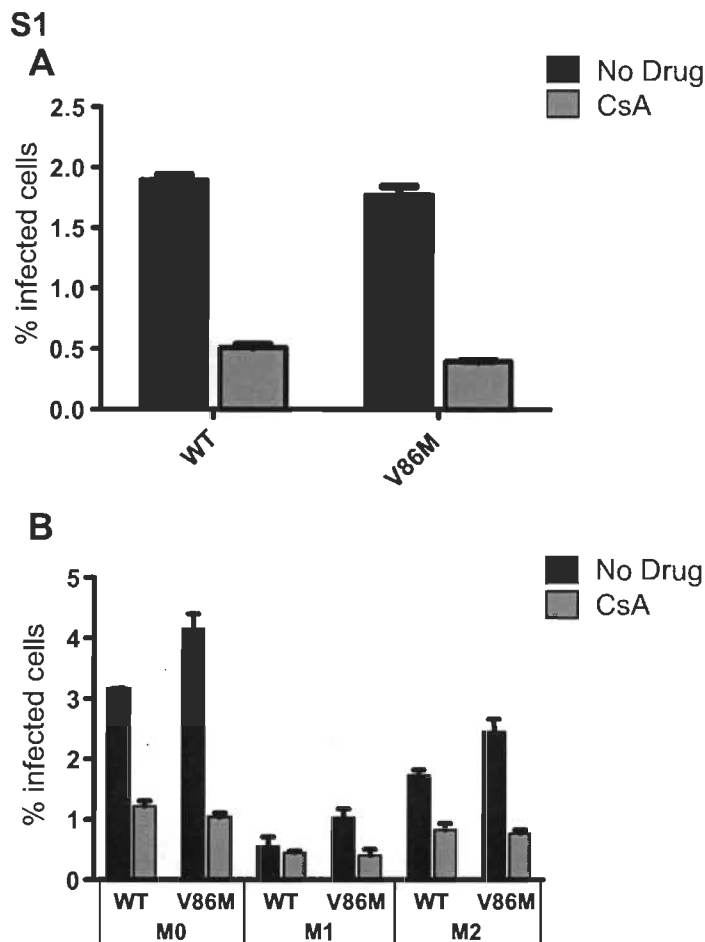


Figure 3.9S CA-V86M HIV-1 is inhibited by CsA in human lymphocytes and macrophages.

(A) Activated human CD4⁺ lymphocytes were challenged with WT or V86M HIV-1NL-GFP at a multiplicity of infection of ~0.1 in the absence or presence of 2 μ M CsA. WT and V86M viral preparations were adjusted by reverse transcriptase assay. The percentage of infected (GFP-positive) cells was determined two days later by flow cytometry. Shown are average values from three independent infections with standard deviations. (B) THP-1 cells differentiated and polarized into either macrophages (M0), pro-inflammatory macrophages (M1) or anti-inflammatory macrophages (M2) were challenged with WT or V86M HIV-1NL-GFP exactly like above but at an M.O.I of ~0.02.

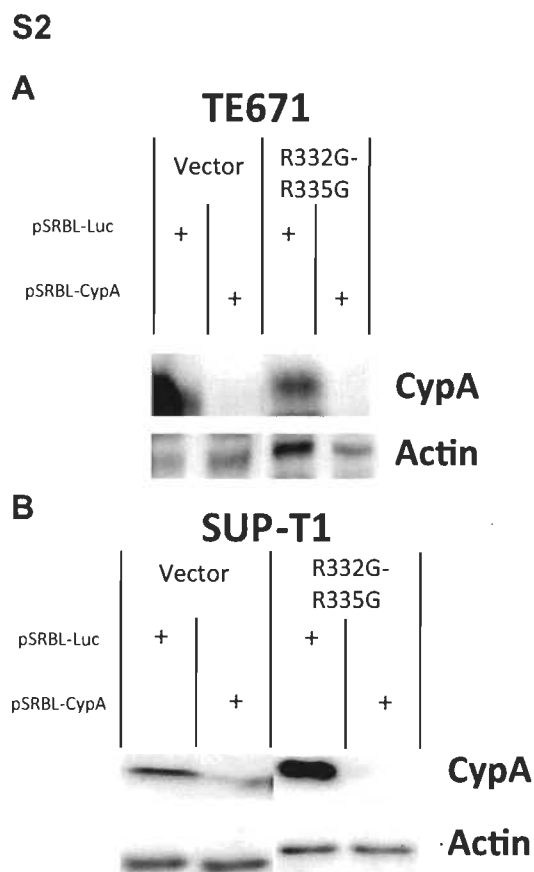


Figure 3.10S Western blotting analysis of CypA knockdown.

TE671 cells (A) and SupT1 cells (B) expressing the indicated TRIM5 α_{Hu} mutants and controls were stably transduced with retroviral vectors expressing shRNAs targeting either CypA or the non-relevant control Luciferase. Untransduced cells were eliminated; then, whole cell lysates were prepared from a similar numbers of cells and processed for western blotting using antibodies against CypA or X-actin as a loading control.

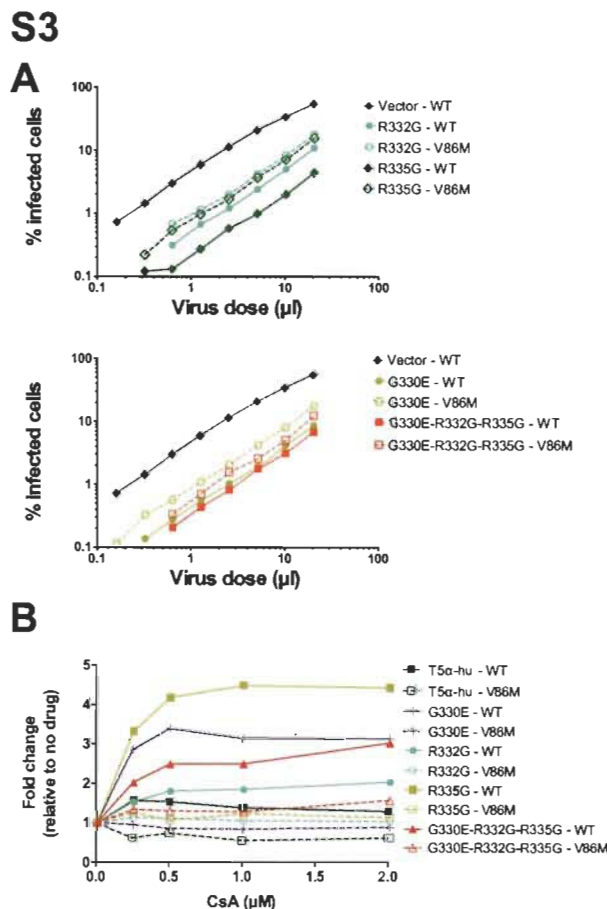


Figure 3.11S Restriction of WT and CA-V86M HIV-1 by a panel of TRIM5 α_{Hu} mutants.

(A) TE671 cells transduced with the indicated TRIM5 α cDNAs were challenged with multiple doses of WT or CA-V86M HIV-1NL-GFP as described in Figure 3.2. Cells were analyzed by FACS 2 days later. Control permissive cells transduced with the “empty” vector and infected with WT HIV-1NL-GFP are included. (B) TE671 cells expressing the indicated TRIM5 α_{Hu} mutants and controls were challenged with WT or V86M HIV-1NL-GFP in the presence of increasing concentrations of CsA. Virus doses were adjusted for each virus-cell combination so that approximately 1% of the cells were infected in the absence of CsA. The percentage of GFP-positive cells was determined by FACS 2 days later and results are shown as –fold increases relative to the no-drug controls.

CHAPITRE IV

RESTRICTION OF A DIVERSE PANEL OF HIV-1 CLINICAL ISOLATES BY A MUTATED HUMAN TRIM5 α

MAXIME VEILLETTE, SOPHIE HAMEL, LIONEL BERTHOUX

Manuscrit en préparation pour soumission dans *Human Gene Therapy*.

Résumé

De récentes avancées dans le domaine de la thérapie génique amènent de nouvelles possibilités d'utiliser des transgènes humains contre le VIH-1. À cette fin, l'identification et la caractérisation des transgènes potentiels sont primordiales. Des mutations ponctuelles dans le domaine de reconnaissance de la capsid virale conférant une activité anti-VIH au facteur de restriction humain TRIM5 α ont été décrites. Puisque ces mutants ont préalablement été testés exclusivement contre des souches virales adaptées à la recherche en laboratoire, nous avons étudié le potentiel du mutant R332G-R335G de TRIM5 α humain à restreindre des souches du VIH-1 hautement divergentes, incluant les membres du groupe O. Nos résultats montrent que toutes les souches testées étaient efficacement restreintes par le transgène. De plus, des isolats cliniques présentant des mutations permettant d'échapper au lymphocyte T cytotoxique (CTL) et qui ont été montrés comme étant plus susceptible à TRIM5 α_{Hu} sauvage étaient également plus sensible à R332G-R335G, suggérant ainsi qu'une thérapie génique basée sur TRIM5 α pourrait réduire la probabilité que le virus acquière des mutations permettant une résistance au CTL *in vivo*.

Abstract

Recent advances in the gene therapy field open up novel possibilities to use human-based transgenes against HIV-1. In order to achieve this, the identification and characterization of potent transgenes is essential. Point mutations in the capsid-recognizing domain of the human restriction factor TRIM5 α have been described as conferring anti-HIV-1 activity. Because those mutants were previously exclusively tested against laboratory-adapted HIV-1 strains, we investigated the ability of the human R332G-R335G TRIM5 α mutant to restrict highly divergent HIV-1 strains, including Group O. Our results show that all of the strains tested were potently inhibited by the transgene. In addition, clinical isolates bearing cytotoxic T lymphocyte (CTL) escape mutations that were previously shown to be more susceptible to the wild-type human TRIM5 α were also found to be more susceptible to restriction by R332G-R335G and rhesus TRIM5 α , suggesting that TRIM5 α -based therapy might decrease the probability of acquiring CTL escape mutations *in vivo*.

Introduction

Recent advances in gene therapy technologies have renewed the interest in developing approaches aimed at inhibiting HIV-1 infection. Of note, several new clinical studies were started in the past years, with the vast majority of those aiming to impede viral entry by disrupting the expression of the coreceptor CCR5 via RNA interference or by direct knockout of the CCR5 locus (Didigu and Doms, 2012; Kiem et al., 2012). Another approach that has been pursued by several groups consists of using human-based transgenes in order to efficiently impede HIV-1 replication. One such candidate is the retroviral restriction factor TRIM5 α , which acts in the immediate post-entry, pre-integration window (Owens et al., 2003; Perez-Caballero et al., 2005; Stremlau et al., 2004). It targets the N-terminal domain of viral capsid proteins (CA-NTD) that form the outer surface of the viral core (Cowan et al., 2002; Forshey et al., 2005; Ikeda et al., 2004; Owens et al., 2003; Shi and Aiken, 2006; Stremlau et al., 2006a). The interaction between the two proteins leads to several blocks to the viral cycle (Anderson et al.,

2006; Berthoux et al., 2004; Black and Aiken, 2010; Campbell et al., 2008; Diaz-Griffero et al., 2006; Perron et al., 2007; Rold and Aiken, 2008; Stremlau et al., 2006a; Zhao et al., 2011) while also promoting innate immunity signaling (Pertel et al., 2011). However, the range of viruses restricted by TRIM5 α varies greatly in a species-specific way. For example, the human ortholog of TRIM5 α (TRIM5 α_{Hu}) only moderately restricts HIV-1 (~2-fold) while its rhesus monkey counterpart (TRIM5 α_{Rh}) is highly active against HIV-1 (50 to 100-fold) (Pham et al., 2010; Stremlau et al., 2004; Yap et al., 2004). Studies have shown that overexpression of TRIM5 α_{Rh} in human cells is dominant over the endogenously expressed protein and leads to a potent block to HIV-1 replication (Berthoux et al., 2005a; Pham et al., 2010; Stremlau et al., 2004), a phenotype also seen in hematopoietic progenitor cells (Anderson and Akkina, 2005). Replacing regions within the CA-targeting domain, called PRYSPRY, of TRIM5 α_{Hu} by the corresponding sequences from its rhesus ortholog has resulted in human/rhesus chimeric TRIM5 α proteins that can efficiently restrict HIV-1 when transduced in human cells (Kambal et al., 2011). Modeling studies and genetic screens have also led to the identification of point mutations in the variable region 1 of the TRIM5 α_{Hu} PRYSPRY domain that allow it to target HIV-1 for restriction (Pham et al., 2010; Pham et al., submitted; Yap et al., 2005). We previously described the R332G-R335G TRIM5 α_{Hu} mutant as especially efficient at restricting HIV-1 (Pham et al., 2010). Because these mutants differ only slightly from the endogenous form of TRIM5 α_{Hu} , they would probably not be immunogenic, thus making them ideal candidates for gene therapy applications.

It is well-established that escape mutations often lead to a fitness cost on viral replication (Friedrich et al., 2004; Liu et al., 2007; Martinez-Picado et al., 2006; Troyer et al., 2009). Interestingly, a study of CA mutations that allow viral escape from cytotoxic T lymphocyte (CTL) has shown that some of these mutations could increase the virus susceptibility to TRIM5 α_{Hu} (Battivelli et al., 2011). Conversely, a single CA mutation at position 86 (V86M) that confers resistance to both TRIM5 α_{Rh} and R332G-R335G TRIM5 α_{Hu} has recently been identified (Pacheco et al., 2010; Veillette et al., submitted). These data raise the possibility that some HIV-1 clinical isolates or

clones highly divergent from clade B laboratory-adapted strains might be poorly sensitive to restriction by TRIM5 α_{Hu} mutants. Potential applications for TRIM5 α_{Hu} mutants necessitate that they show potency on a wide range of HIV-1 strains. Here we investigate the susceptibility of a diverse panel of HIV-1 strains to restriction by R332G-R335G TRIM5 α_{Hu} and the impact of CTL escape mutations on restriction by this mutant.

Results

In order to assay the sensitivity of diverse HIV-1 isolates to mutated human TRIM5 α , we obtained *gag* or *gag-pol* expressing vectors from 14 independently derived isolates. Those vectors were previously constructed in two different backbones. The first set of vectors encodes the full-length *gag* gene from 11 isolates in an otherwise isogenic context (pCMV- Δ 8.91) (Ikeda et al., 2004). The other set is based on a NL4-3 vector that contains a deletion in *env* and expresses *Renilla* luciferase in place of *nef*, and in which the *Gag-PR* sequence is derived from three isolates bearing CTL escape mutations (Battivelli et al., 2011). The two sets were used to produce either GFP- or Renilla-encoding, VSV-G pseudotyped HIV-1 vectors by transient transfection as previously described (Berube et al., 2007; Pham et al., 2010). Strains name, sequence accession numbers, subtypes or clades, and cellular tropism are summarized in Tableau 4.1.

Tableau 4.1
Summary information on viral strains used.

Name of strain	GenBank accession no.	Subtype/clade	Tropism ^a	Reference(s)
p8.9Ex (HXB2)	K03455	B	T	
89.6	U39362	B	U	(Kim et al., 1995)
JRCSF	M38429	B	T	(Chesebro et al., 1991; Gao et al., 1998)
YU-2	M93258	B	M	(Gao et al., 1998; Li et al., 1991)
GUN-1WT	D34592	U	T/M	(Shimizu et al., 1994; Takeuchi et al., 1987)
GUN-1V	D34594	U	T/M	(Fu et al., 1999; Shimizu et al., 1994; Takeuchi et al., 1987)
MVP-5180	L20571	Group O	T/M	(Gurtler et al., 1994)
90CR056	AF005496	H	U	(Gao et al., 1998)
92BR025	U52953	C	U	(Gao et al., 1998)
93BR029	AF005495	B/F	U	(Gao et al., 1998)
94UG114	U88824	D	U	(Gao et al., 1998)
NRC1	JN408075	B	U	(Matsuoka et al., 2009)
NRC2	JN408076	B	U	(Matsuoka et al., 2009)
NRC10	JN408077	B	U	(Matsuoka et al., 2009)

^a T, T-Tropic; M, M-Tropic; U, Unknown.

The genetic diversity of the panel of HIV-1 isolates used in this study was first determined by phylogenetic sequence analysis. For all strains for which information was available, the full-length *gag* nucleotide sequence was retrieved and aligned to randomly chosen reference strains. The alignment was manually checked, and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). As shown in Figure 4.1A, the panel of HIV-1 isolates used in this study includes representatives of different group M subtypes (B, C, D and H) and one evolutionary distant strain from group O. Also, some are known to be macrophage-tropic (CCR5-tropic) while others are T cell-tropic (CXCR4-tropic) (Table 4.1). In order to analyze specific differences in the CA sequence of the strains used, the first 150 amino acids of CA (CA-NTD) were aligned (Figure 4.1B). The isolates in the panel present mutations in all of the seven helical regions except one (helical region 5). Interestingly, three of the strains used

present mutations at the same position as the TRIM5 α -resistant V86M (MVP-5180, V86P; NRC1 and NRC10, V86A). In addition, the mutations at position 86 and two others (96 and 116) are reverted back to wild-type (NL4-3) in the NRC10-VMG strain. Previous studies did not investigate whether other mutations at position 86 could also impact infectivity of HIV-1 under TRIM5 α -mediated restriction.

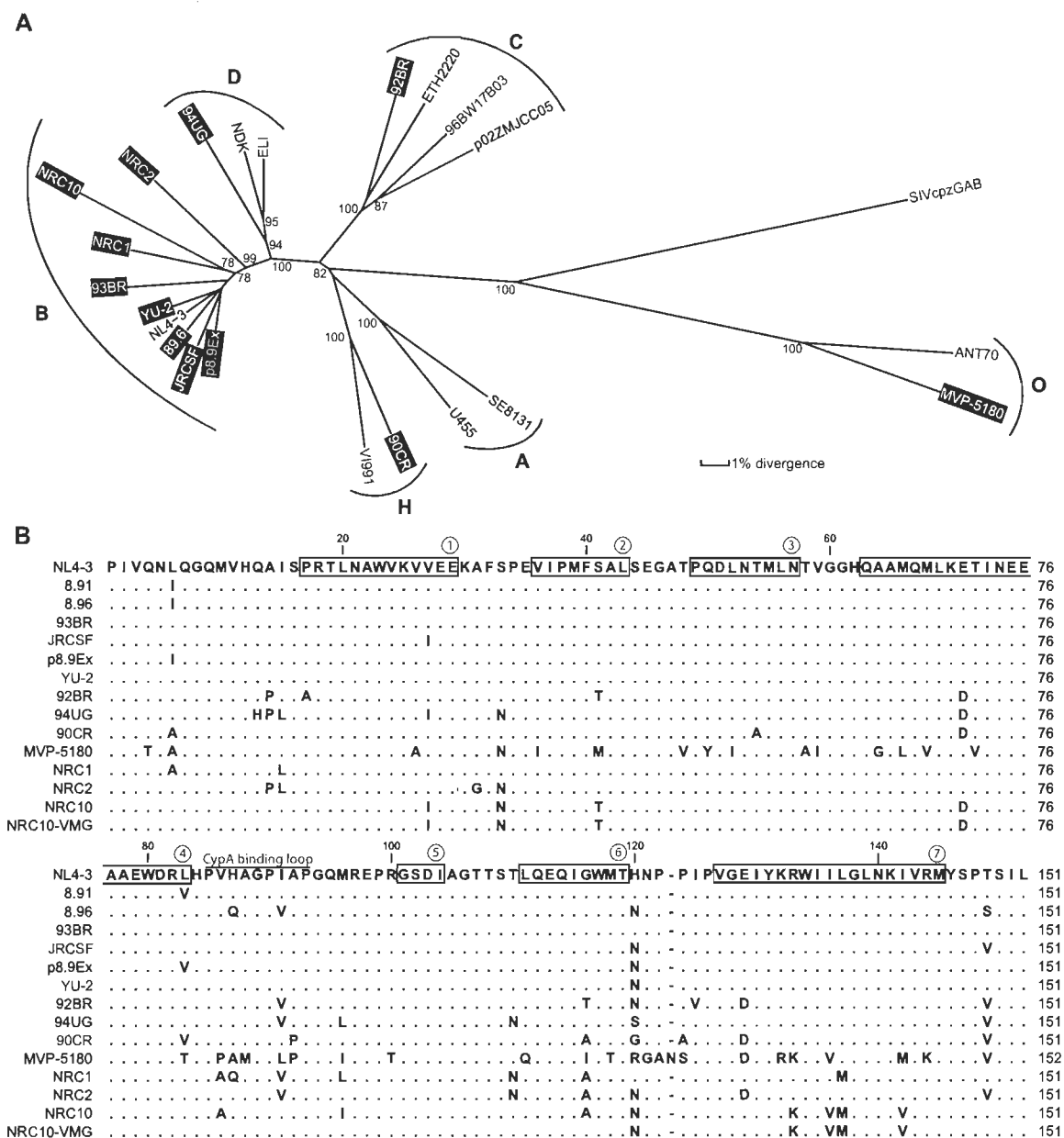


Figure 4.1 Phylogenetic relationships and sequence alignment of the virus strains used in this study.

(A) Phylogenetic relationships of the virus strains used in this study to randomly-chosen reference strains. Trees were constructed from

full-length *gag* nucleotide sequences by using the neighbor-joining method (see Materials & Methods for details). Branch lengths are drawn to scale, with the bar representing 0.01 nucleotide substitution per site. Percent bootstraps values in which the adjacent branching was supported are indicated at the nodes (values of 75% and higher are shown). Strains with a highlighted name were used in this study. Brackets indicate either HIV-1 group O or subtypes of group M. (B) Annotated sequence alignment of the first 150 CA amino acids of strains used in this study and for which sequences were available. The NL4-3 sequence is shown as the reference sequence and the seven helical regions of N-terminal CA are boxed and numbered (numbers in circles). Amino acids are numbered according to the beginning of CA.

Using the GFP-encoding HIV-1 virions produced from the first set of vectors, we infected human TE671 cells expressing either wild-type TRIM5 α_{Hu} , R332G-R335G TRIM5 α_{Hu} or TRIM5 α_{Rh} and quantified GFP-positive cells two days post-infection by flow cytometry. As shown in Figure 4.2, all viruses tested were susceptible to restriction by both TRIM5 α_{Rh} and R332G-R335G TRIM5 α_{Hu} . Although the extent of the restriction varied slightly from strain to strain, R332G-R335G TRIM5 α_{Hu} inhibited all HIV-1 strains by more than 10-fold, with the exception of 93BR. In addition, the pattern of restriction by TRIM5 α_{Rh} was similar to that of R332G-R335G TRIM5 α_{Hu} , supporting the idea that these two orthologs interact with HIV-1 CA in a similar fashion.

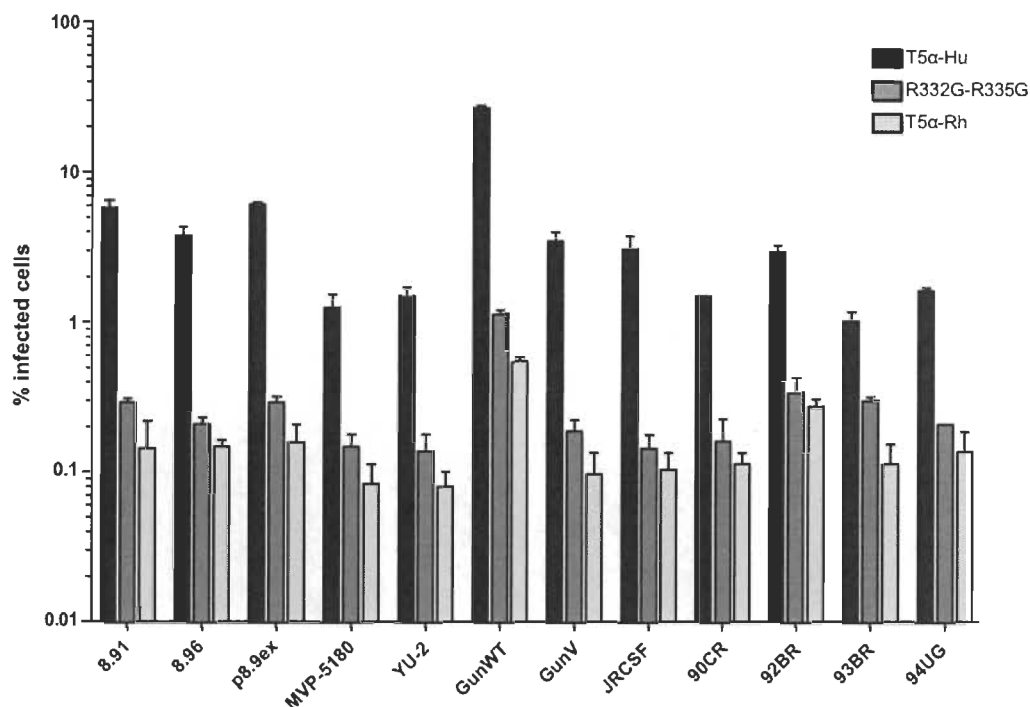


Figure 4.2 Infectivity of different HIV-1 strains in cells expressing a TRIM5 α _{Hu} mutant.

TE671 cells were transduced with the indicated TRIM5 α cDNAs. Cells were then infected at a single MOI with the different GFP-encoding viral vectors. The percentage of infected cells was determined two days post-infection by flow cytometry. Shown are the mean and standard deviation of three independent infections.

Assaying the sensitivity of the strains that used *Renilla* luciferase as a reporter gene required a different approach. We measured the infectivity of those strains in both feline CRFK cells, which do not express any known TRIM5 α ortholog, and in human TE671 cells transduced with the different TRIM5 α cDNAs. Results were normalized to 1 for the NL4-3 virus in each cell type (CRFK and TE671 expressing TRIM5 α _{Hu}, see Figure 4.3). The NRC1 clone had the same restriction pattern as that of NL4-3, although the former was a bit less sensitive to R332G-R335G TRIM5 α _{Hu} (11.7- vs. 4.8-fold). Our results were consistent with what was previously described (Battivelli et al., 2011), as NRC2 and NRC10 showed increased sensitivity to TRIM5 α _{Hu} (13.5- and 56-fold, respectively). Moreover, increased susceptibility to restriction by TRIM5 α _{Hu} was linked with higher restriction by R332G-R335G TRIM5 α _{Hu} and TRIM5 α _{Rh}. The NRC10-VMG clone, in which three mutations (V86A, M96I and G116A) of NRC10 are reverted back

to NL4-3 (Figure 4.1B) showed decreased sensitivity to all three TRIM5 α , supporting the idea that human (mutated or WT) and rhesus TRIM5 α recognize NRC10 CA through, at least in part, the same interaction domains. In addition, the increase in restriction in cells expressing R332G-R335G TRIM5 α _{Hu} or TRIM5 α _{Rh}, compared with cells expressing WT TRIM5 α _{Hu}, was less important for the two strains (NRC2, NRC10) that were already more sensitive to WT TRIM5 α _{Hu}. This again suggests that CA from these two HIV-1 strains bind WT TRIM5 α _{Hu} in a fashion similar to their interaction with R332G-R335G TRIM5 α _{Hu} or TRIM5 α _{Rh}.

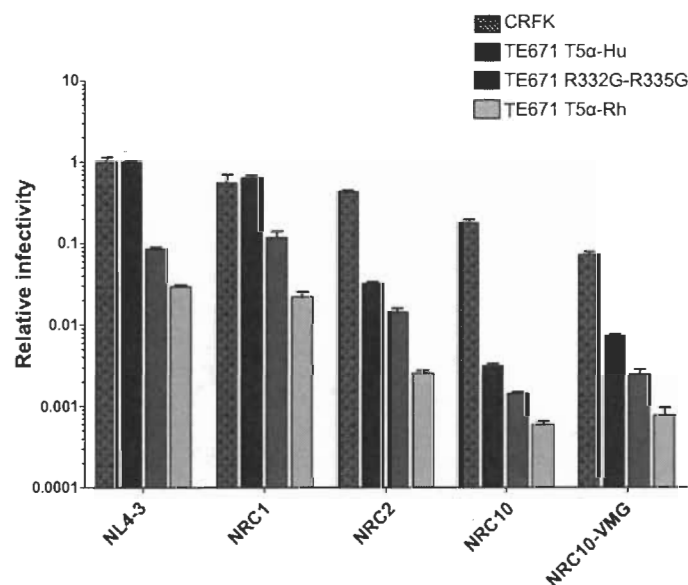


Figure 4.3 Sensitivity of CTL escape mutants to mutated TRIM5 α _{Hu}.

In order to assay the sensitivity of the HIV-1_{NL-Luc} strains to restriction by WT or R332G-R335G TRIM5 α _{Hu}, feline CRFK cells and TE671 cells expressing the indicated TRIM5 α cDNAs were infected with a single dose of the different HIV-1_{NL-Luc} strains. Two days post-infection, cells were lysed and luciferase activity was measured. The relative light units (RLU) values obtained were normalized to NL4-3 in non-restrictive conditions (CRFK and TE671 expressing TRIM5 α _{Hu} WT). Shown are the mean and standard deviation of three independent infections.

Discussion

Restriction factors such as TRIM5 α are thought to play an important role in protecting against non-host retroviruses and limiting cross-species transmission (Cullen,

2006; Hatzioannou et al., 2004; Kirmaier et al., 2010; Mariani et al., 2003). Our lab and others have previously shown that point mutations in the PRYSPRY domain of the human TRIM5 α protein can increase restriction of lab-adapted HIV-1 viral strains (Li et al., 2006; Maillard et al., 2007; Pham et al., 2010; Stremlau et al., 2005; Yap et al., 2005). Because those constructs differ only slightly from their endogenous counterpart, they would probably not be immunogenic, thus making them ideal candidates for therapeutic treatment of HIV-1. However, it was not yet known whether these TRIM5 α variants would restrict clinical isolates of HIV-1. Our results show that all strains of a diverse panel of HIV-1 variants are potently restricted by a mutated TRIM5 α_{Hu} (R332G-R335G) and by TRIM5 α_{Rh} . In addition, the conserved restriction pattern across all strains tested suggests that these two HIV-1 restrictive variants of TRIM5 α share the same CA determinants. However, the HIV-1 strains used here presented variations across almost all of the helical regions of CA-NTD (except helix 5) and we found no correlation between individual mutations and restriction levels.

- In a clinical context, predicting how a treatment will interfere or synergize with other natural processes is of high importance. Some mutations in CA that were associated with escape from CTLs were previously shown to increase sensitivity to TRIM5 α_{Hu} (Battivelli et al., 2010; Battivelli et al., 2011) and our results show that this increased sensitivity can also be extended to other, more restrictive, TRIM5 α variants. This suggests that a highly restrictive TRIM5 α used in gene therapy applications could help mitigate the rate at which CTL escape mutations appears, thus probably making it significantly harder for the virus to escape both the acquired immunity and gene therapy-based protections. CTL escape mutations are often followed by compensatory mutations that reduce the fitness cost of the resistance mutations (Battivelli et al., 2011; Brockman et al., 2007; Brumme et al., 2009). Interestingly, such compensatory mutations in the NRC10 strain directly affect the CypA-binding loop of CA (V86A and M96I), which is known to play an important role in TRIM5 α -mediated restriction (Berthoux et al., 2004, 2005b; Keckesova et al., 2006; Stremlau et al., 2006b). In addition, the residue at position 86 was already shown to play a crucial role in the resistance to both TRIM5 α_{Rh} (Pacheco et al., 2010) and mutated TRIM5 α_{Hu} (Veillette et al., submitted). Although the

NRC1 strain shares some mutations that apparently render NRC10 more susceptible to TRIM5 α restriction (V86A and G116A), the former presents the same restriction pattern as NL4-3. Without excluding the possibility of context-specific effects of other mutations, this phenotype could also be explained by the presence in NRC1 of the H87Q mutation, which was already described as conferring resistance to TRIM5 α (Chatterji et al., 2005; Ikeda et al., 2004). Taken together, this suggests that mutations in or near the CypA-binding loop of CA play an important role in modulating HIV-1 interactions with both innate and acquired immune defenses.

Materials and Methods

Plasmids DNAs

pMIP-TRIM5 α_{Hu} and pMIP-TRIM5 α_{Rh} express C-terminal FLAG-tagged versions of the corresponding proteins and have been extensively described before (Berthoux et al., 2005a; Berthoux et al., 2005b; Berube et al., 2007; Sebastian et al., 2006). For all HIV-1 strains used in this study except NRC1, NRC2 and NRC10, pCMV- Δ 8.91 -based *gag-pol* expressing vectors were described elsewhere and are a kind gift of Greg Towers and Yasuhiro Ikeda (Ikeda et al., 2004). pNL4-3XCS-derived proviral vectors containing the *Renilla* luciferase gene in place of *nef*, that present a 580-bp deletion in *env* and code for either NL4-3, NRC1, NRC2 or NRC10 *gag-pol* were previously described and are a kind gift of Allan Hance (Battivelli et al., 2010). pMD-G and pTRIP_{CMV-GFP} have been described elsewhere (Berthoux et al., 2005a; Berthoux et al., 2004, 2005b; Berthoux et al., 2003; Naviaux et al., 1996; Zufferey et al., 1997).

Cell lines

Human rhabdomyosarcoma TE671 cells, human embryonic kidney 293T cells and feline renal CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

Retroviral vectors production

HIV-1-based vectors were produced through transient transfection of 293T cells and collected as previously described (Berube et al., 2007). To produce strain-specific HIV-1_{TRIP-CMV-GFP}, cells were co-transfected with pTRIP_{CMV-GFP}, the respective pCMV- Δ 8.91 vector and pMD-G. To produce strain-specific HIV-1_{NL-Luc}, cells were co-transfected with the respective pNL4-3XCS vector and pMD-G.

Viral challenges and measurement of sensitivity to TRIM5 α

For viral challenges with HIV-1_{TRIP-CMV-GFP}, cells were plated in 24-wells plates at 50,000 cells/well and infected the next day with the appropriate HIV-1 vectors at an MOI of 0.1. Virus doses were normalized by titering the viral stocks in feline CRFK cells (data not shown). Two days post-infection, cells were trypsinised and fixed in 1 to 2% formaldehyde in a PBS solution. The % of GFP-positive cells were then determined by analyzing 10,000 to 20,000 cells with a FC500 MPL cytometer (Beckman Coulter) using the CXP software (Beckman Coulter).

For viral challenges with HIV-1_{NL-Luc}, cells were plated in 24-wells plates at 50,000 cells/well and infected the next day with 100 μ l of the appropriate HIV-1 vectors, a dose that resulted in measurable luciferase activity in all tested conditions. Two days post-infection, the cells were washed once with PBS then lysed in 100 μ l of Glo Lysis Buffer (Promega, Madison, WI, USA) following manufacturer recommendations. Relative light units (RLU) were measured using the Renilla-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer instructions and read in a Biotek Synergy HT plate-reader.

Phylogenetic tree analysis

Phylogenetic relationships of the strains used in this study were estimated by sequence comparison with randomly-chosen reference strains from the Los Alamos HIV Sequence Database (<http://www.hiv.lanl.gov>). *Gag* sequences of all the strains were

aligned using ClustalX2 (2.0) and a phylogenetic tree was constructed by the neighbor-joining method. The reliability of the topology was estimated by performing bootstrap analysis with 1,000 replicates. iTOL v2.2 was used to draw the tree for illustration purposes (Letunic and Bork, 2007, 2011).

Protein sequence alignment

The first 150 amino acids of the CA protein of each strain were aligned using the CLC Mainworkbench software (v6.5) using default parameters.

Conclusions

The screening of a panel of HIV-1 vectors expressing *gag* sequences derived from different isolates allowed us to determine that a broad range of strains can be restricted by TRIM5 α_{Hu} mutants, re-affirming their interest as gene therapy candidates. Increased sensitivity of CTL escape mutants to TRIM5 α_{Hu} was also shown to correlate with increased sensitivity to both mutated TRIM5 α_{Hu} and TRIM5 α_{Rh} , hinting that clinical applications using TRIM5 α -based transgenes might also help prevent or at least delay acquisition of CTL escape mutations by HIV-1.

Acknowledgements

We thank Greg Towers (University College London, UK), Yasuhiro Ikeda (Mayo Clinic, MN, USA) and Allan Hance (INSERM, France) for the generous gift of plasmids. We are also grateful to Kathleen Riopel and Alexandre Paquin for preparation of plasmid DNA, and to Mélodie B. Plourde for helping with redaction of the manuscript. This work was supported by the Canadian Institutes for Health Research and the Canada Research Chair program. M.V. was supported by a FRQS Master's Training Award.

References

- Anderson, J., Akkina, R., 2005. TRIM5 α expression restricts HIV-1 infection in lentiviral vector-transduced CD34⁺-cell-derived macrophages. *Mol Ther* 12, 687-696.
- Anderson, J.L., Campbell, E.M., Wu, X., Vandegraaff, N., Engelman, A., Hope, T.J., 2006. Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. *J Virol* 80, 9754-9760.
- Battivelli, E., Lecossier, D., Matsuoka, S., Migraine, J., Clavel, F., Hance, A.J., 2010. Strain-specific differences in the impact of human TRIM5 α , different TRIM5 α alleles, and the inhibition of capsid-cyclophilin A interactions on the infectivity of HIV-1. *J Virol* 84, 11010-11019.
- Battivelli, E., Migraine, J., Lecossier, D., Yeni, P., Clavel, F., Hance, A.J., 2011. Gag cytotoxic T lymphocyte escape mutations can increase sensitivity of HIV-1 to human TRIM5 α , linking intrinsic and acquired immunity. *J Virol* 85, 11846-11854.
- Berthoux, L., Sebastian, S., Sayah, D.M., Luban, J., 2005a. Disruption of human TRIM5 α antiviral activity by nonhuman primate orthologues. *J Virol* 79, 7883-7888.
- Berthoux, L., Sebastian, S., Sokolskaja, E., Luban, J., 2004. Lv1 inhibition of human immunodeficiency virus type 1 is counteracted by factors that stimulate synthesis or nuclear translocation of viral cDNA. *Journal of virology* 78, 11739-11750.
- Berthoux, L., Sebastian, S., Sokolskaja, E., Luban, J., 2005b. Cyclophilin A is required for TRIM5 α -mediated resistance to HIV-1 in Old World monkey cells. *Proc Natl Acad Sci U S A* 102, 14849-14853.
- Berthoux, L., Towers, G.J., Gurer, C., Salomoni, P., Pandolfi, P.P., Luban, J., 2003. As(2)O(3) enhances retroviral reverse transcription and counteracts Ref1 antiviral activity. *Journal of virology* 77, 3167-3180.
- Berube, J., Bouchard, A., Berthoux, L., 2007. Both TRIM5 α and TRIMCyp have only weak antiviral activity in canine D17 cells. *Retrovirology* 4, 68.
- Black, L.R., Aiken, C., 2010. TRIM5 α disrupts the structure of assembled HIV-1 capsid complexes *in vitro*. *J Virol* 84, 6564-6569.

- Brockman, M.A., Schneidewind, A., Lahaie, M., Schmidt, A., Miura, T., Desouza, I., Rvkin, F., Derdeyn, C.A., Allen, S., Hunter, E., Mulenga, J., Goepfert, P.A., Walker, B.D., Allen, T.M., 2007. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *J Virol* 81, 12608-12618.
- Brumme, Z.L., John, M., Carlson, J.M., Brumme, C.J., Chan, D., Brockman, M.A., Swenson, L.C., Tao, I., Szeto, S., Rosato, P., Sela, J., Kadie, C.M., Frahm, N., Brander, C., Haas, D.W., Riddler, S.A., Haubrich, R., Walker, B.D., Harrigan, P.R., Heckerman, D., Mallal, S., 2009. HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. *PLoS One* 4, e6687.
- Campbell, E.M., Perez, O., Anderson, J.L., Hope, T.J., 2008. Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha. *J Cell Biol* 180, 549-561.
- Chatterji, U., Bobardt, M.D., Stanfield, R., Ptak, R.G., Pallansch, L.A., Ward, P.A., Jones, M.J., Stoddart, C.A., Scalfaro, P., Dumont, J.M., Besseghir, K., Rosenwirth, B., Gallay, P.A., 2005. Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in Owl monkey cells. *J Biol Chem* 280, 40293-40300.
- Chesebro, B., Nishio, J., Perryman, S., Cann, A., O'Brien, W., Chen, I.S., Wehrly, K., 1991. Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *J Virol* 65, 5782-5789.
- Cowan, S., Hatzioannou, T., Cunningham, T., Muesing, M.A., Gottlinger, H.G., Bieniasz, P.D., 2002. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. *Proc Natl Acad Sci U S A* 99, 11914-11919.
- Cullen, B.R., 2006. Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J Virol* 80, 1067-1076.
- Diaz-Griffero, F., Li, X., Javanbakht, H., Song, B., Welikala, S., Stremmler, M., Sodroski, J., 2006. Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5. *Virology* 349, 300-315.
- Didigu, C.A., Doms, R.W., 2012. Novel approaches to inhibit HIV entry. *Viruses* 4, 309-324.

- Forshey, B.M., Shi, J., Aiken, C., 2005. Structural requirements for recognition of the human immunodeficiency virus type 1 core during host restriction in owl monkey cells. *J Virol* 79, 869-875.
- Friedrich, T.C., Dodds, E.J., Yant, L.J., Vojnov, L., Rudersdorf, R., Cullen, C., Evans, D.T., Desrosiers, R.C., Mothe, B.R., Sidney, J., Sette, A., Kunstman, K., Wolinsky, S., Piatak, M., Lifson, J., Hughes, A.L., Wilson, N., O'Connor, D.H., Watkins, D.I., 2004. Reversion of CTL escape-variant immunodeficiency viruses *in vivo*. *Nat Med* 10, 275-281.
- Fu, D.X., Jinno, A., Shimizu, N., Haraguchi, Y., Hoshino, H., 1999. Isolation and characterization of a monoclonal antibody that inhibits HIV-1 infection. *Microbes Infect* 1, 677-684.
- Gao, F., Robertson, D.L., Carruthers, C.D., Morrison, S.G., Jian, B., Chen, Y., Barre-Sinoussi, F., Girard, M., Srinivasan, A., Abimiku, A.G., Shaw, G.M., Sharp, P.M., Hahn, B.H., 1998. A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. *J Virol* 72, 5680-5698.
- Gurtler, L.G., Hauser, P.H., Eberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J.M., Kaptue, L., 1994. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J Virol* 68, 1581-1585.
- Hatzioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., Bieniasz, P.D., 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. *Proc Natl Acad Sci U S A* 101, 10774-10779.
- Ikeda, Y., Ylinen, L.M., Kahar-Bador, M., Towers, G.J., 2004. Influence of gag on human immunodeficiency virus type 1 species-specific tropism. *Journal of virology* 78, 11816-11822.
- Kambal, A., Mitchell, G., Cary, W., Gruenloh, W., Jung, Y., Kalomoiris, S., Nacey, C., McGee, J., Lindsey, M., Fury, B., Bauer, G., Nolta, J.A., Anderson, J.S., 2011. Generation of HIV-1 resistant and functional macrophages from hematopoietic stem cell-derived induced pluripotent stem cells. *Mol Ther* 19, 584-593.
- Keckesova, Z., Ylinen, L.M., Towers, G.J., 2006. Cyclophilin A renders human immunodeficiency virus type 1 sensitive to Old World monkey but not human TRIM5 alpha antiviral activity. *J Virol* 80, 4683-4690.
- Kiem, H.P., Jerome, K.R., Deeks, S.G., McCune, J.M., 2012. Hematopoietic-stem-cell-based gene therapy for HIV disease. *Cell Stem Cell* 10, 137-147.

- Kim, F.M., Kolson, D.L., Balliet, J.W., Srinivasan, A., Collman, R.G., 1995. V3-independent determinants of macrophage tropism in a primary human immunodeficiency virus type 1 isolate. *J Virol* 69, 1755-1761.
- Kirmaier, A., Wu, F., Newman, R.M., Hall, L.R., Morgan, J.S., O'Connor, S., Marx, P.A., Meythaler, M., Goldstein, S., Buckler-White, A., Kaur, A., Hirsch, V.M., Johnson, W.E., 2010. TRIM5 suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. *PLoS Biol* 8.
- Letunic, I., Bork, P., 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127-128.
- Letunic, I., Bork, P., 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* 39, W475-478.
- Li, Y., Kappes, J.C., Conway, J.A., Price, R.W., Shaw, G.M., Hahn, B.H., 1991. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J Virol* 65, 3973-3985.
- Li, Y., Li, X., Stremlau, M., Lee, M., Sodroski, J., 2006. Removal of arginine 332 allows human TRIM5alpha to bind human immunodeficiency virus capsids and to restrict infection. *J Virol* 80, 6738-6744.
- Liu, Y., McNevin, J., Zhao, H., Tebit, D.M., Troyer, R.M., McSweyn, M., Ghosh, A.K., Shriner, D., Arts, E.J., McElrath, M.J., Mullins, J.I., 2007. Evolution of human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitopes: fitness-balanced escape. *J Virol* 81, 12179-12188.
- Maillard, P.V., Reynard, S., Serhan, F., Turelli, P., Trono, D., 2007. Interfering residues narrow the spectrum of MLV restriction by human TRIM5alpha. *PLoS Pathog* 3, e200.
- Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H., Landau, N.R., 2003. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114, 21-31.
- Martinez-Picado, J., Prado, J.G., Fry, E.E., Pfafferott, K., Leslie, A., Chetty, S., Thobakgale, C., Honeyborne, I., Crawford, H., Matthews, P., Pillay, T., Rousseau, C., Mullins, J.I., Brander, C., Walker, B.D., Stuart, D.I., Kiepiela, P., Goulder, P., 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* 80, 3617-3623.

- Matsuoka, S., Dam, E., Lecossier, D., Clavel, F., Hance, A.J., 2009. Modulation of HIV-1 infectivity and cyclophilin A-dependence by Gag sequence and target cell type. *Retrovirology* 6, 21.
- Naviaux, R.K., Costanzi, E., Haas, M., Verma, I.M., 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *Journal of virology* 70, 5701-5705.
- Owens, C.M., Yang, P.C., Gottlinger, H., Sodroski, J., 2003. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol* 77, 726-731.
- Pacheco, B., Finzi, A., Stremlau, M., Sodroski, J., 2010. Adaptation of HIV-1 to cells expressing rhesus monkey TRIM5alpha. *Virology* 408, 204-212.
- Perez-Caballero, D., Hatzioannou, T., Zhang, F., Cowan, S., Bieniasz, P.D., 2005. Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. *J Virol* 79, 15567-15572.
- Perron, M.J., Stremlau, M., Lee, M., Javanbakht, H., Song, B., Sodroski, J., 2007. The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid. *J Virol* 81, 2138-2148.
- Pertel, T., Hausmann, S., Morger, D., Zuger, S., Guerra, J., Lascano, J., Reinhard, C., Santoni, F.A., Uchil, P.D., Chatel, L., Bisiaux, A., Albert, M.L., Strambio-De-Castillia, C., Mothes, W., Pizzato, M., Grutter, M.G., Luban, J., 2011. TRIM5 is an innate immune sensor for the retrovirus capsid lattice. *Nature* 472, 361-365.
- Pham, Q.T., Bouchard, A., Grutter, M.G., Berthoux, L., 2010. Generation of human TRIM5alpha mutants with high HIV-1 restriction activity. *Gene Ther* 17, 859-871.
- Rold, C.J., Aiken, C., 2008. Proteasomal degradation of TRIM5alpha during retrovirus restriction. *PLoS Pathog* 4, e1000074.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406-425.
- Sebastian, S., Sokolskaja, E., Luban, J., 2006. Arsenic counteracts human immunodeficiency virus type 1 restriction by various TRIM5 orthologues in a cell type-dependent manner. *Journal of virology* 80, 2051-2054.

- Shi, J., Aiken, C., 2006. Saturation of TRIM5 alpha-mediated restriction of HIV-1 infection depends on the stability of the incoming viral capsid. *Virology* 350, 493-500.
- Shimizu, N.S., Shimizu, N.G., Takeuchi, Y., Hoshino, H., 1994. Isolation and characterization of human immunodeficiency virus type 1 variants infectious to brain-derived cells: detection of common point mutations in the V3 region of the env gene of the variants. *J Virol* 68, 6130-6135.
- Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848-853.
- Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I., Sodroski, J., 2006a. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* 103, 5514-5519.
- Stremlau, M., Perron, M., Welikala, S., Sodroski, J., 2005. Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. *J Virol* 79, 3139-3145.
- Stremlau, M., Song, B., Javanbakht, H., Perron, M., Sodroski, J., 2006b. Cyclophilin A: an auxiliary but not necessary cofactor for TRIM5alpha restriction of HIV-1. *Virology* 351, 112-120.
- Takeuchi, Y., Inagaki, M., Kobayashi, N., Hoshino, H., 1987. Isolation of human immunodeficiency virus from a Japanese hemophilia B patient with AIDS. *Jpn J Cancer Res* 78, 11-15.
- Troyer, R.M., McNevin, J., Liu, Y., Zhang, S.C., Krizan, R.W., Abraha, A., Tebit, D.M., Zhao, H., Avila, S., Lobritz, M.A., McElrath, M.J., Le Gall, S., Mullins, J.I., Arts, E.J., 2009. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathog* 5, e1000365.
- Yap, M.W., Nisole, S., Lynch, C., Stoye, J.P., 2004. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc Natl Acad Sci U S A* 101, 10786-10791.
- Yap, M.W., Nisole, S., Stoye, J.P., 2005. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr Biol* 15, 73-78.

- Zhao, G., Ke, D., Vu, T., Ahn, J., Shah, V.B., Yang, R., Aiken, C., Charlton, L.M., Gronenborn, A.M., Zhang, P., 2011. Rhesus TRIM5alpha disrupts the HIV-1 capsid at the inter-hexamer interfaces. PLoS Pathog 7, e1002009.
- Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L., Trono, D., 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. Nature biotechnology 15, 871-875.

CHAPITRE V

DISCUSSION ET PERSPECTIVES

S'inscrivant dans l'effort concerté de lutte contre la pandémie de VIH-1, l'identification et la caractérisation de transgènes humains efficaces contre le VIH-1 est un enjeu de taille. L'un d'entre eux, le facteur de restriction TRIM5 α , a déjà fait l'objet d'efforts qui ont permis d'identifier des mutations lui conférant une activité anti-VIH. Entre autres, notre laboratoire a identifié le mutant R332G-R335G qui permet une diminution d'environ 20 fois de l'infectivité du VIH-1. La possibilité d'avoir un transgène encore plus actif nous a poussés à réaliser un crible par mutagenèse aléatoire de la région variable 1 du domaine de reconnaissance de la capsid virale de TRIM5 α . Ceci nous a permis d'identifier une autre mutation, G330E, qui permet de restreindre le VIH-1. Cependant, la combinaison de cette nouvelle mutation avec R332G-R335G n'est pas parvenue à augmenter le potentiel de ce transgène. Au contraire, une réduction de l'efficacité a été observée sous certaines conditions. Le meilleur candidat, R332G-R335G, a donc été retenu pour nos études subséquentes.

Le VIH-1 étant un virus hautement mutagénique, il est normal et prévisible d'identifier des virions ayant acquis des mutations leur permettant d'échapper à une pression sélective. Pour TRIM5 α , la littérature a présenté une mutation de la capsid virale, V86M, qui permet une résistance à ce facteur de restriction chez le singe rhésus [74]. Considérant que ce TRIM5 α a le même mécanisme d'action que notre transgène, nous avons étudié si cette mutation pouvait également conférer une résistance aux mutants de TRIM5 α_{Hu} , dont le mutant R332G-R335G. Nos résultats ont confirmé notre hypothèse et nous avons ainsi voulu déterminer le mécanisme de cette résistance. L'étude de l'effet de V86M sur le transport nucléaire du PIC et de l'implication de la CypA a permis de suggérer que V86M résiste à TRIM5 α par l'abrogation des mécanismes de restriction CypA-dépendant, rétablissant ainsi le transport nucléaire du PIC.

Alors que les études sur l'activité de restriction des transgènes de TRIM5 α _{Hu} n'avaient été effectuées qu'avec des souches du VIH-1 adaptées au laboratoire, aucune donnée n'était disponible sur l'efficacité de ces transgènes contre des souches cliniques. Nous avons donc testé cette activité contre un vaste éventail de souches dérivées d'isolats cliniques avec des représentants de plusieurs groupes et sous-groupes du VIH. Ces expériences ont montré que R332G-R335G permet la restriction efficace de toutes les souches testées, suggérant ainsi que la restriction par ce mutant n'est pas limitée aux souches de laboratoire ou quelques sous-groupes du VIH.

5.1 L'identification de transgènes aux propriétés antivirales

Depuis l'essor des technologies de biologie moléculaire, de plus en plus de techniques différentes sont disponibles pour identifier, modifier et améliorer des gènes d'intérêts et les protéines qui en découlent. Ainsi, les scientifiques ont pris avantage de ces technologies pour améliorer divers aspects de la condition humaine, ce qui inclut autant des applications médicales qu'industrielles. D'un point de vue médical, les maladies infectieuses demeurent à ce jour un problème d'envergure et la biologie médicale offre des approches novatrices afin d'identifier de nouvelles stratégies thérapeutiques. De plus, elle permet de mieux caractériser ces nouvelles stratégies, accélérant ainsi le processus vers une application clinique.

5.1.1 Transgènes de TRIM5 α

Alors que la comparaison de la séquence d'un orthologue restrictif de TRIM5 α avec celle de la protéine humaine a permis l'identification d'une mutation, deux approches différentes par mutagenèse aléatoire ont résulté en l'identification de deux autres de ces mutations. Malgré le fait qu'elles proviennent de trois stratégies différentes, ces mutations sont situées au sein d'une même portion de la région variable 1 de TRIM5 α (acides aminés 330 à 335). Récemment, des expériences de résonance magnétique nucléaire (NMR) ont permis d'affirmer que le domaine PRYSPRY de TRIM5 α interagit par ses quatre régions variables avec la CA virale [36].

Or, de simples mutations ponctuelles dans la région v1 permettent une restriction du VIH-1. Ce qui semble à priori contradictoire est en partie expliqué par le modèle structural présenté en Figure 2.7 qui suggère que ces mutations ponctuelles peuvent avoir un effet répandu sur les trois autres régions variables sans toutefois affecter le repliement de la protéine. Il est possible que le mécanisme par lequel TRIM5 α reconnaît la capsid virale, via un assemblage multimérique et de faibles interactions, puisse permettre à l'activité du facteur de restriction de résister à des mutations induisant des changements conformationnels marqués. De plus, les mêmes travaux de NMR suggèrent que l'interaction PRYSPRY-CA est dominée par une région v1 mobile, donnant ainsi une flexibilité au domaine PRYSPRY ce qui lui permet de s'adapter aux diverses conformations du cœur viral. Il n'est donc pas surprenant que des mutations ponctuelles dans cette région qui semble si importante induisent de tels changements sur le potentiel de restriction.

Cette flexibilité pourrait également être impliquée dans le potentiel qu'a TRIM5 α de restreindre différents virus ou encore de s'adapter aux différences présentes chez un même virus (différents groupes et sous-groupes du VIH, par exemple). Une telle flexibilité est d'ailleurs observée dans d'autres composantes du système immunitaire tels que les IgM [36, 75]. C'est en effet cette flexibilité qui leur permet de réagir à différents antigènes.

Bien que la région v1 semble être un facteur déterminant dans la spécificité de la restriction, il n'est pas exclu que d'autres mutations dans les autres régions variables du domaine PRYSPRY puissent conduire à la restriction du VIH-1 par TRIM5 α_{Hu} . D'ailleurs, il est important de rappeler que le crible présenté dans cet ouvrage était limité à une mutagenèse de la région v1. Cependant, le crible par Pham *et al.* [70] a plutôt utilisé une mutagenèse du domaine PRYSPRY. Bien qu'il ait identifié qu'une mutation dans la région v1, il est possible que la méthodologie utilisée dans le crible ait favorisé une identification des mutations dans les régions les plus déterminantes seulement. Néanmoins, la réussite de ces deux cribles montre bien le potentiel qu'ont ces approches d'identifier de nouveaux transgènes humains à caractère antiviral.

5.1.2 Transgènes d'autres facteurs de restriction

Alors que notre étude se limite à TRIM5 α , d'autres facteurs de restriction font néanmoins l'objet de travaux dans d'autres laboratoires. L'un d'entre eux, APOBEC3G, est une cytidine déaminase qui induit une hypermutation de l'ADN virale en interférant avec le processus de la transcription inverse. Avec l'évolution, le VIH-1 a cependant développé un mécanisme de résistance à ce facteur de restriction : Vif. En effet, la protéine virale Vif est capable d'interagir et de lier APOBEC3G et favorise sa dégradation par le protéasome. La forme humaine d'APOBEC3G n'est donc pas efficace contre le VIH-1.

À l'instar de TRIM5 α , on retrouve chez le singe rhésus un orthologue d'APOBEC3G qui est actif contre le VIH-1. Un groupe de recherche a donc introduit des mutations chez la protéine humaine par comparaison de séquence avec la version simienne. Cette approche leur a permis d'identifier une mutation (D128K) qui rend APOBEC3G résistant à l'action de Vif, rétablissant ainsi son activité antivirale [76]. Bien que cette approche ait porté fruit, il est probable qu'un crible par mutagenèse aléatoire, tel que nous l'avons réalisé pour TRIM5 α , serait un outil précieux pour l'identification de mutations supplémentaires conférant une résistance à Vif.

5.1.3 D'autres candidats se cachent dans le génome

Bien qu'il est utile d'utiliser des cribles par mutagenèse aléatoire pour modifier des facteurs de restriction connus, d'importants travaux ont été réalisés afin d'identifier de nouveaux facteurs de restriction ou protéines cellulaires essentielles à la réplication virale. En ce sens, un crible ciblant plus de 19 000 gènes humains a permis d'identifier 114 facteurs qui présentaient un impact significatif sur l'infection du VIH-1 [77]. De ce nombre, les auteurs ont choisi de cibler leurs expériences supplémentaires sur un seul d'entre eux, laissant donc un vaste nombre de possibilités inexplorées. Considérant qu'il faut énormément d'efforts et de temps pour caractériser de nouveaux facteurs de restriction, il est probable que de mieux comprendre les cofacteurs cellulaires essentiels à l'infection du VIH, tels la protéine LEDGF qui est essentielle à l'intégration optimale

de l'ADN virale dans le génome cellulaire, puisse ouvrir de nouvelles voies vers des stratégies thérapeutiques, qu'elles soient pharmacologiques ou via thérapie génique [78].

5.2 La résistance virale et le cas de CA-V86M

5.2.1 Transduction, virus répliatif et TRIM5 α : ce que nous apprend V86M

D'un point de vue pratique, il existe principalement deux stratégies différentes pour analyser la restriction de facteurs tels que TRIM5 α . Afin de permettre une manipulation plus aisée et sécuritaire, et puisque TRIM5 α agit aux étapes précoces du cycle de réplication, la plupart des expériences sont réalisées avec des souches non-répliatives du VIH. Le génome de ces souches ne code pas pour l'enveloppe virale, laquelle est introduite sur un autre vecteur utilisé lors de la production des virions. L'enveloppe naturelle du VIH, qui limite l'infection aux cellules exprimant CD4 et un corécepteur spécifique, est bien souvent remplacée par l'enveloppe du virus de la stomatite vésiculaire (VSV) qui elle permet l'entrée du virus dans plusieurs types cellulaires. Utilisant un tel virus modifié, l'analyse de la restriction par TRIM5 α se fait alors en observant la diminution du nombre de cellules infectées dans l'échantillon. La deuxième stratégie, qui reflète un peu plus la réalité du virus, utilise un génome viral complet. Ces virus seront donc répliatifs et l'analyse de la restriction par TRIM5 α se fera indirectement en mesurant la quantité de virus produits par ces cellules.

Bien que l'on puisse observer l'efficacité des mutants de TRIM5 α dans ces deux contextes expérimentaux, d'importantes différences restent à prendre en considération et sont soulevées par les expériences réalisées avec CA-V86M. En effet, une étude antérieure démontrant que la mutation V86M confère une résistance à TRIM5 α_{Rh} présente l'observation de cette résistance sous les deux contextes expérimentaux, non- et répliatif [74]. Or, nous n'avons pas observé la résistance de V86M aux mutants de TRIM5 α_{Hu} lors d'expériences de réplication. Il y a donc disparité entre i) les résultats des deux contextes et ii) nos résultats et ceux déjà publiés. L'explication du premier point est plus aisée, puisque malgré que TRIM5 α agisse aux

étapes précoces du cycle de réplication virale, il n'est pas exclu qu'il puisse exercer une activité à une autre étape du cycle viral. En ce sens, certaines études ont suggéré que TRIM5 α puisse jouer un rôle inhibiteur dans l'assemblage des nouvelles particules virales [79, 80].

Pour ce qui est de la disparité observée entre nos résultats et ceux du groupe ayant identifié V86M, il est important de noter que nous n'avons pas utilisé le même type cellulaire qu'eux. En effet, leur système était basé sur l'infection de cellules HeLa-CD4 exprimant TRIM5 α_{Rh} . Pour notre part, nous avons privilégié un modèle basé sur l'infection d'une lignée cellulaire de lymphocytes T (Sup-T1). Un des facteurs ayant pu résulter en la disparité observée est la transmission virale par ce qui est appelé les synapses virales. Ce type de transmission est différent de la transmission classique en ce sens qu'il se fait de cellule à cellule, sans que les virus aient besoin d'être relâchés dans le milieu extracellulaire. À ce jour, peu est connu sur les conséquences qu'a la transmission synaptique sur le virus. Considérant que le temps de maturation du virus et que la méthode d'entrée cellulaire sont différents, il est possible que ce type de transmission vienne modifier le déroulement des étapes post-entrée, pouvant donc ainsi altérer la susceptibilité à la restriction par TRIM5 α .

5.2.2 La résistance virale dans un contexte clinique

Il est également important de reconnaître et d'évaluer la signification d'une mutation de résistance isolée *in vitro* sur l'efficacité potentielle des traitements thérapeutiques qui découleraient d'une utilisation de TRIM α . Premièrement, nous n'avons pas observé de réplication accrue du virus V86M dans un contexte répliatif en présence des transgènes de TRIM5 α , ce qui tend à diminuer l'impact clinique qu'aurait cette mutation. De plus, le contexte clinique est de plusieurs ordres de grandeur plus complexe, faisant intervenir les fonctions immunes du patient et les drogues antirétrovirales qui seraient toujours utilisées. La probabilité de voir resurgir une telle mutation résistante à TRIM5 α en est donc d'autant réduite. Néanmoins, il ne faut pas

sous-estimer le potentiel mutagénique du virus, alors que l'une des principales causes de l'échec des traitements actuels est la résistance virale.

De plus, à la lumière des analyses d'isolats cliniques effectués au chapitre IV (voir Figure 4.1), la région de la boucle de liaison à CypA de la CA virale semble être importante pour diverses fonctions virales. En effet, alors que la mutation V86M procure une résistance à TRIM5 α , la mutation V86A permet d'échapper aux CTL, mais induit une sensibilité plus grande à TRIM5 α [81]. Les effets opposés observés par des mutations à cette position pourraient rendre la résistance virale à l'un ou l'autre des facteurs plus difficile.

5.3 Thérapie génique : implications des résultats obtenus et perspectives

5.3.1 Transgènes de TRIM5 α : leur potentiel clinique

Sans expériences concrètes, l'étude des transgènes de TRIM5 α dans un contexte clinique n'est que spéculative. Cependant, puisque TRIM5 α agit aux étapes précoces de la réplication virale il réduit donc dramatiquement les niveaux de transcrits viraux produits. La synthèse des nouvelles protéines virales en est donc réduite et le risque de voir surgir une mutation de résistance est faible. De plus, la thérapie génique vise à repeupler le patient de cellules immunitaires résistantes au virus, rétablissant ainsi les fonctions immunes. S'il demeure actif, le système immunitaire est capable de bâtir une forte réponse contre le virus. En effet, plusieurs études ont présenté des données suggérant que les anticorps neutralisants et la réponse cytotoxique dépendante des anticorps exercent une forte pression sélective sur le virus [82, 83]. Combinée à la restriction par un transgène de TRIM5 α , il est probable que le potentiel qu'aurait le virus d'échapper à toutes ces pressions simultanées soit dramatiquement réduit.

Un autre déterminant essentiel au succès d'une thérapie génique impliquant TRIM5 α est la survie et la prolifération des cellules modifiées. Les déterminants de ce point sont répartis en deux facettes. Premièrement, afin que les cellules modifiées

puissent être acceptées et non rejetées par l'hôte, leur présentation antigénique ne devrait pas être modifiée d'une manière à présenter des antigènes du non-soi. Spéculativement, l'utilisation de transgènes modifiés par quelques mutations devrait réduire les probabilités que les cellules modifiées ne soient rejetées par l'hôte. Deuxièmement, peu est connu sur le devenir des antigènes du VIH après restriction par TRIM5 α . Théoriquement, il est possible que des antigènes du virus soient incorporés et présentés par le complexe majeur d'histocompatibilité. Cette présentation d'antigène pourrait résulter en une activation de cellules cytotoxiques contre la cellule ayant interrompu l'infection par le VIH, diminuant ainsi l'avantage sélectif des cellules modifiées.

Une équipe de recherche a par ailleurs utilisé une nouvelle approche de modification génique afin de réaliser l'introduction de plusieurs gènes anti-VIH tout en empêchant l'expression du corécepteur CCR5 [84]. À cette fin, ils ont utilisé des nucléases à doigt de zinc (ZFN) afin de cibler spécifiquement le gène CCR5 et y induire un bris du double brin d'ADN. Tirant profit de la machinerie de réparation de l'ADN par homologie, ils ont introduit un vecteur exogène où étaient placés des bras d'homologie pour CCR5 entourant une cassette d'expression pour une chimère humaine – rhesus de TRIM5 α , APOBEC3G D128K et Rev M10. Ce dernier est une version dominante négative de la protéine virale Rev, et lorsqu'exprimée empêche l'activité de la protéine Rev virale, ce qui inhibe la réplication en altérant l'export nucléaire des transcrits viraux non épissés. Cette approche a résulté en l'insertion permanente de ces transgènes dans le gène CCR5 et a rendu les cellules modifiées à première vue totalement résistantes à l'infection par le VIH-1. Néanmoins, cette stratégie demeure loin d'être complètement viable cliniquement puisqu'elle emploie une surexpression des facteurs de restrictions alors que TRIM5 α peut engendrer de graves dérèglements de la signalisation de l'immunité innée dans ce contexte [56].

5.4 Perspectives générales

Bien que les travaux présentés dans cet ouvrage pavent la voie vers de potentielles approches cliniques utilisant des transgènes de TRIM5 α , plusieurs étapes et analyses

restent à faire. Tout d'abord, mieux comprendre les modifications à la structure de TRIM5 α qu'apportent les mutations dans le domaine PRYSPRY : ceci pourrait aider à identifier avec précision les déterminants de l'interaction TRIM5 α – CA. Pour ce faire, l'utilisation de techniques de biologie structurale telles que la cristallographie protéique serait un atout.

Concernant la résistance de V86M à TRIM5 α , nous avons pu cibler le mécanisme de cette résistance à l'interaction CypA – CA modifiée. Cependant, le rôle exact de CypA dans la réplication virale n'est pas connu. Les récentes études et la nôtre tendent à suggérer que CypA pourrait favoriser l'import nucléaire du PIC [68, 85]. Puisque CypA joue également un rôle important dans la restriction par TRIM5 α , mieux comprendre le rôle de CypA dans la réplication virale pourrait aider à mieux cerner les mécanismes de restriction et de résistance à TRIM5 α . Également, puisque la mutation de résistance étudiée ici a été identifiée après passage en série du virus dans des cellules exprimant TRIM5 α_{Rh} , il serait pertinent d'essayer d'identifier de nouvelles mutations conférant une résistance à TRIM5 α_{Hu} R332G-R335G. Ceci pourrait permettre de mieux prédire des mutations de résistance qui pourraient se développer *in vivo*.

La dernière facette de cet ouvrage concernait la restriction de différents isolats cliniques du VIH-1 par nos transgènes de TRIM5 α . Bien que nous ayons analysé la restriction de souches représentant les divers groupes et sous-groupes du VIH, effectuer cette expérience sur un vaste nombre d'échantillons isolés d'une même cohorte de patients infectés permettrait de mieux cerner les variations de restriction auxquelles on pourrait s'attendre dans un contexte clinique.

CONCLUSION

La recherche de nouvelles approches thérapeutiques contre le VIH/SIDA est un enjeu de taille. Les travaux présentés dans cet ouvrage permettent d'avancer les connaissances sur le facteur de restriction TRIM5 α , notamment sur sa spécificité d'action et les mécanismes de résistance virale. Nous avons également démontré le potentiel qu'a la mutagenèse aléatoire d'identifier de nouveaux transgènes à activité antivirale. Somme toute, l'ensemble de ces travaux continue de paver la voie vers une thérapie génique utilisant des transgènes de TRIM5 α , laquelle semble être un objectif de plus en plus réaliste.

RÉFÉRENCES BIBLIOGRAPHIQUES

1. Organisation mondiale de la santé, **Rapport ONUSIDA sur l'épidémie mondiale de SIDA 2010**, ONUSIDA, 2010
2. Hamers, R.L., et al., **The status of HIV-1 resistance to antiretroviral drugs in sub-Saharan Africa**. *Antiviral therapy*, 2008. **13**(5): p. 625-39.
3. Obiako, O.R., H.M. Murktar, and D. Ogoina, **Antiretroviral drug resistance implications for HIV/AIDS reduction in sub-Saharan Africa and other developing countries**. *Nigerian journal of medicine: journal of the National Association of Resident Doctors of Nigeria*, 2010. **19**(3): p. 302-10.
4. Murnane, P.M., et al., **Using plasma viral load to guide antiretroviral therapy initiation to prevent HIV-1 transmission**. *PloS one*, 2012. **7**(11): p. e51192.
5. Sung, H., et al., **High frequency of drug resistance mutations in human immunodeficiency virus type 1-infected Korean patients treated with HAART**. *AIDS research and human retroviruses*, 2007. **23**(10): p. 1223-9.
6. Uy, J., et al., **Initiation of HAART at higher CD4 cell counts is associated with a lower frequency of antiretroviral drug resistance mutations at virologic failure**. *Journal of acquired immune deficiency syndromes*, 2009. **51**(4): p. 450-3.
7. Maia Teixeira, S.L., et al., **Trends in drug resistance mutations in antiretroviral-naïve intravenous drug users of Rio de Janeiro**. *Journal of medical virology*, 2006. **78**(6): p. 764-9.
8. Frentz, D., C.A. Boucher, and D.A. van de Vijver, **Temporal changes in the epidemiology of transmission of drug-resistant HIV-1 across the world**. *AIDS reviews*, 2012. **14**(1): p. 17-27.
9. Marmor, M., et al., **Homozygous and heterozygous CCR5-Delta32 genotypes are associated with resistance to HIV infection**. *Journal of acquired immune deficiency syndromes*, 2001. **27**(5): p. 472-81.
10. Samson, M., et al., **Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene**. *Nature*, 1996. **382**(6593): p. 722-5.

11. Dean, M., et al., **Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene.** Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*, 1996. **273**(5283): p. 1856-62.
12. Hutter, G., et al., **Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation.** *The New England journal of medicine*, 2009. **360**(7): p. 692-8.
13. Okano, H., et al., **Steps toward safe cell therapy using induced pluripotent stem cells.** *Circulation research*, 2013. **112**(3): p. 523-33.
14. Cheng, G., et al., **IL-2R signaling is essential for functional maturation of regulatory T cells during thymic development.** *Journal of immunology*, 2013. **190**(4): p. 1567-75.
15. Hacein-Bey-Abina, S., et al., **Efficacy of gene therapy for X-linked severe combined immunodeficiency.** *The New England journal of medicine*, 2010. **363**(4): p. 355-64.
16. Hacein-Bey-Abina, S., et al., **Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1.** *The Journal of clinical investigation*, 2008. **118**(9): p. 3132-42.
17. Shimizu, A., et al., **Novel gene therapy viral vector using non-oncogenic lymphotropic herpesvirus.** *PloS one*, 2013. **8**(2): p. e56027.
18. Fang, Y., et al., **A self-deletion lentiviral vector to reduce the risk of replication-competent virus formation.** *The journal of gene medicine*, 2013. **15**(2): p. 102-12.
19. Craigie, R. and F.D. Bushman, **HIV DNA Integration.** *Cold Spring Harbor perspectives in medicine*, 2012. **2**(7): p. a006890.
20. Malbec, M., **Implication de MDM2 dans la restriction des rétrovirus par TRIM5a selon un mécanisme dépendant du protéasome.** Mémoire de maîtrise en biophysique et biologie cellulaires, 2011, Université du Québec à Trois-Rivières.
21. Forshey, B.M., et al., **Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication.** *Journal of virology*, 2002. **76**(11): p. 5667-77.

22. DesGroseillers, L. and P. Jolicoeur, **Physical mapping of the Fv-1 tropism host range determinant of BALB/c murine leukemia viruses.** *Journal of virology*, 1983. **48**(3): p. 685-96.
23. Cowan, S., et al., **Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism.** *Proceedings of the National Academy of Sciences of the United States of America*, 2002. **99**(18): p. 11914-9.
24. Munk, C., et al., **A dominant block to HIV-1 replication at reverse transcription in simian cells.** *Proceedings of the National Academy of Sciences of the United States of America*, 2002. **99**(21): p. 13843-8.
25. Sayah, D.M., et al., **Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1.** *Nature*, 2004. **430**(6999): p. 569-73.
26. Stremlau, M., et al., **The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys.** *Nature*, 2004. **427**(6977): p. 848-53.
27. Meroni, G. and G. Diez-Roux, **TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases.** *BioEssays: news and reviews in molecular, cellular and developmental biology*, 2005. **27**(11): p. 1147-57.
28. Reymond, A., et al., **The tripartite motif family identifies cell compartments.** *The EMBO journal*, 2001. **20**(9): p. 2140-51.
29. Luban, J., **Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection.** *Journal of virology*, 2007. **81**(3): p. 1054-61.
30. Lienlaf, M., et al., **Contribution of E3-ubiquitin ligase activity to HIV-1 restriction by TRIM5alpha(rh): structure of the RING domain of TRIM5alpha.** *Journal of virology*, 2011. **85**(17): p. 8725-37.
31. Diaz-Griffero, F., et al., **Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5.** *Virology*, 2006. **349**(2): p. 300-15.
32. Kim, J., C. Tipper, and J. Sodroski, **Role of TRIM5alpha RING domain E3 ubiquitin ligase activity in capsid disassembly, reverse transcription blockade, and restriction of simian immunodeficiency virus.** *Journal of virology*, 2011. **85**(16): p. 8116-32.
33. Diaz-Griffero, F., et al., **Modulation of retroviral restriction and proteasome inhibitor-resistant turnover by changes in the TRIM5alpha B-box 2 domain.** *Journal of virology*, 2007. **81**(19): p. 10362-78.

34. Diaz-Griffero, F., et al., **A B-box 2 surface patch important for TRIM5alpha self-association, capsid binding avidity, and retrovirus restriction.** *Journal of virology*, 2009. **83**(20): p. 10737-51.
35. Ganser-Pornillos, B.K., et al., **Hexagonal assembly of a restricting TRIM5alpha protein.** *Proceedings of the National Academy of Sciences of the United States of America*, 2011. **108**(2): p. 534-9.
36. Biris, N., et al., **Structure of the rhesus monkey TRIM5alpha PRYSPRY domain, the HIV capsid recognition module.** *Proceedings of the National Academy of Sciences of the United States of America*, 2012. **109**(33): p. 13278-83.
37. Nakayama, E.E., et al., **A specific region of 37 amino acid residues in the SPRY (B30.2) domain of African green monkey TRIM5alpha determines species-specific restriction of simian immunodeficiency virus SIVmac infection.** *Journal of virology*, 2005. **79**(14): p. 8870-7.
38. Stremlau, M., et al., **Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction.** *Journal of virology*, 2005. **79**(5): p. 3139-45.
39. Kar, A.K., et al., **Biochemical and biophysical characterization of a chimeric TRIM21-TRIM5alpha protein.** *Journal of virology*, 2008. **82**(23): p. 11669-81.
40. Stremlau, M., et al., **Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor.** *Proceedings of the National Academy of Sciences of the United States of America*, 2006. **103**(14): p. 5514-9.
41. Langelier, C.R., et al., **Biochemical characterization of a recombinant TRIM5alpha protein that restricts human immunodeficiency virus type 1 replication.** *Journal of virology*, 2008. **82**(23): p. 11682-94.
42. Sawyer, S.L., et al., **Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain.** *Proceedings of the National Academy of Sciences of the United States of America*, 2005. **102**(8): p. 2832-7.
43. Bieniasz, P.D., **Restriction factors: a defense against retroviral infection.** *Trends in microbiology*, 2003. **11**(6): p. 286-91.

44. Song, B., et al., **Retrovirus restriction by TRIM5alpha variants from Old World and New World primates.** *Journal of virology*, 2005. **79**(7): p. 3930-7.
45. Soares, E.A., et al., **Evolution of TRIM5alpha B30.2 (SPRY) domain in New World primates.** *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases*, 2010. **10**(2): p. 246-53.
46. Johnson, W.E. and S.L. Sawyer, **Molecular evolution of the antiretroviral TRIM5 gene.** *Immunogenetics*, 2009. **61**(3): p. 163-76.
47. Sebastian, S. and J. Luban, **TRIM5alpha selectively binds a restriction-sensitive retroviral capsid.** *Retrovirology*, 2005. **2**: p. 40.
48. Black, L.R. and C. Aiken, **TRIM5alpha disrupts the structure of assembled HIV-1 capsid complexes in vitro.** *Journal of virology*, 2010. **84**(13): p. 6564-9.
49. Perron, M.J., et al., **The human TRIM5alpha restriction factor mediates accelerated uncoating of the N-tropic murine leukemia virus capsid.** *Journal of virology*, 2007. **81**(5): p. 2138-48.
50. Zhao, G., et al., **Rhesus TRIM5alpha disrupts the HIV-1 capsid at the inter-hexamer interfaces.** *PLoS pathogens*, 2011. **7**(3): p. e1002009.
51. Yang, Y., T. Fricke, and F. Diaz-Griffero, **Inhibition of reverse transcriptase activity increases stability of the HIV-1 core.** *Journal of virology*, 2013. **87**(1): p. 683-7.
52. Hulme, A.E., O. Perez, and T.J. Hope, **Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription.** *Proceedings of the National Academy of Sciences of the United States of America*, 2011. **108**(24): p. 9975-80.
53. Wu, X., et al., **Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection.** *Proceedings of the National Academy of Sciences of the United States of America*, 2006. **103**(19): p. 7465-70.
54. Kutluay, S.B., D. Perez-Caballero, and P.D. Bieniasz, **Fates of Retroviral Core Components during Unrestricted and TRIM5-Restricted Infection.** *PLoS pathogens*, 2013. **9**(3): p. e1003214.

55. Anderson, J.L., et al., **Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins.** *Journal of virology*, 2006. **80**(19): p. 9754-60.
56. Pertel, T., et al., **TRIM5 is an innate immune sensor for the retrovirus capsid lattice.** *Nature*, 2011. **472**(7343): p. 361-5.
57. Siren, J., et al., **Cytokine and contact-dependent activation of natural killer cells by influenza A or Sendai virus-infected macrophages.** *The Journal of general virology*, 2004. **85**(Pt 8): p. 2357-64.
58. Carthagen, L., et al., **Human TRIM gene expression in response to interferons.** *PloS one*, 2009. **4**(3): p. e4894.
59. Carthagen, L., et al., **Implication of TRIM alpha and TRIMCyp in interferon-induced anti-retroviral restriction activities.** *Retrovirology*, 2008. **5**: p. 59.
60. Battivelli, E., et al., **Strain-specific differences in the impact of human TRIM5alpha, different TRIM5alpha alleles, and the inhibition of capsid-cyclophilin A interactions on the infectivity of HIV-1.** *Journal of virology*, 2010. **84**(21): p. 11010-9.
61. Mohanram, V., et al., **IFN-alpha Induces APOBEC3G, F, and A in Immature Dendritic Cells and Limits HIV-1 Spread to CD4+ T Cells.** *Journal of immunology*, 2013. **190**(7): p. 3346-53.
62. Bosco, D.A., et al., **Catalysis of cis/trans isomerization in native HIV-1 capsid by human cyclophilin A.** *Proceedings of the National Academy of Sciences of the United States of America*, 2002. **99**(8): p. 5247-52.
63. Gamble, T.R., et al., **Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid.** *Cell*, 1996. **87**(7): p. 1285-94.
64. Sokolskaja, E., L. Berthoux, and J. Luban, **Cyclophilin A and TRIM5alpha independently regulate human immunodeficiency virus type 1 infectivity in human cells.** *Journal of virology*, 2006. **80**(6): p. 2855-62.
65. Towers, G.J., et al., **Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors.** *Nature medicine*, 2003. **9**(9): p. 1138-43.
66. Keckesova, Z., L.M. Ylinen, and G.J. Towers, **Cyclophilin A renders human immunodeficiency virus type 1 sensitive to Old World monkey but not human TRIM5 alpha antiviral activity.** *Journal of virology*, 2006. **80**(10): p. 4683-90.

67. Shah, V.B., et al., **The host proteins transportin SR2/TNPO3 and cyclophilin A exert opposing effects on HIV-1 uncoating.** *Journal of virology*, 2013. **87**(1): p. 422-32.
68. Ambrose, Z., et al., **Human immunodeficiency virus type 1 capsid mutation N74D alters cyclophilin A dependence and impairs macrophage infection.** *Journal of virology*, 2012. **86**(8): p. 4708-14.
69. Yap, M.W., S. Nisole, and J.P. Stoye, **A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction.** *Current biology: CB*, 2005. **15**(1): p. 73-8.
70. Pham, Q.T., et al., **Generation of human TRIM5alpha mutants with high HIV-1 restriction activity.** *Gene therapy*, 2010. **17**(7): p. 859-71.
71. Kambal, A., et al., **Generation of HIV-1 resistant and functional macrophages from hematopoietic stem cell-derived induced pluripotent stem cells.** *Molecular therapy: the journal of the American Society of Gene Therapy*, 2011. **19**(3): p. 584-93.
72. Monaco, C., et al., **Canonical pathway of nuclear factor kappa B activation selectively regulates proinflammatory and prothrombotic responses in human atherosclerosis.** *Proceedings of the National Academy of Sciences of the United States of America*, 2004. **101**(15): p. 5634-9.
73. Escarcega, R.O., et al., **The transcription factor nuclear factor-kappa B and cancer.** *Clinical oncology*, 2007. **19**(2): p. 154-61.
74. Pacheco, B., et al., **Adaptation of HIV-1 to cells expressing rhesus monkey TRIM5alpha.** *Virology*, 2010. **408**(2): p. 204-12.
75. Notkins, A.L., **Polyreactivity of antibody molecules.** *Trends in immunology*, 2004. **25**(4): p. 174-9.
76. Xu, H., et al., **A single amino acid substitution in human APOBEC3G antiretroviral enzyme confers resistance to HIV-1 virion infectivity factor-induced depletion.** *Proceedings of the National Academy of Sciences of the United States of America*, 2004. **101**(15): p. 5652-7.
77. Liu, L., et al., **A whole genome screen for HIV restriction factors.** *Retrovirology*, 2011. **8**: p. 94.

78. Christ, F. and Z. Debyser, **The LEDGF/p75 integrase interaction, a novel target for anti-HIV therapy.** *Virology*, 2013. **435**(1): p. 102-9.
79. Sakuma, R., S. Ohmine, and Y. Ikeda, **Determinants for the rhesus monkey TRIM5alpha-mediated block of the late phase of HIV-1 replication.** *The Journal of biological chemistry*, 2010. **285**(6): p. 3784-93.
80. Zhang, X., et al., **Inhibitory effect of human TRIM5alpha on HIV-1 production.** *Microbes and infection/Institut Pasteur*, 2010. **12**(10): p. 768-77.
81. Battivelli, E., et al., **Gag cytotoxic T lymphocyte escape mutations can increase sensitivity of HIV-1 to human TRIM5alpha, linking intrinsic and acquired immunity.** *Journal of virology*, 2011. **85**(22): p. 11846-54.
82. Overbaugh, J. and L. Morris, **The Antibody Response against HIV-1.** *Cold Spring Harbor perspectives in medicine*, 2012. **2**(1): p. a007039.
83. Chung, A.W., et al., **Immune escape from HIV-specific antibody-dependent cellular cytotoxicity (ADCC) pressure.** *Proceedings of the National Academy of Sciences of the United States of America*, 2011. **108**(18): p. 7505-10.
84. Voit, R.A., et al., **Generation of an HIV Resistant T-cell Line by Targeted "Stacking" of Restriction Factors.** *Molecular therapy: the journal of the American Society of Gene Therapy*, 2013.
85. Schaller, T., et al., **HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting and replication efficiency.** *PLoS pathogens*, 2011. **7**(12): p. e1002439.