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CARACTÉRISATION DE LA RESTRICTION DES RÉTROVIRUS  
PAR LES PROTÉINES DE LA FAMILLE TRIM5 ET RÔLES  
DES MOTIFS RELATIFS À LA SUMOYLATION

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## RÉSUMÉ

La lutte contre le virus du VIH demeure un enjeu d'actualité puisqu'il n'existe, encore à ce jour, aucune cure efficace. Avec plus de 35 millions de personnes infectées à travers le monde, le développement d'une cure est primordial. Vu l'apparition de virus résistants avec les traitements actuels, le développement d'une thérapie génique impliquant des facteurs de restriction, protéines naturellement exprimées chez l'hôte possédant une activité antivirale, s'avère une alternative intéressante. TRIM5 $\alpha$  est une protéine exprimée chez beaucoup de mammifères et elle permet d'inhiber la transmission inter-espèces de plusieurs rétrovirus, notamment le VIH-1. La caractérisation de TRIM5 $\alpha$  et la compréhension des mécanismes impliqués dans la restriction des rétrovirus sont nécessaires au développement d'une thérapie impliquant cette protéine.

TRIM5 $\alpha$  inhibe le VIH peu de temps après l'entrée du virus dans la cellule en interagissant avec la capsid virale via le domaine PRYSPRY situé en C-terminal. Ce dernier détermine le tropisme de TRIM5 $\alpha$  pour les différents rétrovirus susceptibles à sa restriction. Les protéines TRIM5 $\alpha$  possèdent 3 domaines en N-terminal : RING, B-Box et coiled-coil. Le domaine RING possède une activité E3 ubiquitine-ligase importante pour l'auto-ubiquitination de la protéine. Quant aux domaines B-Box et coiled-coil, ils sont impliqués dans la formation de multimères et de dimères de TRIM5 $\alpha$ , respectivement. L'activité de TRIM5 $\alpha$  en réponse aux infections virales a lieu à différentes étapes du cycle viral et empêche différents mécanismes essentiels à une réplication efficace. Premièrement, l'interaction de TRIM5 $\alpha$  avec la capsid virale déstabilise cette dernière en dirigeant TRIM5 $\alpha$  vers une dégradation par le protéasome suite à son auto-ubiquitination. Par conséquent, la transcription inverse de l'ARN viral en ADN est inhibée. De plus, l'activité de TRIM5 $\alpha$  empêcherait le transport du cœur viral vers le noyau en séquestrant le cœur viral dans ce qu'on appelle des corps cytoplasmiques de TRIM5 $\alpha$ . Récemment, l'implication des protéines TRIM5 $\alpha$  dans l'activation de la signalisation de l'immunité innée a été mise en évidence. La formation de chaînes d'ubiquitines liées à K63 via l'activité du domaine RING de TRIM5 $\alpha$  permet d'activer TAK1, une composante clé dans l'activation de NF- $\kappa$ B et AP-1.

Malgré la compréhension de nombreux processus déclenchés par TRIM5 $\alpha$ , l'incapacité d'extraire et de purifier la protéine « pleine longueur » par des techniques conventionnelles et la faible expression des protéines endogènes peuvent compliquer sa caractérisation. Néanmoins, la transduction de protéines TRIM5 $\alpha$  étiquetées permet l'expression stable de TRIM5 $\alpha$  dans différents types cellulaires. Le premier objectif de cette étude fut de caractériser la multimérisation de TRIM5 $\alpha$  en traitant des cellules exprimant TRIM5 $\alpha$  de façon stable avec différentes doses d'un agent de « *cross-linking* » qui permet l'association entre protéines. L'exposition à des virus sensibles à TRIM5 $\alpha$  ou à des drogues reconnues pour inhiber la restriction dépendante de TRIM5 $\alpha$  n'a pas affecté le patron de multimérisation de TRIM5 $\alpha$ , ce qui suppose que la capacité à multimériser est indépendante de l'interaction avec la capsid virale. Toutefois, nous

avons identifié la présence d'hexamères de TRIM5 $\alpha$  qui sont maintenant reconnus pour interagir avec la capsid hexamérique du virus.

En plus des fonctions nommées ci-haut, la restriction des rétrovirus dépendante de TRIM5 $\alpha$  impliquerait une protéine nommée SUMO. Celle-ci modifie sa cible en s'attachant de façon covalente à une lysine présente dans une séquence consensus ( $\psi$ -K-X-D/E). Il existe des motifs qui permettent l'interaction avec des protéines SUMOylées que l'on nomme SIMs. TRIM5 $\alpha$  possède un site de SUMOylation potentiel, soit la lysine en amont du domaine RING (K10), et 4 SIMs potentiels situés dans le domaine PRYSPRY, dont un fût identifié dans cette étude (SIM4). Le second objectif de cette étude a été de caractériser les rôles de la lysine en position 10 et du motif SIM4 de TRIM5 $\alpha$  dans la restriction du VIH et l'activation des voies de signalisation de l'immunité innée. La mutagenèse dirigée a permis d'identifier le rôle de ces motifs. La lysine en position 10 permet à TRIM5 $\alpha$  de recruter l'E2 Ubc13/Uev1A pour favoriser la formation de chaînes d'ubiquitines liées à K63 qui, par conséquent, permettent une activation optimale de NF- $\kappa$ B et AP-1. Quant à SIM4, il aurait évolué chez les primates de façon à reconnaître différentes capsides virales rencontrées au cours de l'évolution, tout en permettant à TRIM5 $\alpha$  de garder sa capacité à activer l'immunité innée via l'interaction entre SIM4 et des protéines SUMOylées.

**Mots-clés :** TRIM5 $\alpha$ , multimères, HIV-1, SUMO, ubiquitine, immunité innée, NF- $\kappa$ B, AP-1.

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## LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
AP-1	<i>activating protein 1</i>
BRET	<i>Bioluminescence resonance energy transfer</i>
CA	Capside
CCR5	<i>CC-chemokine receptor 5</i>
CRM1	<i>chromosomal region maintenance 1</i>
IFN	interféron
LTR	<i>Long terminal repeat</i>
MA	Protéine de matrice
NC	Nucléocapside
NES	<i>Nuclear export signal</i>
NLS	<i>Nuclear localization signal</i>
NEMO	<i>NF-<math>\kappa</math>B essential modifier</i>
NF- $\kappa$ B	<i>nuclear factor kappa B</i>
PML	<i>promyelocytic leukemia</i>
SIDA	Syndrome de l'immunodéficience acquise
SIM	<i>SUMO interacting motif</i>
SUMO	<i>small ubiquitin-like modifier</i>
TAB1/2/3	<i>TAK-1 binding protein-1/-2/-3</i>
TAK1	<i>Transforming growth factor <math>\beta</math> activated kinase-1</i>
TRIM	<i>TRIPartite Motif</i>
VIH	Virus de l'immunodéficience humaine

# CHAPITRE I

## INTRODUCTION

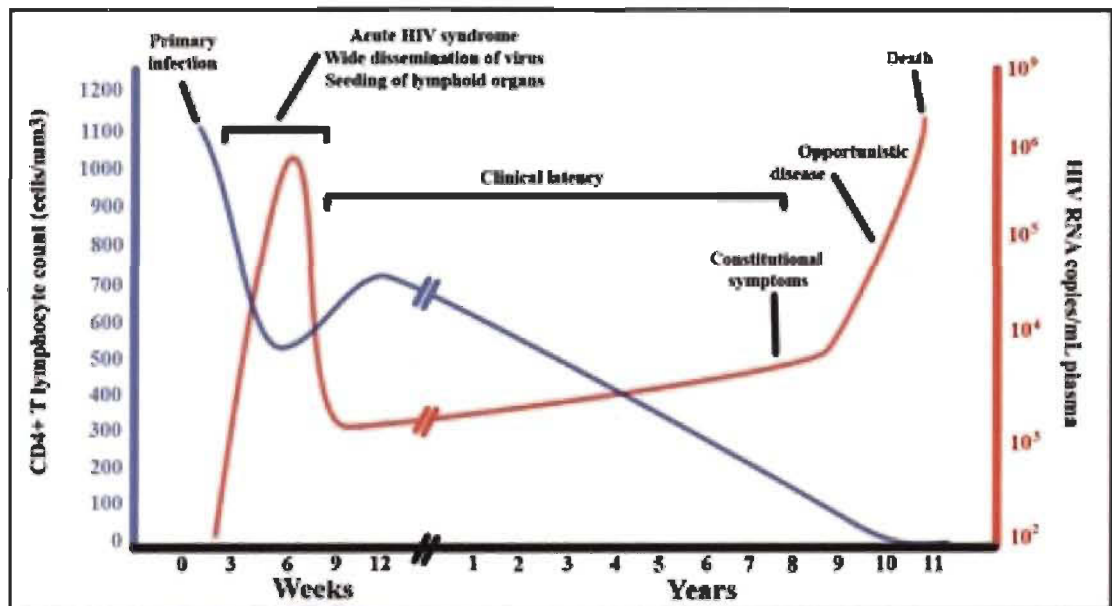
### 1.1 Virus de l'immunodéficience humain (VIH)

Isolé pour la première fois en 1983 par des chercheurs de l'institut Pasteur, ce rétrovirus connu sous le nom de virus de l'immunodéficience humaine (VIH) depuis l'année 1986 (30, 31) est l'agent causal d'une maladie toujours incurable à ce jour, le syndrome de l'immunodéficience acquise (SIDA). Il existe deux sous-types de virus : le VIH de type 1 (VIH-1) et le VIH de type 2 (VIH-2). Le VIH-1 est le résultat de plusieurs transmissions interspèces, au moins quatre, du virus de l'immunodéficience simienne (VIS) du chimpanzé et du gorille chez l'humain, tandis que le VIH-2 est le résultat d'au moins huit transmissions indépendantes provenant de singes sooty mangabey infectés par le VIS (164). Le VIH-2, moins virulent, est confiné principalement aux régions de l'Afrique de l'Ouest et du sud-ouest de l'Inde (213). Le VIH-1 est classifié en groupes (M, N, O et P) et les membres du groupe M sont responsables de l'actuelle pandémie qui touche plusieurs millions d'individus à travers le monde (101, 138, 177).

#### 1.1.1 La pathogénèse du VIH

Le VIH est un virus à acide ribonucléique (ARN) qui a comme cible les cellules du système immunitaire, notamment les lymphocytes CD4<sup>+</sup> (110, 111). La primo-infection au VIH est souvent asymptomatique, mais certaines personnes infectées vont présenter des symptômes similaires à ceux d'une grippe (*p. ex.* fièvre, douleurs musculaires, ganglions enflés) dans le mois suivant le contact initial avec le virus. La séroconversion, qui est caractérisée par la présence d'anticorps spécifiques au VIH, a lieu jusqu'à trois mois après l'exposition d'un individu au virus. La phase chronique asymptomatique ou phase de latence clinique se caractérise par l'absence de symptômes chez l'individu porteur du VIH alors que le virus continue de se développer dans son

organisme, et cette phase peut durer plusieurs années (Figure 1.1). L'affaiblissement immunitaire mène généralement à la phase clinique symptomatique que l'on nomme SIDA (152, 155). Celle-ci est caractérisée par une augmentation rapide de la charge virale et une diminution des lymphocytes  $CD4^+$  sous un seuil de 200 cellules par microlitre ( $\mu\text{l}$  ou  $\text{mm}^3$ ) de sang (141). Les individus sidéens sont plus susceptibles aux maladies opportunistes dues à des bactéries, des champignons, des virus ou des parasites qui sont normalement inoffensifs chez des individus avec un système immunitaire compétent. L'apparition de cancers est également fréquente chez les patients atteints du SIDA, notamment le sarcome de Kaposi (51). Les patients sidéens succombent généralement aux maladies opportunistes qu'ils ont contractées.



**Figure 1.1 La progression du VIH chez un individu infecté.**  
 Courbe en bleu : décompte des lymphocytes  $CD4^+$  (cellules/mm<sup>3</sup>);  
 Courbe en rouge : nombre de copies d'ARN viral (copies/ml de plasma).  
 (L'illustration provient de cette publication : (155).)

### 1.1.2 Épidémiologie

Selon les derniers rapports de l'ONUSIDA datant de 2013, le nombre total d'individus vivant avec le VIH, incluant les adultes et les enfants, est passé de

29,4 millions en 2001 à 35,3 millions en 2012, soit 5,9 millions de plus en une décennie. Ce chiffre peut sembler élevé, mais il est important de mentionner que de 1991 à 2001, le nombre d'individus nouvellement infectés avait quasiment triplé, passant d'approximativement 10 millions en 1991 à 29,4 millions en 2001. Le développement de programmes d'intervention et l'accessibilité à la trithérapie ont grandement contribué à la diminution du nombre d'individus nouvellement infectés passant de 3,2 millions en 2001 à 2,3 millions en 2012. Par contre, le nombre de décès reliés à cette infection est toujours élevé, soit de 1,6 million d'individus en 2012, faisant de cette maladie un problème majeur de santé publique à l'échelle mondiale (99).

### 1.1.3 Les thérapies anti-rétrovirales ou trithérapies

Malgré les importants progrès réalisés dans le domaine des traitements anti-rétroviraux, il n'existe pas de vaccin efficace ou de thérapie capable d'éradiquer définitivement le virus ou de contrôler à long terme sa réplication après l'interruption des médicaments. Les thérapies disponibles actuellement sont efficaces pour diminuer la réplication virale et retarder la progression de la maladie liée à l'infection au VIH. Depuis 1995, les cliniciens utilisent comme traitement la trithérapie ou multi-thérapie qui combine 3 ou plusieurs drogues ciblant différentes protéines virales essentielles à la réplication du virus (*p. ex.* transcriptase inverse, intégrase, protéase) (70, 84). Certaines drogues ciblent l'entrée du virus dans la cellule en inhibant la fusion membranaire (48) ou en ciblant CCR5 (*CC-chemokine receptor 5*), un co-récepteur important pour l'entrée du VIH dans les lymphocytes T CD4<sup>+</sup> (180). En plus de nombreux effets secondaires indésirables, l'apparition de virus résistants demeure le problème majeur rencontré avec ces thérapies (175, 214). En effet, les virus à ARN sont reconnus pour insérer fréquemment des erreurs lors de la transcription; malgré le fait que la majorité des transcrits s'avèrent improductifs, ceux qui sont viables permettent d'engendrer des mutants résistants (135, 153). Pour la même raison, il est difficile de développer un vaccin étant donné sa grande variabilité antigénique. La lutte anti-VIH est d'autant plus difficile vu la nature du virus qui est capable de latence; la réactivation de cellules

infectées dites latentes et la présence de multiples réservoirs constituent des obstacles majeurs dans l'éradication de cette maladie (136).

Néanmoins, de récentes études proposent que l'utilisation de la trithérapie à un stade très précoce de l'infection, soit durant les premiers jours de vie des nouveau-nés infectés de leur mère durant la grossesse, constitue une cure possible pour ces enfants. En effet, deux cas d'enfants vraisemblablement guéris ont été répertoriés et ces études stipulent que le VIH n'était plus détectable, et ce, même après l'arrêt de la trithérapie (167-169). Il est important de préciser que dans tous les cas où il est question de cure ou de guérison, il s'agit d'une cure dite « fonctionnelle » puisque le niveau de virus est sous le seuil de détection, seuil déterminé avec des outils standard de détection, conséquemment, cela ne signifie pas qu'il y a élimination complète du virus. Il n'en demeure pas moins que ces études sont très encourageantes, mais elles ne s'appliquent malheureusement pas dans le cas d'individus plus âgés qui sont infectés par le VIH.

#### **1.1.4 La thérapie génique**

Vu la limitation de la trithérapie en terme de cure potentielle, une autre approche s'avère très prometteuse, la thérapie génique. Le cas du « patient de Berlin » a démontré qu'il était possible de « guérir » d'une infection au VIH suite à une greffe de moelle osseuse. En effet, le niveau de virus dans le sang de ce patient est demeuré indétectable, et ce, après plusieurs années sans trithérapie. Lors de la greffe, le patient a reçu la moelle osseuse d'un individu qui portait des allèles homozygotes pour CCR5 $\Delta$ 32, une délétion de 32 paires de bases qui survient naturellement dans le gène codant pour CCR5 (94). Vu les risques importants encourus lors de ce genre d'intervention et la faible prévalence d'individus portant cette mutation (fréquence de 0.0808 chez les caucasiens), il est peu probable que cette technique soit utilisée à grande échelle (29). Une approche plus sensée en termes de faisabilité est l'utilisation de cellules souches hématopoïétiques, notamment l'utilisation des cellules CD34<sup>+</sup>. Cette technique implique l'isolement des cellules CD34<sup>+</sup> du sang du patient que l'on modifie ensuite génétiquement *ex vivo* pour les introduire chez le patient (43, 108). Une étude récente a testé la faisabilité d'une



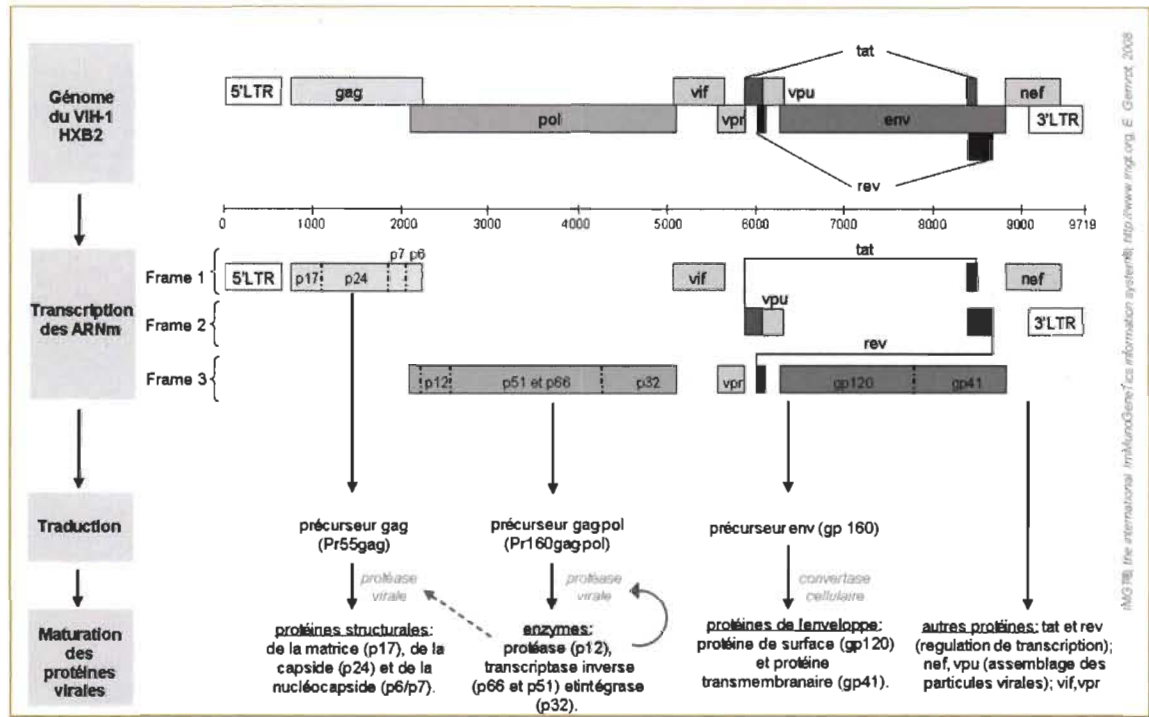
technique semblable qui utilisait plutôt des lymphocytes T CD4<sup>+</sup> prélevés chez 12 patients infectés. Une semaine après la perfusion de lymphocytes T CD4<sup>+</sup> transduits avec un co-récepteur CCR5 non fonctionnel, les résultats ont démontré une diminution du niveau d'ARN viral chez la plupart des patients, atteignant même un niveau indétectable chez un patient. Malgré des résultats intéressants, il s'agit d'une seule étude avec seulement 12 patients. De plus, la population de cellules génétiquement modifiées s'est épuisée après environ 96 semaines (215). L'épuisement des cellules modifiées est un problème important rencontré avec ces techniques. Que ce soit l'utilisation de cellules hématopoïétiques CD34<sup>+</sup> ou de lymphocytes T CD4<sup>+</sup>, les deux techniques doivent permettre d'obtenir un pourcentage de cellules transduites chez le patient assez important pour avoir un effet sur la réplication du VIH, et ce, à long terme (219). De toute évidence, il reste encore beaucoup à faire afin de mettre au point une thérapie génique au point possédant un rapport-bénéfices/risques avantageux pour le patient atteint du VIH. Pour ce faire, il faut, de prime abord, avoir une compréhension et une connaissance approfondie du virus et particulièrement de son interaction avec son hôte.

### 1.1.5 Classification

Les virus membres de la famille des *Retroviridae* sont caractérisés par des virus enveloppés à ARN monocaténaire (simple brin), non-segmentés, de polarité positive. Ils ont pour particularité leur stratégie de réplication qui comprend une étape de transcription inverse de l'ARN en ADN double-brin et l'intégration de cet ADN dans le génome de la cellule-hôte (32). Le VIH est une espèce de virus faisant partie de la sous-famille *Orthoretrovirinae* qui comprend 7 genres incluant le genre *Lentivirus* (95). Ceux-ci sont caractérisés par une longue période d'incubation et la capacité d'infecter des cellules qui ne se divisent pas, contrairement aux autres membres de la famille des *Retroviridae*. Cette dernière caractéristique s'explique par la capacité des lentivirus à pénétrer le noyau de la cellule via les pores nucléaires, contrairement aux autres rétrovirus qui requièrent la mitose (103). De plus, les *Lentivirus* sont cytotoxiques donc ils vont causer la mort des cellules qu'ils infectent, par conséquent les lymphocytes CD4<sup>+</sup> dans le cas du HIV. Ces virus sont dits complexes, car leur génome contient, en

plus des gènes nécessaires à la réplication, des gènes codant pour des protéines accessoires augmentant ainsi leur virulence et leur caractère infectieux.

### 1.1.6 Organisation du génome du VIH



**Figure 1.2 Organisation du génome du VIH-1.**

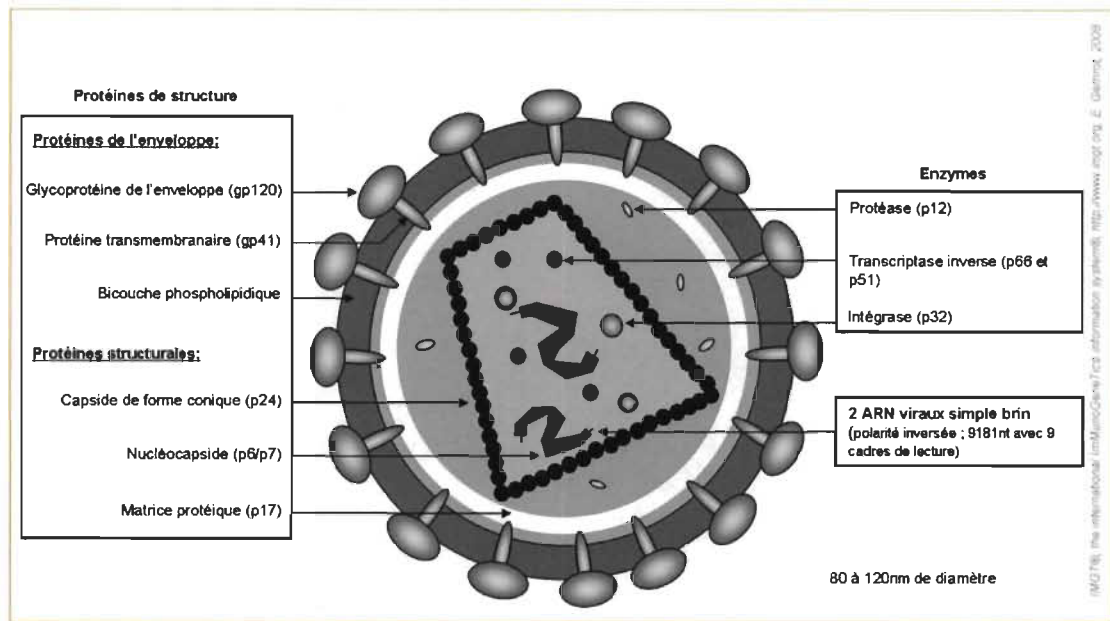
Le génome du VIH-1 et les différents gènes le composant (*gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *tat*, *rev* et *nef*) sont présentés dans le haut de la figure. Les différentes étapes de réplication (transcription des ARN messager (ARNm), traduction et maturation des protéines virales) sont également représentées. Le bas de la figure présente les différentes protéines virales matures : protéines structurales, enzymes, protéines d'enveloppe et les autres protéines dites accessoires ([www.imgt.org](http://www.imgt.org)).

Le génome du VIH-1 est représenté à la figure 1.2 et l'expression de ces différents gènes est régulée par le centre de contrôle qui constitue les deux séquences terminales en 5' et 3' que l'on nomme *long terminal repeat* (LTR). Le génome contient les gènes communs aux rétrovirus qui sont capables de réplication efficace : *gag*, *pol* et *env* (58). Le gène *gag* code pour les protéines de la matrice (MA), la capsid (CA) et la nucléocapsid (NC). Le gène *pol* permet la synthèse des différentes enzymes virales : la

protéase, la transcriptase inverse et l'intégrase. La protéase permet l'expression des différents gènes et le clivage adéquat des protéines. La reverse transcriptase est l'enzyme définissant les rétrovirus et transcrit les simples brins d'ARN en ADN double-brin et finalement, l'intégrase permet l'intégration de l'ADN viral à l'intérieur du génome de la cellule-hôte. Les glycoprotéines de l'enveloppe gp120 et gp41 sont produites par le gène *env* (181). En plus de ces trois gènes, le génome du VIH-1 code pour plusieurs autres protéines de régulation et de protéines dites accessoires. La protéine Tat est cruciale pour la transcription promue par les LTRs et elle est impliquée dans la régulation de la réplication (171). Quant à la protéine Rev, elle aurait un rôle important dans le transport de l'ARN viral provenant du noyau vers le cytoplasme (52). Vpu, Vif, Vpr et Nef ont été nommées protéines accessoires ou auxiliaires puisqu'elles ne sont pas nécessaires à une réplication productive dans certaines lignées cellulaires (58).

### 1.1.7 Structure du virus

La figure 1.3 représente la structure d'un virion mature. Les deux brins d'ARN constituant le génome viral sont protégés par la nucléocapside. La capsid virale de forme conique contient le génome viral et les trois enzymes virales essentielles à la réplication. La capsid et son contenu sont appelés cœur viral. La capsid virale est constituée de sous-unités hexamériques de protéines p24. La capsid est entourée de la matrice protéique et de l'enveloppe qui provient de la membrane cellulaire à bicouche lipidique de la cellule préalablement infectée. L'enveloppe contient les glycoprotéines de surface gp120 et les protéines transmembranaires gp41.



**Figure 1.3 Représentation schématique d'un virion mature.**

À gauche, sont indiquées les différentes protéines de l'enveloppe : glycoprotéine (gp120), protéine transmembranaire (gp41) et la bicouche phospholipidique, et les protéines structurales : capside (p24), nucléocapside (p6/7) et la matrice protéique (p17). À droite, sont indiqués les enzymes : protéase (p12), transcriptase inverse (p66 et p51) et l'intégrase (p32), et les 2 ARN viraux simple brin ([www.imgt.org](http://www.imgt.org)).

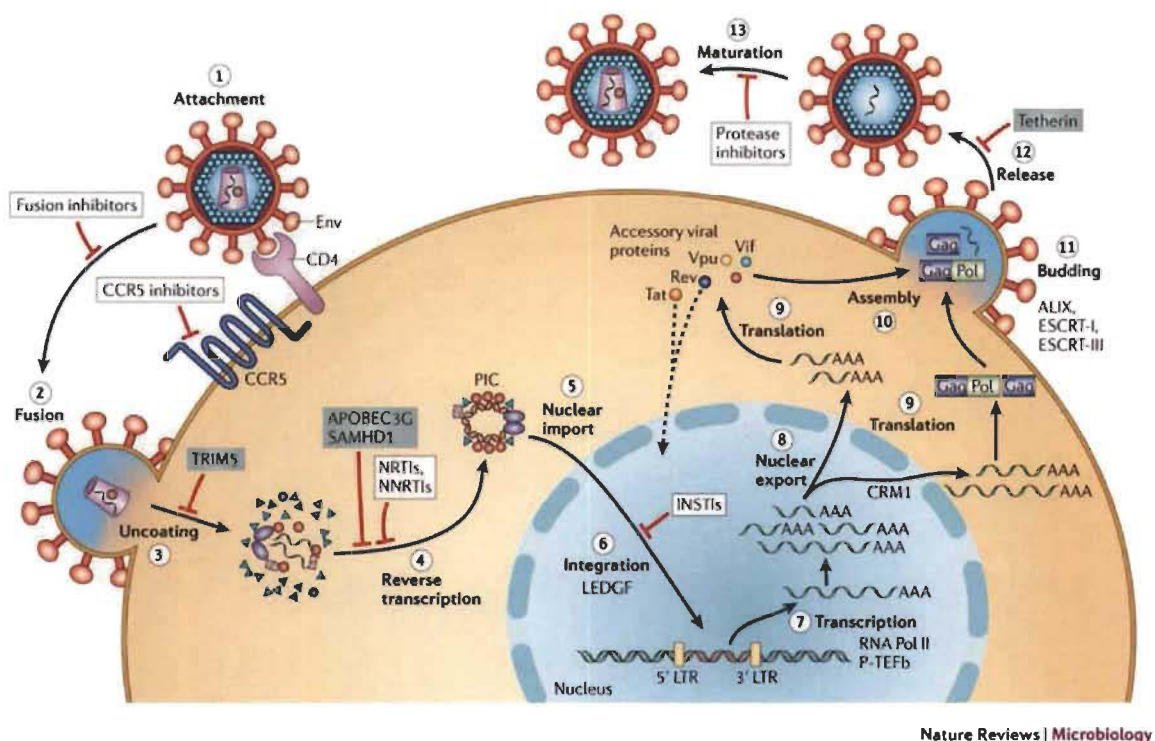
### 1.1.8 Cycle de réplication viral

On divise le cycle de réplication du VIH en 2 phases : la phase précoce qui inclue les étapes précédant et incluant l'intégration de l'ADN viral dans le génome de la cellule-hôte et la phase tardive qui représente les étapes suivant l'intégration jusqu'à la maturation du virus.

#### 1.1.8.1 Phase précoce

L'étape 1 (Figure 1.4) constitue l'attachement du VIH à la cellule cible via la protéine virale gp120 (env) et le récepteur cellulaire CD4. Cependant, l'entrée du virus dans la cellule dépend également de la présence d'un co-récepteur, soit CCR5 chez les lymphocytes T et les macrophages, ou CXCR4 (*CXC chemokine receptor 4*) chez les lymphocytes T principalement (13, 46). Quant aux cellules dendritiques, elles expriment

les deux co-récepteurs à de faibles niveaux (66). L'étape 2 représente la fusion membranaire entre la bicouche lipidique de l'enveloppe virale et la membrane plasmique de la cellule-hôte, qui permet l'entrée subséquente du cœur viral dans le cytoplasme. La prochaine étape (étape 3) est la décapsidation et cette étape nécessite une attention particulière puisque celle-ci est ciblée par la protéine de restriction à l'étude, TRIM5 $\alpha$  (discuté plus loin).



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**Figure 1.4 Schéma représentant le cycle de réplication viral du VIH.**  
**Encadrés blancs :** drogues utilisées lors de la trithérapie (inhibiteurs de la fusion, drogues ciblant CCR5, inhibiteurs nucléosidiques et non-nucléosidiques de la transcriptase inverse (NRTIs et NNRTIs), inhibiteurs de l'intégrase (INSTIs : *Integrase Strand Transfer Inhibitors*) et inhibiteurs de la protéase. **Encadrés gris :** facteurs cellulaires de restriction (discutés plus loin). (L'image provient de cet article : (49).)

Le processus de décapsidation est défini par le désassemblage contrôlé de la capsid virale permettant ainsi l'entrée du génome et des protéines virales dans le cytoplasme (56, 67). Cette étape survient juste après l'entrée du cœur viral dans la cellule. La décapsidation constitue une étape cruciale de l'infection, cependant, elle demeure la moins caractérisée du cycle viral et fait l'objet de nombreuses études (197,

233, 244). C'est une étape hautement régulée, car une décapsidation prématurée ou retardée affecte considérablement le cycle viral de réplication qui peut s'avérer non productif. L'insertion de mutations altérant la stabilité de la capsidie peut affecter la transcription inverse (56), ici représentée dans la figure 1.4 par l'étape 4 où l'ARN viral est rétrotranscrit en ADN viral. Des mutations semblables affectent également l'entrée de l'ADN viral dans le noyau (44, 235, 236). Le processus de décapsidation et de transcription inverse sont des processus intimement liés (56, 208), et certaines études suggèrent même que la décapsidation et l'étape de transcription inverse se produiraient simultanément (89, 185, 209, 238). La transcription inverse pourrait nécessiter la dissociation de la capsidie (224), toutefois il n'est pas exclu que le processus de transcription inverse puisse provoquer le désassemblage de la capsidie (89). On considèrerait que la décapsidation est un processus qui se déroulait dans le cytoplasme peu de temps après l'entrée du virus dans la cellule, mais certaines études proposent plutôt que le cœur viral se rendrait intact au nucléopore, endroit où auraient lieu la décapsidation et la transcription inverse (6, 88). De toute évidence, le processus de décapsidation et son rôle dans la transcription inverse nécessitent une meilleure compréhension au niveau moléculaire et structural.

Suite à la transcription inverse, il y a formation du complexe de pré-intégration (PIC) qui se compose principalement de l'ADN viral, la nucléocapsidie, la transcriptase inverse, la protéine Vpr et l'intégrase (233). Le PIC est ensuite transporté au noyau avec l'aide des microtubules et des filaments d'actine présents dans le cytoplasme (7, 144). Il est important de préciser ici que les étapes comprenant la décapsidation, la transcription inverse et le transport du complexe viral vers le noyau sont actuellement peu caractérisées et certains suggèrent que la majorité de ces étapes auraient lieu près du noyau (6, 88). Le complexe viral entre ensuite dans le noyau (étape 5) via des mécanismes qui impliquent notamment des importines (117, 245), des nucléoporines (*p. ex.* la nucléoporine Nup358/RanBP2) (146) et des transportines (*p. ex.* la transportine 3 (TNPO3)) (79). La dernière étape de la phase précoce du cycle viral du VIH est l'intégration de l'ADN dans le génome de la cellule catalysé par l'intégrase (étape 6).

### ***1.1.8.2 Phase tardive***

Suite à l'intégration, le provirus sert de matrice pour la synthèse des ARNs viraux (étape 7) qui codent pour la totalité des protéines virales nécessaires à une réplication productive. Les LTRs du VIH servent de site d'initiation de la transcription et contiennent les éléments nécessaires à la synthèse d'ARN (53) incluant, entre autres, deux sites de liaison à NF- $\kappa$ B (nuclear factor kappa B) et un site de liaison à Sp1 en amont de la boîte TATA. Ces sites sont très importants pour la réplication, car la délétion de ceux-ci prévient complètement la réplication virale (188). Comme mentionné précédemment, la protéine Tat est impliquée dans le processus de transcription. L'activité basale de transcription des LTRs est très faible et la synthèse d'ARN est grandement augmentée en présence de la protéine trans-activatrice de transcription Tat (35, 55). Les transcrits d'ARN viral codant pour les protéines régulatrices Tat, Rev et Nef sont exportés via un mécanisme d'export nucléaire dépendant de Tap, tandis que les transcrits codant pour les protéines d'assemblage et l'ARN génomique sont exportés (étape 8) à l'aide, principalement, de la protéine virale Rev, qui se fixe au RRE, (176) et d'une exportine nommée CRM1 (chromosomal region maintenance 1) (128).

Dans le cytoplasme, les transcrits d'ARN sont traduits en protéines virales (étape 9) à l'aide de la machinerie cellulaire de l'hôte. Il y a ensuite assemblage du virus, étape qui repose sur l'activité de la protéine de structure Gag (148, 211). Lors de l'assemblage (étape 10), il y a encapsidation des 2 brins d'ARN génomique (58). Le bourgeonnement (étape 11) est l'étape finale dans le processus de l'assemblage viral et est nécessaire à la libération du virus. Le bourgeonnement est dépendant des ESCRT (endosomal sorting complex required for transport) (148, 196). Ce sont des complexes protéiques qui sont recrutés via un domaine spécifique des protéines Gag et permettent la scission membranaire, processus essentiel pour le relâchement du virus (étape 12)(22, 91). Par la même occasion, le virus acquiert la membrane cellulaire, étape déterminante des virus enveloppés (196). La dernière étape est la maturation du virus (étape 13) et elle représente le clivage par la protéase virale de Gag et GagPol en protéines matures Gag et Pol (58). Le virus ainsi produit est prêt à infecter une autre cellule.

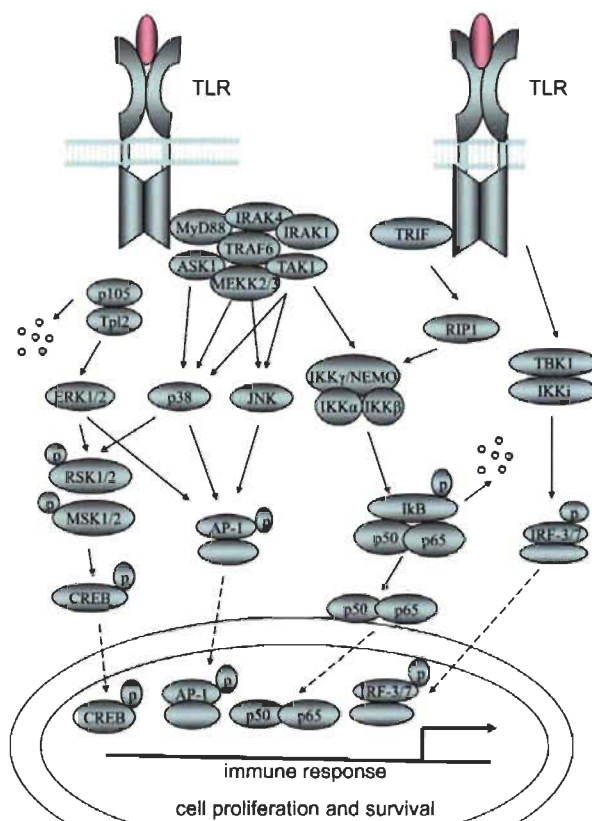
### 1.1.9 La réponse immunitaire anti-VIH

Le VIH est un virus qui cible spécifiquement les cellules du système immunitaire de l'hôte, soit les lymphocytes T CD4<sup>+</sup>, les macrophages et les cellules dendritiques. L'interaction entre le VIH et ces cellules-cibles va mener à l'activation de différents mécanismes de défense. La première ligne de défense est l'immunité innée et quoique non spécifique, elle s'avère importante dans l'infection au VIH. Cette première réponse inclut aussi différentes protéines que l'on nomme facteurs de restriction. Ceux-ci sont généralement inductibles par les interférons de type I sécrétés lors de la réponse innée. Il s'en suit l'immunité dite adaptative qui implique notamment, la production d'anticorps par les lymphocytes B et elle constitue une réponse plus spécifique à l'attaque virale.

#### 1.1.9.1 Immunité innée et adaptative

La réponse du système immunitaire inné a lieu quelques minutes à quelques jours après l'infection (118). Les composantes actives de l'immunité innée sont nombreuses (*p. ex.* cellules dendritiques, macrophages, neutrophiles, cytokines, chimiokines) et leur spécificité d'action est variée. En ce qui a trait au VIH, la production de cytokines (*p. ex.* interférons (IFN) de type I  $\alpha$  et  $\beta$ ) est cruciale dans la réponse anti-rétrovirale. Tout commence par la reconnaissance d'un agent pathogène spécifique, soit les PAMPs (*pathogen-associated molecular patterns*), par des récepteurs spécifiques nommés PRRs (*pattern-recognition receptors*) qui incluent, entre autres, les TLRs (*Toll-like receptors*) qui peuvent être situés à la surface de la cellule ou à l'intérieur des endosomes (105). Les cellules exprimant ces récepteurs sont les macrophages, les lymphocytes B et T, les cellules dendritiques, les neutrophiles, les cellules épithéliales et endothéliales (86). Par exemple, la liaison d'un ARN viral (PAMP) à ce récepteur va activer plusieurs voies de signalisation intracellulaires qui vont permettre l'activation de facteurs de transcription (*p. ex.* NF- $\kappa$ B, AP-1, IRF-3 (*IFN regulatory factor 3*) (Figure 1.5)). Ces derniers sont capables d'induire la réponse inflammatoire et d'activer le système immunitaire via la production de cytokines et de chimiokines (2).





**Figure 1.5** Voies de signalisation NF- $\kappa$ B et AP-1 dépendantes des Toll-Like Receptors.

Les TLRs reconnaissent les PAMPs (*p. ex.* LPS, flagelline, ARN simple brin). L'activation de la signalisation dépendante des TLRs s'initie lorsqu'il y a association de la région cytoplasmique des TLRs avec l'adaptateur MyD88. Le recrutement de la kinase IRAK4 (IL-1 receptor-associated kinase-4) par MyD88 va permettre la phosphorylation d'IRAK-1 qui s'associe ensuite avec TRAF6 et permet ainsi l'activation du complexe IKK. Conséquemment, il y a activation de AP-1 par les MAP kinases (JNK, p38 MAPK) et de NF- $\kappa$ B (p50/p65) (121).

Les cellules dendritiques sont des cellules présentatrices d'antigènes et elles jouent un rôle important dans l'induction de la réponse immunitaire adaptative. Ces cellules sont distribuées partout dans le corps à l'exception du cerveau. La nature des cellules dendritiques et leur présence au niveau des muqueuses génitale et rectale suggèrent que celles-ci seraient potentiellement les premières cellules à interagir avec le VIH lors d'une transmission sexuelle (92, 174). Le VIH se lie aux cellules dendritiques via le récepteur CD4 et les co-récepteurs CCR5 ou CXCR4, qui sont par ailleurs, tous exprimés à de faibles niveaux dans ces cellules (66). Les cellules dendritiques migrent

ensuite vers les organes lymphoïdes pour présenter leur antigène aux lymphocytes T qui, par le fait même, pourront être infectés. Ceci aura pour conséquence d'activer la réponse immunitaire spécifique (174, 231). Il est important de mentionner que la réplication virale est peu productive dans les cellules dendritiques en raison d'un facteur de restriction récemment identifié, SAMHD1 (*SAM domain and HD domain-containing protein 1*) (115) (discuté plus loin).

La réponse immunitaire adaptative repose principalement sur la réponse humorale. La production d'anticorps a lieu environ une à deux semaines après la phase aigüe de l'infection (119). Les immunoglobulines (Ig) sont sécrétées par les lymphocytes B en réponse à l'infection. Selon l'avancement de la maladie, différentes sous-classes d'immunoglobulines sont exprimées. Par exemple, le niveau d'IgG dans le sérum est inférieur dans les patients sidéens que les patients asymptomatiques (10, 145). De façon générale les anticorps produits lors de l'infection au VIH vont s'attacher au virus et vont essayer de l'inactiver et de le neutraliser. La neutralisation se fait majoritairement par les IgG (113) et les anticorps neutralisants vont cibler les glycoprotéines d'enveloppe gp120 et gp41. Néanmoins, la neutralisation du VIH par les anticorps est très inefficace (3, 205).

En second lieu, il y a la réponse des lymphocytes T. Les lymphocytes CD4<sup>+</sup> répondent à l'infection en produisant des cytokines (*p. ex.* interleukine-4 (IL-4), IL-6) qui ont un rôle à jouer dans l'activation, la stimulation et la différenciation des lymphocytes B. Les lymphocytes CD4<sup>+</sup> sécrètent également des interférons de type I (IFN $\alpha$ ) et de type II (IFN $\gamma$ ), autres cytokines largement impliquées dans la réponse antivirale (106). Quant aux lymphocytes CD8<sup>+</sup>, ils sont cytotoxiques et vont tuer les cellules infectées en exprimant, entre autres, des perforines (119). Il est important de mentionner que l'infection au VIH provoque également une baisse dans la sécrétion de certaines cytokines par les lymphocytes T, comme c'est le cas pour l'IL-2 qui voit sa production diminuée chez les patients infectés; toutefois, son niveau est rétabli lors de l'utilisation de la trithérapie (50).

En somme, le système immunitaire est capable de répondre à l'attaque du VIH, mais celui-ci s'avère inefficace pour éliminer l'infection et la maladie prend le dessus tôt ou tard. Il existe d'autres barrières au rétrovirus que l'on nomme facteurs de restriction et ceux-ci sont très étudiés vu leur potentiel thérapeutique, spécialement dans l'optique d'une thérapie génique.

### ***1.1.9.2 Mécanismes intracellulaires de restriction virale***

L'étude des rétrovirus a permis d'identifier certaines protéines cellulaires, possédant une activité antivirale, que l'on nomme facteurs de restriction (161). Ceux-ci font partie de la réponse immunitaire innée et certains inhibent spécifiquement les rétrovirus tandis que d'autres ont un spectre d'activité antivirale plus large. Les facteurs de restriction permettent, entre autres, d'inhiber la transmission virale d'une espèce à une autre (75, 98). Les rétrovirus ont évolué en réponse à ce système intrinsèque de restriction et ont développé certaines stratégies pour contourner ces obstacles. Malgré la divergence dans leur mécanisme d'action, ces facteurs ont en commun quelques caractéristiques : ils sont généralement codés par des gènes individuels, la plupart sont inductibles aux interférons de type I ( $\alpha$  et  $\beta$ ) dans certains types cellulaires et les rétrovirus ont évolué pour résister à ces facteurs dans leur hôte naturel (75).

Les protéines membres de la famille APOBEC (*apolipoprotein B mRNA-editing, enzyme-catalytic*) sont des facteurs de restriction présents dans les lymphocytes T (74). APOBEC3G (*APOBEC polypeptide-like 3G*) est incorporé dans les particules rétrovirales lors de l'assemblage en interagissant avec l'ARN (246). Son activité cytidine désaminase change les résidus cystidines (C) en uraciles (U). Ceci affecte la transcription inverse en insérant des mutations dans le transcrit d'ADN viral qui a pour conséquence d'inhiber la réplication (Figure 1.4). Le VIH contourne cette activité antivirale en exprimant la protéine virale Vif (200) qui bloque l'incorporation d'APOBEC3G dans les virions en diminuant son niveau d'expression dans la cellule, notamment en provoquant la dégradation d'APOBEC3G par le protéasome (137).

Un second facteur de restriction a été identifié récemment, il s'agit de SAMHD1 (115). Cette protéine est exprimée notamment dans les cellules dendritiques humaines et inhibe le VIH-1. Son mécanisme d'action se résume à son activité dNTPase qui empêche la transcription inverse (Figure 1.4) en limitant l'accès aux dNTPs (64). Son activité est inhibée par une protéine rétrovirale produite par le VIH-2 que l'on nomme Vpx. Cette dernière permet la dégradation, via le protéasome, du facteur de restriction SAMHD1.

La tétherine/BST2 (*bone marrow stromal antigen 2*) est une protéine transmembranaire (Figure 1.4) qui empêche la libération des virions nouvellement formés (166). Tout comme les précédents facteurs de restriction, cette protéine est inhibée par un facteur viral produit par le VIH-1 : la protéine accessoire Vpu qui diminue l'expression de BST2 à la surface de la cellule (218).

La famille des protéines TRIM, que l'on nomme ainsi en raison de leur motif tripartite RBCC (discuté plus loin), a fait l'objet de plusieurs études dans la dernière décennie vu l'implication de plusieurs membres de cette famille dans la restriction des rétrovirus (159). Par exemple, la protéine TRIM28/KAP1 supprime la transcription des rétrovirus (228, 229). La protéine TRIM19/PML (*promyelocytic leukemia*) possède également une activité antivirale contre certains virus comme l'influenza et le virus de la stomatite vésiculaire (VSV) (28), mais son rôle précis n'a pas été identifié. On suggère que TRIM19 agirait surtout au niveau de la transcription virale dans le noyau, comme c'est le cas avec un membre de la famille des rétrovirus, le spumavirus (182). La protéine la plus étudiée de cette famille est, sans contredit, la protéine TRIM5 $\alpha$  qui est capable d'inhiber plusieurs rétrovirus tels que le VIH-1.

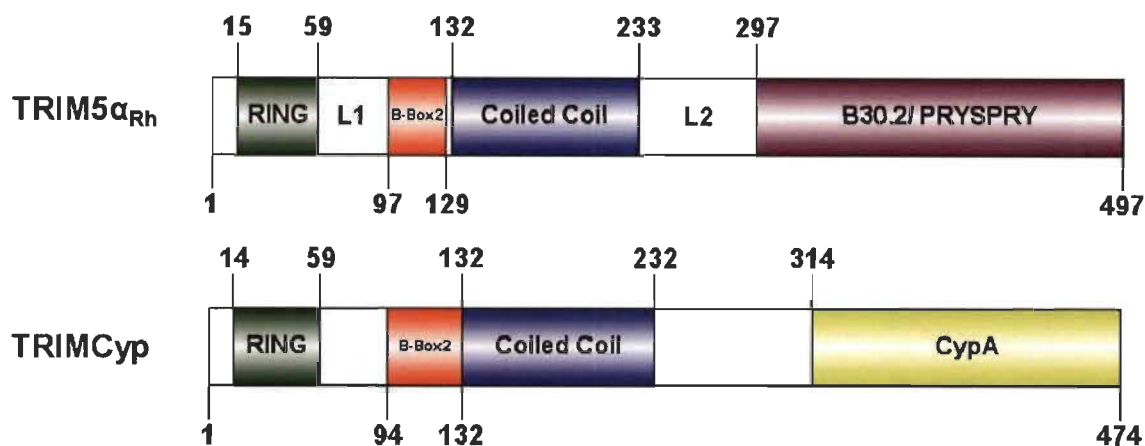
## 1.2 Les protéines de la famille TRIM5

Ces protéines sont présentes chez beaucoup de mammifères, mais celles qui font l'objet de cette étude sont exprimées chez les primates humains et non humains. Elles sont codées par le gène *TRIM5* et les cellules humaines expriment différents isoformes :

alpha, gamma, delta, kappa et iota (12). L'isoforme alpha est celui qui possède une activité anti-rétrovirale et il est exprimé au niveau du cytoplasme. Néanmoins, certains orthologues de TRIM5 $\alpha$  sont capables de migrer vers le noyau (39). Comme mentionné précédemment, les gènes codant pour les facteurs de restriction sont inductibles aux interférons et dans le cas de TRIM5 $\alpha$ , la transcription va augmenter en réponse aux interférons de type I  $\alpha$  (IFN $\alpha$ ) et  $\beta$  (IFN $\beta$ ) (9, 23). L'intérêt majeur de cette protéine est sa capacité d'inhiber certains rétrovirus, peu de temps après l'entrée du virus dans la cellule, en interagissant spécifiquement avec la capsid virale. De ce fait, TRIM5 $\alpha$  humain (TRIM5 $\alpha_{Hu}$ ) est incapable d'inhiber le VIH-1 tandis que TRIM5 $\alpha$  du singe macaque Rhésus (TRIM5 $\alpha_{Rh}$ ) en a la capacité (208). Une autre protéine de la même famille est également capable d'inhiber le VIH-1, il s'agit de la protéine TRIMCyp. Cette protéine fusionnée est issue d'une rétrotransposition du gène codant pour la cyclophiline A (CypA) dans le locus du gène *TRIM5* (195).

### 1.2.1 Structure des protéines TRIM5 $\alpha$ et TRIMCyp

Ce qui définit les protéines TRIM est la présence d'au moins deux des trois domaines situés en N-terminal que l'on nomme RING, B-Box (B-Box1 et/ou B-Box-2) et Coiled-coil (RBCC). Ce sont des domaines très conservés parmi les protéines de cette famille (159, 184). En C-terminal, TRIM5 $\alpha_{Rh}$  et TRIMCyp possèdent un domaine qui permet l'interaction spécifique avec la capsid virale soit le domaine B30.2/PRYSPRY et CypA, respectivement (Figure 1.6). Ces quatre domaines contribuent à une activité de restriction optimale, ainsi la mutation d'un domaine peut affecter l'efficacité de restriction ou engendrer la perte totale de restriction (126).



**Figure 1.6** Schéma des protéines TRIM5 $\alpha_{Rh}$  et TRIMCyp. (Illustré à partir du logiciel *Domain Graph* DOG1.0 (183) et des séquences de références NCBI : NM\_0.1032910 pour TRIM5 $\alpha$  du singe macaque rhésus et AAT73777 pour TRIMCyp du singe-hibou; L1 = linker 1 et L2 = linker 2.)

### 1.2.1.1 Le domaine RING et l'ubiquitination

Le domaine RING (*Really Interesting New Gene*) contient dans sa structure des résidus très conservés de cystéines et d'histidines nécessaires au maintien de deux atomes de zinc (60). Cette structure est spécifique aux domaines RING et confère une activité E3 ubiquitine-ligase impliquée dans la modification post-traductionnelle nommée ubiquitination. Ainsi, la présence de ce domaine dans la protéine TRIM5 $\alpha$  lui permet de s'auto-ubiquitiner, étape importante dans le processus de restriction des rétrovirus (41, 109, 126, 237).

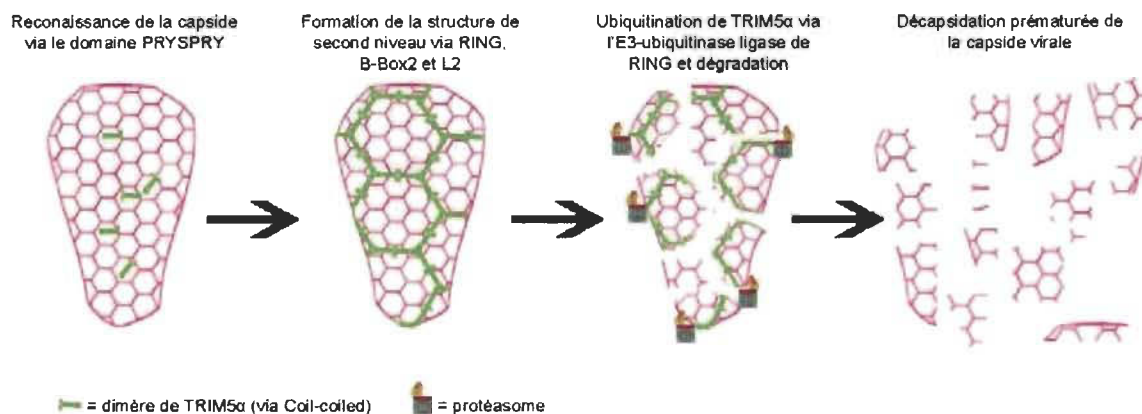
L'ubiquitination est impliquée dans de nombreux processus cellulaires, mais le plus caractérisé demeure son rôle dans la dégradation des protéines dépendante du protéasome (27, 59, 216). On lui attribue maintenant d'autres fonctions de régulation comme le transport intracellulaire, la réparation de l'ADN et la signalisation cellulaire (65, 149). Cette modification post-traductionnelle se définit par la liaison covalente de la glycine C-terminale de l'ubiquitine (Ub), un polypeptide de 76 acides aminés (8kDa), à une lysine (K) présente sur la protéine cible, dans ce cas-ci, TRIM5 $\alpha$ . L'ubiquitination

implique 3 enzymes : E1 active l'ubiquitine en formant une liaison thiol-ester avec celle-ci en présence d'ATP; l'ubiquitine activée va ensuite former une liaison thiol-ester avec l'enzyme de conjugaison E2 et l'ubiquitine sera attachée de façon covalente à la lysine de la protéine-cible à l'aide de l'enzyme E3 ubiquitin-ligase (241). Il y a différents types d'ubiquitination : la mono-ubiquitination, qui consiste à l'ajout d'une seule ubiquitine sur la lysine cible, a été reliée à l'endocytose des protéines, le transport intracellulaire et la réparation de l'ADN (72, 139, 207), et en second lieu, la poly-ubiquitination via l'une des sept lysines de l'ubiquitine (K6, K11, K27, K29, K33, K48 et K63). On appelle ces formations des chaînes de polyubiquitine (81) et les plus connues sont les chaînes d'ubiquitines liées à K48 et K63 (36, 222). Le type d'ubiquitination dépend notamment du recrutement des enzymes E2, étape contrôlée en grande partie par l'E3 ubiquitine-ligase (36).

Les chaînes d'ubiquitines liées à K48 sont formées par l'enzyme E2 Ubc5Hb (54, 220) et elles sont spécifiques à la dégradation des protéines dépendante du protéasome (62). La dégradation de TRIM5 $\alpha$  est dépendante du protéasome et l'ajout d'un virus sensible à TRIM5 $\alpha$  accélère la dégradation de la protéine (187). Ce mécanisme implique l'auto-ubiquitination de TRIM5 $\alpha$  via son activité E3 ubiquitine-ligase du domaine RING et l'enzyme de conjugaison E2, UbcH5b (116, 237). Ce domaine serait impliqué dans l'activité de restriction des protéines TRIM5 $\alpha$  car l'insertion de mutations ponctuelles, notamment dans la région permettant l'interaction avec l'enzyme de conjugaison E2 (*p. ex.* la mutation de l'isoleucine 17 et l'arginine 60 dans TRIM5 $\alpha$ ), affecte non seulement sa capacité à s'auto-ubiquitiner, mais aussi la capacité de TRIM5 $\alpha_{Rh}$  à inhiber efficacement le VIH-1 (41, 126). Une autre fonction a été récemment attribuée à l'activité du domaine RING de TRIM5 $\alpha$  : il est capable d'activer la formation de chaînes d'ubiquitines liées à K63 en interagissant avec l'enzyme de conjugaison Ubc13/Uev1a, étape importante dans l'activation de certaines voies de signalisation telle que NF- $\kappa$ B (discuté plus loin).

En plus de son activité E3 ubiquitine-ligase, le domaine RING, en association avec le domaine B-Box2, serait également impliqué dans l'association de second niveau ou multimérisation de TRIM5 $\alpha$ , étape permettant une interaction optimale avec la capsid virale (125). L'ubiquitination subséquente des multimères de TRIM5 $\alpha$ , qui sont liés à la capsid virale, provoquerait une décapsidation prématurée (Figure 1.7) qui affecte conséquemment la réplication virale (38).

Il est important de mentionner que la suppression du domaine RING ou l'insertion de mutations ponctuelles, telles que la cystéine 15 et la cystéine 18 (C15A/C18A), affecte la restriction des rétrovirus dépendante de TRIM5 $\alpha$ , sans toutefois rétablir totalement l'infection (96). De plus, malgré la possible implication du protéasome dans la restriction du VIH-1 (187), l'utilisation d'inhibiteur du protéasome, tel que le MG132, ne semble pas affecter significativement la restriction dépendante de TRIM5 $\alpha$  (232). Ces observations suggèrent que TRIM5 $\alpha$  inhibe différents mécanismes de la réplication virale (discuté plus loin).



**Figure 1.7** Schéma illustrant les différents rôles des domaines de TRIM5 $\alpha$  dans l'interaction avec la capsid virale et sa décapsidation. (Image modifiée à partir de cette publication : (193).)



### ***1.2.1.2 Le domaine B-Box2***

Contrairement au domaine RING, la délétion du domaine B-Box2 ou l'insertion de mutations élimine complètement l'activité anti-rétrovirale de TRIM5 $\alpha$  (40, 96). B-Box2 est impliqué dans l'association de second niveau qui permet la multimérisation de TRIM5 $\alpha$  et la formation de corps cytoplasmiques de TRIM5 $\alpha$  (123, 125). Toutefois, les mécanismes exacts sont toujours à l'étude et l'incapacité de purifier TRIM5 $\alpha$ , vu son faible niveau d'expression endogène, sa tendance à s'agglomérer lorsque surexprimée et le manque d'anticorps spécifiques, ont compliqué sa caractérisation biophysique et structurale (102). Des études fonctionnelles ont démontré que le domaine B-Box2, en coopération avec le domaine RING, augmente l'efficacité d'interaction avec la capsid virale en formant des structures de second niveau (42, 61, 125) (Figure 1.7). La construction d'une protéine recombinante (chimère entre TRIM21 et TRIM5 $\alpha$ ) a permis sa purification et la tenue d'études plus poussées au niveau structural. Il a été proposé que la structure de second niveau de TRIM5 $\alpha$  serait un multimère d'hexamères qui serait capable d'interagir avec la capsid hexamérique du virus (61). Par contre, pour obtenir cette structure de second niveau, il faut au préalable que la protéine TRIM5 $\alpha$  forme des dimères, fonction attribuable au domaine coiled-coil (125).

### ***1.2.1.3 Le domaine coiled-coil***

Pour interagir avec la capsid virale, TRIM5 $\alpha$  doit former des dimères (Figure 1.7) et cette association sera ensuite stabilisée par la formation de structures de second niveau. La dimérisation relève principalement du domaine coiled-coil, mais le linker 2 (Figure 1.6) aurait un rôle essentiel à jouer dans la dimérisation (97, 125). En effet, le domaine coiled-coil seul n'est pas suffisant pour la formation de dimères et le linker 2 (L2) contribuerait à une conformation propice pour l'association des deux protéines (97). L'étape de dimérisation serait essentielle à une interaction efficace avec la capsid virale puisqu'un monomère de TRIM5 $\alpha$  est incapable d'interagir avec la capsid (45, 102, 116). Tous les mutants pour le domaine coiled-coil qui sont incapables de dimériser n'inhibent pas efficacement le VIH (97).

#### ***1.2.1.4 Les domaines B30.2/PRYSPRY et Cyclophiline A***

Ces domaines en C-terminal sont responsables de l'interaction directe avec la capsid virale et confèrent le tropisme de TRIM5 $\alpha$  pour les différents rétrovirus (172, 210). Pour inhiber différents rétrovirus, cette région a subi une pression sélective lors de l'évolution qui a mené notamment à la rétrotransposition de la cyclophiline A dans le gène TRIM5 de différents singes (18, 195, 242) et à la présence de régions variables dans le domaine PRYSPRY (129, 194, 202). Il y a quatre régions variables (203) et elles seraient toutes impliquées, à un certain niveau, dans la restriction virale (160). La région V1 est impliquée dans l'interaction directe avec la capsid virale puisque l'insertion de mutations, notamment la mutation d'un seul acide aminé, permet à l'orthologue humain de TRIM5 $\alpha$  d'inhiber le VIH-1 (173, 210, 240).

#### **1.2.2 Mécanismes d'action des protéines TRIM5 $\alpha$ et TRIMCyp**

Tous les domaines de TRIM5 $\alpha$  et TRIMCyp sont nécessaires à une activité de restriction optimale. Cependant, c'est l'interaction spécifique avec la capsid virale qui va déclencher les différents mécanismes de restriction, et ce, de façon séquentielle ou simultanée.

##### ***1.2.2.1 Décapsidation prématurée et inhibition de la transcription inverse***

L'attachement de dimères de TRIM5 $\alpha$  à la capsid virale va entraîner, de prime abord, la formation d'un treillis composé de plusieurs hexamères autour de la capsid virale. Ultiment, ceci provoquera la déstabilisation de la capsid qui aura comme conséquence une décapsidation prématurée (209). Cette étape est déclenchée par l'auto-ubiquitination de TRIM5 $\alpha$  qui mène à sa dégradation par le protéasome (41). Puisque la décapsidation et la transcription inverse sont des processus intimement liés, une décapsidation prématurée provoque, subséquentement ou simultanément, l'inhibition de la transcription inverse (238). Comme mentionné ci-haut, l'ajout d'inhibiteurs du protéasome n'affecte pas significativement la capacité d'inhiber le VIH-1, mais restaure

la transcription inverse indiquant que la restriction s'effectue à plusieurs étapes du cycle viral (232).

### **1.2.2.2 Inhibition du transport de la capsid virale vers le noyau**

TRIM5 $\alpha$  inhibe le transport du cœur viral vers le noyau (4, 232). Certaines études proposent que le cœur viral soit séquestré à l'intérieur des corps cytoplasmiques de TRIM5 $\alpha$  (21, 33). De plus, comme les composantes du cytosquelette sont fréquemment utilisées par les virus comme transport jusqu'au noyau (133), et c'est le cas du VIH (144, 189), certains suggèrent, notamment notre laboratoire, que TRIM5 $\alpha$  serait capable d'interagir avec le cytosquelette, plus précisément les microtubules, pour empêcher ainsi le transport du cœur viral vers le noyau (163).

### **1.2.2.3 Activation des voies de signalisation NF- $\kappa$ B et AP-1**

TRIM5 $\alpha$  est un facteur important de l'immunité innée et sa transcription est inductible aux interférons, mais de récentes études suggèrent que TRIM5 $\alpha$  serait aussi un PRR : son interaction spécifique avec la capsid virale déclencherait diverses cascades de signalisations, notamment celles activant les facteurs de transcriptions NF- $\kappa$ B et AP-1 (*activating protein 1*) (69, 170). Comme mentionné précédemment, cette fonction serait attribuable, notamment, à la fonction E3 ubiquitine-ligase du domaine RING de TRIM5 $\alpha$ . En effet, le domaine RING qui est responsable de l'auto-ubiquitination de TRIM5 $\alpha$  activerait la formation de chaînes d'ubiquitines liées à K63 en recrutant l'hétérodimère Ubc13/Uev1a (170). Ces chaînes sont spécifiquement responsables de l'activation du complexe composé de TAK1 (*Transforming growth factor  $\beta$  (TGF- $\beta$ ) activated kinase-1*), TAB1 (*TAK1 binding protein 1*) et TAB2/3, qui agit comme régulateur clé des voies de signalisation NF- $\kappa$ B et AP-1 (1). L'insertion de mutations dans le domaine RING entraîne l'incapacité d'activer la voie de signalisation NF- $\kappa$ B (170). TRIM5 $\alpha$  agirait de façon similaire à TRAF6 (*TNF receptor-associated factor 6*), une autre E3 ubiquitine-ligase impliquée dans l'activation de TAK1 (Figure 1.5). D'ailleurs, tout comme TRAF6, TRIM5 $\alpha$  interagirait avec TAK1 et

d'autres protéines de ce complexe (170). L'activation de TAK1 stimule à son tour le complexe IKK (*I-kappa B kinase*) et les MAP (*mitogen-activated proteins*) kinases, résultant en l'activation de NF- $\kappa$ B et AP-1, respectivement (1).

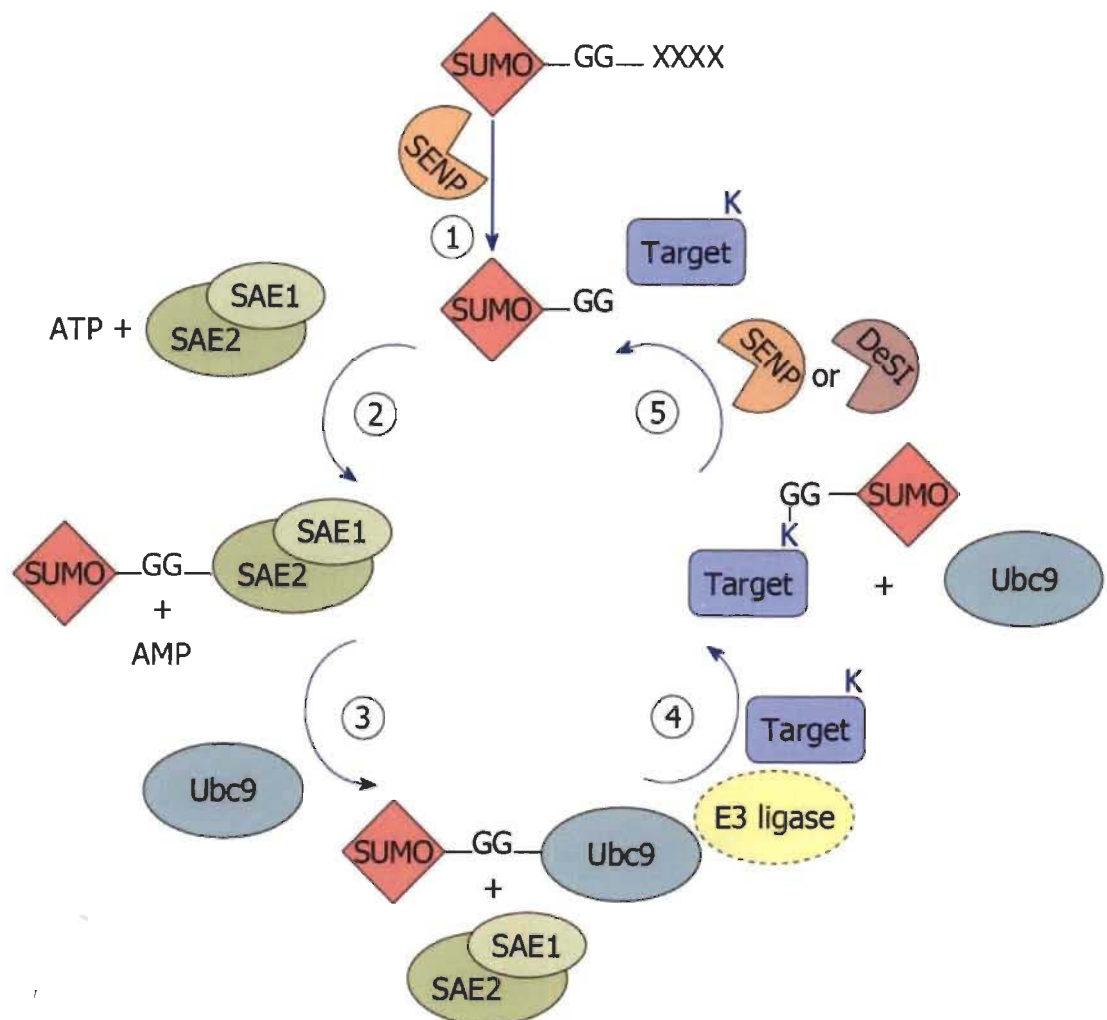
De façon plus détaillée (se référer à la Figure 1.5), le facteur de transcription NF- $\kappa$ B (p50/p65) est maintenu dans le cytoplasme de cellules inactivées à l'aide de son inhibiteur spécifique I $\kappa$ B $\alpha$  (*inhibitory kappa B alpha*) qui, lorsque lié à NF- $\kappa$ B, cache son signal de translocation nucléaire (NLS), jusqu'au moment où cet inhibiteur est dégradé via sa phosphorylation et sa subséquente dégradation par le protéasome. La phosphorylation des protéines I $\kappa$ Bs est dépendante du complexe IKK qui est composé de deux sous-unités catalytiques IKK $\alpha$  et IKK $\beta$ , et une sous-unité régulatrice nommée NEMO (NF- $\kappa$ B *essential modifier* ou IKK $\gamma$ ) (77, 104). Quant au facteur de transcription AP-1 (Figure 1.5), il s'agit d'un hétérodimère formé de deux sous-unités (jun/fos) (100) qui est activé suite à la phosphorylation des MAP kinases (JNK ou p38). Comme mentionné ci-haut, l'activation de ces facteurs de transcription va permettre leur translocation vers le noyau et ainsi initier l'expression de cytokines pro-inflammatoires, d'interférons et de gènes inductibles aux interférons (2).

TRIM5 $\alpha$  interagit avec de nombreuses protéines cellulaires (*p. ex.* ubiquitine, protéasome, microtubules, TAK1) qui lui permettent, entre autres, d'inhiber le virus à différentes étapes de la réplication, faisant de TRIM5 $\alpha$  une protéine multifonctionnelle. Depuis quelques années, certains ont proposé que la protéine SUMO (*Small Ubiquitin-like Modifier*) ait également un rôle important à jouer dans la restriction des rétrovirus dépendante de TRIM5 $\alpha$  (8, 131).

### 1.3 La SUMOylation

Cette modification post-traductionnelle réversible est très semblable à l'ubiquitination et est impliquée dans une panoplie de processus cellulaires incluant le transport nucléaire, la régulation de la transcription, l'apoptose et la stabilité des protéines (76, 147, 154, 227). Les protéines modifiées par SUMO sont majoritairement

présentes dans le noyau, toutefois, il existe des substrats dans le cytoplasme (107). Il existe 4 isoformes de SUMO chez les mammifères : SUMO-1 à SUMO-4 (226). SUMO-1 est une protéine de 11kDa qui va mono-SUMOylée sa protéine cible, contrairement à SUMO-2 et SUMO-3 qui sont reconnus pour former des chaînes (poly-SUMOylation) en se liant de façon covalente à des lysines internes (142). D'ailleurs SUMO-2 et -3 ne diffèrent que par 3 acides aminés et sont souvent référés comme étant SUMO2/3. SUMO-1 partage 50 % de similitude avec SUMO2/3 (226). Pour ce qui est de *SUMO-4*, il s'agit en réalité d'un pseudogène (16).



**Figure 1.8** Schéma illustrant la voie de SUMOylation.  
(L'image provient de cette publication (143).)

La SUMOylation (Figure 1.8) commence par l'étape de maturation (étape 1) qui consiste à cliver la séquence C-terminale (-XXXX) de la protéine SUMO à l'aide de protéases spécifiques faisant partie de la famille des SENP (*sentrin/SUMO specific protease*). Cette étape permet d'exposer les deux glycines (-GG) responsables de la conjugaison avec la lysine de la protéine cible (226). Les protéines SUMO doivent être activées (étape 2) d'une façon dépendante à l'adénosine triphosphate (ATP) par l'enzyme activatrice formée de l'hétérodimère SAE1/SAE2 (*SUMO-activating enzyme E1/E2*). Ensuite, la protéine SUMO est transférée à l'enzyme de conjugaison Ubc9 (*Ubiquitin-conjugating 9*) via un lien thioester (étape 3). Ubc9 va se lier directement à la lysine présente dans la séquence consensus  $\psi$ -K-X-D/E, où  $\psi$  constitue un gros résidu hydrophobe, X désigne un acide aminé quelconque, et D/E désignent les acides aspartique et glutamique, respectivement. Ubc9 va ensuite transférer SUMO sur la protéine cible (étape 4) via l'attachement à la lysine cible (190). Dans un contexte *in vitro*, la SUMOylation ne nécessite pas de ligase E3, mais elle serait nécessaire *in vivo* (143). Il est important de noter que malgré le fait que ~75 % des protéines SUMOylées le sont lorsque SUMO s'attache à cette lysine consensus, il arrive que certaines protéines sont SUMOylées via d'autres lysines et que plusieurs motifs consensus  $\psi$ -K-X-D/E ne sont pas nécessairement SUMOylés (234). La SUMOylation est un processus très dynamique et peut être rapidement renversée à l'aide des mêmes enzymes qui permettent la maturation de SUMO, les SENPs, ou les *DeSUMOylating-isopeptidases* (DeSI), et ceci provoque ce qu'on appelle la déSUMOylation (étape 5) (226).

### 1.3.1 Les sites d'interactions avec SUMO (SIMs)

En plus de la SUMOylation qui requiert la liaison covalente de SUMO à une lysine spécifique, certaines protéines contiennent des sites spécifiques d'interactions avec SUMO qui ne nécessitent pas de liaison covalente et ils sont nommés SIMs (*SUMO-interacting motifs*) ou parfois SBM (*SUMO-binding motif*) (107). Les SIMs sont représentés par des séquences riches en résidus hydrophobiques, composées d'au moins 3 à 4 résidus aliphatiques (c.-à-d. glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), proline (P) et méthionine (M)) et ces séquences sont régulièrement

entourées ou juxtaposées à un acide aminé chargé négativement (acide glutamique (E) ou acide aspartique (D)) (78). Dans plusieurs cas, on retrouve des résidus serine (S) et/ou thréonine (T) adjacents à la séquence hydrophobe et leur phosphorylation pourrait apporter la charge négative nécessaire à l'interaction avec les lysines (K) chargées positivement de SUMO. Cette interaction électrostatique détermine l'affinité, l'orientation et les fonctions de l'association entre les SIMs et SUMO (78, 204). Il y a une grande variabilité dans les séquences hydrophobes et dans les résidus les juxtaposant, et ceci permettrait aux différents SIMs de dicter leur spécificité d'interaction. En plus d'aider la modification covalente d'un substrat par SUMO (107, 150), les SIMs permettent l'interaction avec soit SUMO libre ou des protéines SUMOylées. Ces interactions auraient plusieurs rôles à jouer dans les fonctions cellulaires, notamment au niveau de la réplication et la réparation de l'ADN (82, 162) et dans la formation des corps nucléaires de PML/TRIM19 (201). Les virus peuvent également bénéficier des SIMs comme c'est le cas avec le virus du papillome humain (VPH) qui exprime une protéine (L2) possédant un SIM qui lui permettait d'interagir avec des protéines SUMOylées présentes dans les corps nucléaires de PML, incluant PML, processus nécessaire à une réplication efficace du virus (19).

#### **1.4 Problématiques et objectifs en découlant**

Malgré les récents progrès effectués ces deux dernières décennies, notamment l'amélioration de la trithérapie et de son accessibilité, il n'existe toujours pas de cure permettant l'élimination définitive du VIH chez un individu infecté. La présence de réservoirs, la latence du virus et l'apparition de virus résistants nous poussent à développer d'autres alternatives comme la thérapie génique. TRIM5 $\alpha$  est un candidat idéal vu qu'il est exprimé chez les lymphocytes T et les macrophages, cellules ciblées par le VIH. Bien que l'orthologue humain soit incapable d'inhiber le VIH, la mutation d'un seul acide aminé (R332) permet de remédier à cette situation (240). De plus, les protéines TRIM5 $\alpha$  ne sont pas, contrairement aux autres facteurs de restriction, inhibées par des protéines accessoires du virus (Vif, Vpu, Vpx). Leur caractérisation est donc

essentielle pour prédire leur mode d'action dans la cellule, et ce, avant même de pouvoir les utiliser comme traitement potentiel chez les humains.

### 1.4.1 Objectif 1

#### **Caractériser la capacité de TRIM5 $\alpha$ à multimériser**

Pour remettre en contexte, au début de mes études doctorales, seulement cinq années s'étaient écoulées depuis l'identification de TRIM5 $\alpha$  comme étant le facteur de restriction capable d'inhiber le VIH-1 chez les singes macaques Rhésus (208). La tenue d'études comparatives avec d'autres orthologues de TRIM5 $\alpha$  (63, 122, 239) et l'utilisation de mutants ont permis de caractériser différentes fonctions attribuables aux différents motifs de TRIM5 $\alpha$  : RING (41), B-Box (96), Coiled-coil et PRYSPRY (194, 210). Néanmoins, l'incapacité de purifier la protéine « pleine longueur » pour des études structurales (cristallographie aux rayons X ou résonance magnétique nucléaire NMR) et l'incapacité d'étudier les protéines endogènes, vu leur faible niveau d'expression et l'absence d'anticorps spécifiques efficaces, ont limité leur caractérisation, notamment au niveau structurel et biophysique. La multimérisation de TRIM5 $\alpha$  a été observée plusieurs fois (97, 123, 151) et la formation de structures de second niveau a été attribuée à l'activité du domaine B-Box2 (123). De plus, l'accumulation de ces protéines dans ce qu'on appelle les corps cytoplasmiques de TRIM5 $\alpha$  (20, 21) suggérait la formation d'une structure composée de multimères. En 2008, la construction d'une protéine chimère entre TRIM5 $\alpha$  et le domaine RING de TRIM21 (TRIM5-21R) a permis la purification à partir de cellules d'insectes d'une protéine capable d'inhiber le VIH-1 de façon similaire à TRIM5 $\alpha$  (116). Ces études ont également permis de déterminer que la forme oligomérisée la plus courante était des dimères de TRIM5-21R et que ceux-ci étaient essentiels à l'interaction avec la capsid virale (102, 116). Cependant, peu de choses étaient connues sur les facteurs qui influençaient cette disposition de TRIM5 $\alpha$  à multimériser. Le premier objectif s'est avéré plus général et il consistait à caractériser la capacité de TRIM5 $\alpha$  à former des structures de second niveau dans des conditions dites restrictives, en présence de virus sensibles à l'activité de TRIM5 $\alpha$ , ou non-restrictives,



en traitant avec des drogues reconnues pour diminuer ou inhiber la restriction. Le chapitre II présente l'article publié en 2009 qui détaille les différentes techniques utilisées et les résultats obtenus. Il est important ici d'expliquer une technique couramment utilisée dans notre laboratoire, l'essai de restriction : il consiste à infecter avec le VIH-1-GFP pseudotypé VSV-G des cellules qui expriment de façon endogène ou suite à une surexpression (transitoire par transfection ou permanente par transduction) un facteur de restriction, dans ce cas-ci TRIM5 $\alpha$ . Le pseudotypage du virus permet d'infecter différentes cellules (telles que les cellules HeLa, MDTF, TE671 et CRFK) qui ne sont normalement pas infectées par le VIH-1 sauvage, puisque ce dernier nécessite le récepteur CD4 et un co-récepteur (CCR5 ou CXCR4) pour infecter une cellule. Ainsi, pour un titre infectieux égal de VIH-1, les cellules exprimant la protéine TRIM5 $\alpha_{Rh}$  ne seront pas ou peu infectées comparativement aux cellules qui n'expriment pas le facteur de restriction en question, ces dernières seront, au contraire, dites permissives et exprimeront la GFP. La plupart des cellules utilisées dans les expériences sont transduites avec des vecteurs rétroviraux pour permettre l'expression constitutive de TRIM5 $\alpha$  et pour ce faire, on utilise le vecteur rétroviral pMIP-TRIM5 $\alpha$ WT. Le vecteur pMIP vide est utilisé pour transduire les cellules contrôles qui seront quant à elles permissives à l'infection. Pour revenir à l'article présenté au chapitre II, nos expériences ont permis de mettre en évidence la présence d'hexamères de TRIM5 $\alpha$ , structures de second niveau maintenant reconnues pour permettre la formation de treillis autour de la capsid virale qui est également formée d'hexamères (61). Selon nos résultats, la formation de multimères n'est cependant pas dépendante de l'interaction avec la capsid et ce processus aurait également lieu en absence de virus.

#### 1.4.2 Objectif 2

##### **Identifier le rôle de la lysine potentiellement SUMOylée de TRIM5 $\alpha$ dans la restriction du VIH-1 et la signalisation de l'immunité innée**

Les deux prochains objectifs découlent d'une hypothèse commune qui stipule que SUMO aurait un rôle à jouer dans la restriction des rétrovirus dépendante de TRIM5 $\alpha$ . Cependant, l'implication de cette protéine demeure très controversée. Aucune équipe, y

compris notre laboratoire, n'a pu démontrer que les protéines TRIM5 $\alpha$  étaient SUMOylées. C'est en 2011 qu'une équipe a proposé pour la première fois un rôle pour SUMO dans la restriction des rétrovirus par TRIM5 $\alpha$  (8). Cependant, ce rôle se limitait à la restriction par l'orthologue humain de TRIM5 $\alpha$  du rétrovirus de la leucémie murine (N-MLV), dont la capsid est fort possiblement SUMOylée (243), et l'effet de SUMO se limitait à certaines cellules uniquement (8). Une autre étude a suggéré l'implication de SUMO dans la restriction du VIH-1 dans un contexte où les cellules exprimaient, cette fois-ci, TRIM5 $\alpha_{Rh}$  (131). Par contre, une autre étude a contredit en partie ces précédents résultats puisque l'utilisation d'un inhibiteur de la SUMOylation, la protéine adénovirale Gam1, n'a aucunement affecté la restriction dépendante de TRIM5 $\alpha_{Hu}$  et cette équipe a conclu que la SUMOylation n'avait aucun rôle précis dans la restriction dépendante des protéines TRIM5 $\alpha$  (17). Pourtant, les protéines TRIM5 $\alpha$  contiennent une lysine potentiellement SUMOylée en position 10 (K10), juste en amont du domaine RING, et celle-ci est très conservée parmi les différents orthologues des primates humains et non-humains. Aucun rôle spécifique n'a été attribué à cette lysine dans les précédentes études portant sur SUMO et TRIM5 $\alpha$  (8, 17, 131). Pour cette raison, nous avons étudié le rôle de la lysine potentiellement SUMOylée de TRIM5 $\alpha_{Rh}$  dans la restriction du VIH-1 et l'activation de la signalisation de l'immunité innée. Le chapitre III présente l'article publié en 2014 qui détaille les différentes techniques utilisées et les résultats en découlant. Sommairement, nos expériences ont permis de déterminer que la lysine en position 10 modulait l'activité du domaine RING adjacent probablement en favorisant le recrutement de l'E2 Ubc13/Uev1A par l'E3 ubiquitine-ligase, étape nécessaire à la formation de chaînes d'ubiquitine liées à K63. Conséquemment, la mutation de cette lysine affectait la capacité de TRIM5 $\alpha_{Rh}$  à former des chaînes spécifiques liées à K63 et à activer les voies de signalisations NF- $\kappa$ B et AP-1.

### 1.4.3 Objectif 3

#### **Identifier le rôle de SIM4 dans la restriction du VIH-1 et la signalisation de l'immunité innée médiées par TRIM5 $\alpha_{Rh}$**

En plus de la lysine potentiellement SUMOylée, les protéines TRIM5 $\alpha$  contiennent dans leur domaine PRYSPRY trois séquences qui ont été prédites comme étant des SIMs potentiels et ont été nommées SIM1, SIM2 et SIM3 (8). Des fonctions importantes ont été attribuées à SIM1 et SIM2 dans la restriction médiée par TRIM5 $\alpha$ , tandis que SIM3 ne semble posséder aucun rôle particulier (8, 131). Cependant, une équipe a démontré que les motifs SIM1 et SIM2 étaient enfouis à l'intérieur du cœur hydrophobe de la région PRYSPRY de la protéine et que l'interaction avec d'autres protéines était peu probable. Les phénotypes observés étaient vraisemblablement le résultat d'un repliement anormal de la protéine qui empêchait ainsi le domaine PRYSPRY d'interagir avec la capsid du virus (17). À la lumière de ces résultats, une ré-analyse du domaine PRYSPRY de TRIM5 $\alpha_{Rh}$  a permis d'identifier un nouveau SIM potentiel qui fût nommé SIM4. Le chapitre IV présente l'article en préparation qui porte sur les rôles de SIM4 dans la restriction du VIH-1 et l'activation de l'immunité innée. Pour résumer, nos résultats suggèrent que ce motif est important pour l'interaction de TRIM5 $\alpha_{Rh}$  avec la capsid virale et permet l'activation des voies de signalisation NF- $\kappa$ B et AP-1 par un mécanisme qui ne dépend pas de la formation de chaînes d'ubiquitine liées à K63. Nos résultats proposent plutôt que SIM4 serait important pour interagir avec des protéines SUMOylées impliquées dans ces voies de signalisations. De plus, une étude phylogénétique impliquant plus de 35 espèces de primates humains et non-humains révèle que ce motif a subi des modifications significatives qui sont possiblement le résultat d'un acquis pour ces singes lors de l'évolution, notamment pour interagir avec d'autres capsides rétrovirales, sans toutefois affecter leur capacité à activer les voies de signalisation NF- $\kappa$ B et AP-1.

## CHAPITRE II

### **TRIM5 $\alpha$ AND TRIMCYP FORM APPARENT HEXAMERS AND THEIR MULTIMERIC STATE IS NOT AFFECTED BY EXPOSURE TO RESTRICTION-SENSITIVE VIRUSES OR BY TREATMENT WITH PHARMACOLOGICAL INHIBITORS**

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#### **2.1 Contribution des auteurs**

MÉNT, JB et LB ont conçu cette étude. MÉNT et JB ont effectué les expériences. LB a écrit le manuscrit. Tous les auteurs ont lu et approuvé la version finale du manuscrit.

#### **2.2 Résumé de l'article**

Les protéines de la famille TRIM5 telles que TRIM5 $\alpha$  et TRIMCyp sont des facteurs de restriction reconnus pour inhiber plusieurs rétrovirus peu de temps après l'entrée du virus dans la cellule. La restriction est dépendante de l'interaction spécifique entre les protéines TRIM5 et les protéines (p24) de la capsid qui, lorsqu'elle est intacte et mature, est nommée cœur viral. Ce dernier est composé d'unités hexamériques de protéines p24. Plusieurs mécanismes impliqués dans la restriction des protéines TRIM5 suggèrent que celles-ci interagiraient avec le cœur viral en se multimérisant. De ce fait, l'utilisation d'agents de réticulation ou de « cross-linking » ont permis de démontrer que TRIM5 $\alpha$  et TRIMCyp sont présents dans les cellules de mammifères sous la forme de

trimères. Dans cette étude, nous rapportons que les protéines TRIM5 semblent former des dimères, trimères, hexamères et multimères de grande complexité dans les cellules de mammifères. La multimérisation ne nécessite pas de ponts disulfures et la capacité à former des multimères n'a pas été affectée ni par la présence de virus sensible à la restriction, ni par des traitements reconnus pour inhiber la restriction de TRIM5 $\alpha$ , notamment le trioxido d'arsenic, le MG132 et la cyclosporine A. Pour conclure, la multimérisation des protéines TRIM5 est le résultat d'une interaction de plus d'une interface protéine-protéine et elle n'est apparemment pas déclenchée au contact des cœurs rétroviraux.

### 2.3 Premier article scientifique

#### **TRIM5 $\alpha$ and TRIMCyp form apparent hexamers and their multimeric state is not affected by exposure to restriction-sensitive viruses or by treatment with pharmacological inhibitors**

##### **Abstract**

Proteins of the TRIM5 family, such as TRIM5 $\alpha$  and the related TRIMCyp, are cytoplasmic factors that can inhibit incoming retroviruses. This type of restriction requires a direct interaction between TRIM5 proteins and capsid proteins that are part of mature, intact retroviral cores. In such cores, capsids are arranged as hexameric units. Multiple lines of evidence imply that TRIM5 proteins themselves interact with retroviral cores as multimers. Accordingly, stabilization by crosslinking agents has revealed that TRIM5 $\alpha$  and TRIMCyp are present as trimers in mammalian cells. We report here that TRIM5 proteins seem to form dimers, trimers, hexamers and multimers of higher complexity in mammalian cells. The hexameric form in particular seems to be the most abundant multimer. Multimerization did not involve disulfide bridges and was not affected by infection with restriction-sensitive viruses or by treatment with the known TRIM5 inhibitors arsenic trioxide, MG132 and cyclosporine A. We conclude that TRIM5 multimerization results from more than one protein-protein interface and that it is seemingly not triggered by contact with retroviral cores.

## Findings

TRIM proteins form a family with dozens of members, most of them bearing a tripartite motif composed of a RING, B-box and Coiled-coil domains (1). Restriction of retroviruses by members of the TRIM5 subfamily of TRIM proteins, which comprises the primate proteins TRIM5 $\alpha$  and TRIMCyp (2-4), is initiated by physical recognition of the incoming retrovirus by TRIM5 proteins. This interaction occurs within the first hours following virus entry (5) and involves determinants present in the N-terminal domain of the capsid proteins which constitute the retroviral outer core structure (6-8). Retroviral capsid cores are assembled from hundreds of capsid proteins and the basic capsomer is a hexamer (9-11). Restriction necessitates capsid proteins of the incoming retrovirus to be correctly matured by the retroviral protease (12, 13). This is a required step for the core to adopt its final structure. In addition, mutations that affect the stability of the retroviral core interfere with the efficiency of restriction (12, 13). Virus-free capsid proteins, which do not multimerize to form cores, do not interact with TRIM5 proteins in cells (14). That TRIM5-mediated restriction requires assembled retroviral cores brings the question of whether TRIM5 proteins themselves must be present as multimers. TRIM proteins are known to homomultimerize through their coiled-coil domain (1), which is required for restriction (15). TRIM5 proteins from different species can interact with each other and in doing so can interfere with each other's restriction activity (16). TRIM5 $\alpha$  has also been shown to trap incoming retroviral particles inside cytoplasmic bodies, which further suggests that TRIM5 proteins interact with their targets as multimers (17). TRIM5 $\alpha$  and TRIMCyp have been stabilized as trimers by treatment with cross-linking agents (18-23). Some undefined higher-order multimers have been occasionally observed (18, 19). The relevance of trimerization was confirmed by the fact that modified TRIMCyp, in which the coiled-coil domain is substituted by that of a trimeric heterologous protein, restricted HIV-1, although at much lower levels than wild-type TRIMCyp did (19). A recombinant TRIM5 protein expressed in insect cells was observed as dimers (21) and minor amounts of dimeric TRIM5 $\alpha$  have been observed in cells (23). However, dimerization/trimerization of TRIM5 proteins fails to explain the formation of cytoplasmic bodies or the sequestration of incoming restricted virus in such structures. Thus, we analyzed TRIM5 $\alpha$ /TRIMCyp multimerization in the

presence or absence of restriction-sensitive viruses and upon treatment with various drugs that inhibit the restriction process.

We first analyzed TRIM5 multimerization in stably transduced *Mus dunni* tail fibroblast (MDTF) cell lines (24). Multimers were stabilized by treatment with glutaraldehyde as first described by Mische and collaborators (23). Surprisingly, TRIM5 $\alpha_{rh}$  was not present as a trimer in these cells. Rather, we observed a band with a size in the 300-400 kDa range (Figure 1), and subsequent experiments that used a different molecular weight marker confirmed this apparent weight. Since the TRIM5 $\alpha_{rh}$  monomer migrates at 55 to 60 kDa, this multimer may be a hexamer. Higher-order multimers were also seen but their size could not be estimated. These high molecular weight multimers were present in the stacking gel when they were seen; and in some experiments they were found to have barely penetrated the acrylamide. We cannot exclude that they might be aggregates rather than genuine higher-order assemblies of TRIM5 $\alpha$ . TRIMCyp was found in MDTF cells as dimers and trimers and also as higher-order multimers that included a band slightly heavier than the 250 kDa marker (Figure 1). Since monomeric TRIMCyp migrates at about 45 kDa, the multimer seen is most likely a hexamer (although the migration pattern of multimeric complexes might be different from those of linear proteins). Higher amounts of glutaraldehyde were required to reveal the presence of hexamers and higher-order multimers, compared with dimers or trimers. Thus, TRIM5 $\alpha$  and TRIMCyp can have distinct multimerization profiles despite both being fully active in MDTF cells. They also share the capacity to form apparent hexamers. Because coiled-coil domains can dimerize through the formation of covalent disulfide bridges between cysteine residues in some instances (25), we performed a Western blot analysis of TRIM5 $\alpha$  and TRIMCyp in reducing and nonreducing conditions. In the absence of  $\beta$ -mercaptoethanol, both TRIM5 $\alpha$  and TRIMCyp were less easily detected, but migrated at the expected size; and no dimer or more complex multimers were visible (Figure 1B), with the exception of very high molecular weight structures which seemed to be present in higher amounts compared to the reducing conditions. Thus, it appears that disulfide bridges do not induce TRIM5 protein dimers,



trimers or hexamers, but perhaps they are involved in the formation of non-specific aggregates.

To investigate the possibility that TRIMCyp multimerization was induced or modulated by exposure to a restriction-sensitive virus, we repeated the glutaraldehyde crosslinking assay after 6 hours of continuous infection with TRIP-CMV-GFP, which is an HIV-1 vector encoding GFP (24, 26). Approximately 1% of the MDTF-TRIMCyp cells were infected in these conditions, versus more than 50% of the same cells not expressing TRIMCyp (not shown). Thus, TRIMCyp restriction activity was not saturated at this multiplicity of infection (MOI), yet cells were exposed to large amounts of HIV-1 virions in order to maximize the frequency of TRIMCyp: capsid interaction. However, HIV-1 infection did not noticeably modify the relative amounts of TRIMCyp trimers, hexamers and higher-order multimers (Figure 2A). We repeated the experiment in the presence of cyclosporine A (CsA), which completely abrogates the restriction mediated by TRIMCyp as it binds to the same CypA domain that recognizes HIV-1 capsid proteins (27, 28). CsA treatment, however, had no effect on TRIMCyp multimerization profiles, further implying that multimerization was independent of specific virus recognition.

It was recently reported that TRIM5 $\alpha$  and TRIMCyp are degraded in a proteasome-dependent pathway following infection with a restriction-sensitive retrovirus (29). Thus, it was conceivable that in our previous experiment HIV-1 modulated the multimerization of only a part of the cellular TRIMCyp proteins which were then degraded by the proteasome. To address that possibility, we repeated the experiment in the presence of the proteasomal inhibitor MG132, thereby preventing virus-induced TRIMCyp targeting to the proteasome (not shown). In addition we infected with a higher dose of the HIV-1 vector, leading to 20% infected cells. TRIMCyp restriction activity was significantly saturated at this MOI, implying that most TRIMCyp proteins that were restriction-competent at the time of infection were indeed engaging incoming HIV-1 (24). However, MG132 did not appreciably modify the multimerization profile of TRIMCyp in the absence or presence of HIV-1 (Figure 2B). Like before, dimers, trimers

and higher-order multimers were formed. The band corresponding to putative hexamers was less well-defined compared with previous experiments, but this is probably due to technical reasons unrelated to the effects of MG132 on TRIM5.

We used MDTF cells expressing TRIM5 $\alpha$  cloned from Vero cells (African green monkey) (24) to investigate whether, unlike that of TRIMCyp, TRIM5 $\alpha$  multimerization could be modulated upon infection with a restricted virus. This orthologue of TRIM5 $\alpha$  decreases HIV-1 replication by about 100-fold (30) and also inhibits the N-tropic strains of the murine leukemia virus (MLV), although to a smaller extent (10-fold or less) (24). As in Figure 2, we challenged these cells with restricted (HIV-1 and N-MLV) or non-restricted (B-MLV) viruses at relatively high doses and in presence of MG132 (Figure 3). Under these conditions, the inhibition of N-MLV by TRIM5 $\alpha$ <sub>AGM</sub> was lower than previously observed, a likely consequence of the MG132 treatment and of the high MOI (not shown). TRIM5 $\alpha$ <sub>AGM</sub> formed apparent trimers and hexamers in these cells but no dimers were observed (Figure 3A), nor did we see multimers of very high molecular weight in this particular experiment. Challenges with the different viruses had little effect on the multimerization pattern. The relative number of hexamers stabilized at the highest glutaraldehyde concentration used, decreased slightly in cells infected with one of the restricted viruses (HIV-1; Figure 3A), but increased slightly in cells infected with the other restricted virus (N-MLV; Figure 3B). No notable differences were found at the other glutaraldehyde concentrations. Data from Figure 2 and Figure 3 together suggest that the multimerization of TRIM5 proteins is not modulated by retroviral infections. A caveat in these experiments, however, is that the percentage of TRIM5 proteins actually engaged in the restriction process at any given time is not known. Even at high multiplicities of infection, it is still possible that modulation of multimerization occurs at levels undetectable in our assays.

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) inhibits the restriction activity of TRIM5 proteins in a virus-independent, TRIM5 orthologue-independent, cell type-dependent manner (30, 31). The mechanism of action of this drug on TRIM5 proteins is at present unknown. Thus, it was of interest to analyze whether it could affect the capacity of

TRIM5 proteins to multimerize. We found that  $As_2O_3$  did not affect TRIM5-mediated restriction in MDTF cells (not shown), and thus we used human HeLa cells for this particular experiment. As expected, stable expression of TRIM5 $\alpha_{rh}$  and of TRIMCyp in HeLa cells resulted in an approximately 100-fold reduction in permissiveness to transduction with an HIV-1 virus expressing GFP (not shown). We found that both restriction activities were partly suppressed by  $As_2O_3$  treatment (Figure 4A). More precisely, a short (10 hours) treatment with  $As_2O_3$  at the time of infection increased permissiveness to HIV-1 by up to 15-fold in cells expressing TRIM5 $\alpha_{rh}$  and 20-fold in cells expressing TRIMCyp, while having a smaller, "background" effect of about 4-fold in the control untransduced cells. Crosslinking assays yielded slightly different results in HeLa cells compared with what had been observed in MDTF cells. TRIM5 $\alpha_{rh}$  did not dimerize but trimers were visible, as well as apparent hexamers and higher-order multimers (Figure 4B, upper panel). TRIMCyp was found as dimers, trimers, and hexamers (Figure 4B, lower panel). An additional band migrating faster than the hexamer was visible and could be a pentamer. In both cases, the experiment was done in the absence or presence of  $As_2O_3$ ; and no differences were observed. Therefore, arsenic trioxide does not influence the multimeric state of TRIM5 $\alpha$  and TRIMCyp.

We find that in addition to the dimeric and trimeric forms previously described, TRIM5 $\alpha$  and TRIMCyp can form apparent hexamers and more complex multimers. Why discrete hexamers were not previously seen by others is probably only related to the difficulty of resolving high molecular weight complexes in acrylamide gels, although we cannot totally exclude that the C-terminus FLAG tag used in our constructs may somehow interfere with protein multimerization. Because of low expression levels in mammalian cells, it is not possible at this point to perform the biochemical experiments that would be needed to ascertain that the various multimers seen here are composed of TRIM5 proteins only. For instance, a trimer of TRIM5 could associate with a heterologous cellular protein, yielding a band resembling a TRIM5 hexamer. Thus, other approaches will be needed. The hexamer model is obviously appealing because capsid proteins are themselves organized as hexamers in mature retroviral cores. Thus, a hexamer of TRIM5 proteins could be needed to recognize a retroviral capsomer.

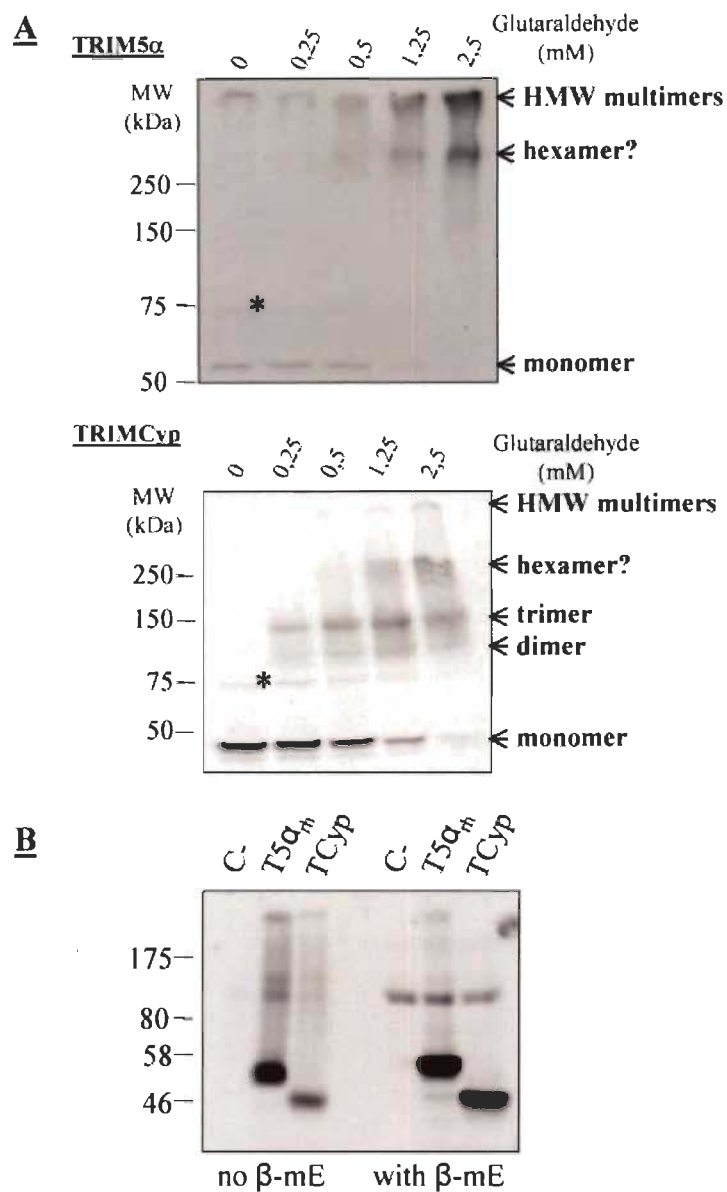
Formation of dimers, trimers and hexamers, however, does not seem to be triggered by contact with a restricted retrovirus. It remains possible that the nature and number of some specific higher order multimers not resolved in our gels could be modulated during the restriction process. Not surprisingly, the coiled-coil domain of TRIM5 proteins has been found to be required for the formation of trimers (18, 23). However, this does not imply that a single protein:protein interface present in this domain is responsible for the various multimeric forms observed. Rather, it is more likely that one interface would lead to dimerization and another one to trimerization; together they would be responsible for hexamerization. Perhaps yet other determinants within TRIM5 $\alpha$  and TRIMCyp lead to the formation of very high molecular weight multimers. Consistent with the existence of more than one molecular site of TRIM5:TRIM5 interactions, Li and Sodroski have recently reported that point mutants in the B-Box domain show normal multimerization patterns in crosslinking assays while being less efficient at engaging in protein:protein interactions through co-immunoprecipitation assays (22). Regardless of what the exact molecular mechanism of TRIM5 multimerization is, our data suggest that TRIM5 multimerization is complex but that formation of low molecular weight multimers is not influenced by contact with a restricted retrovirus.

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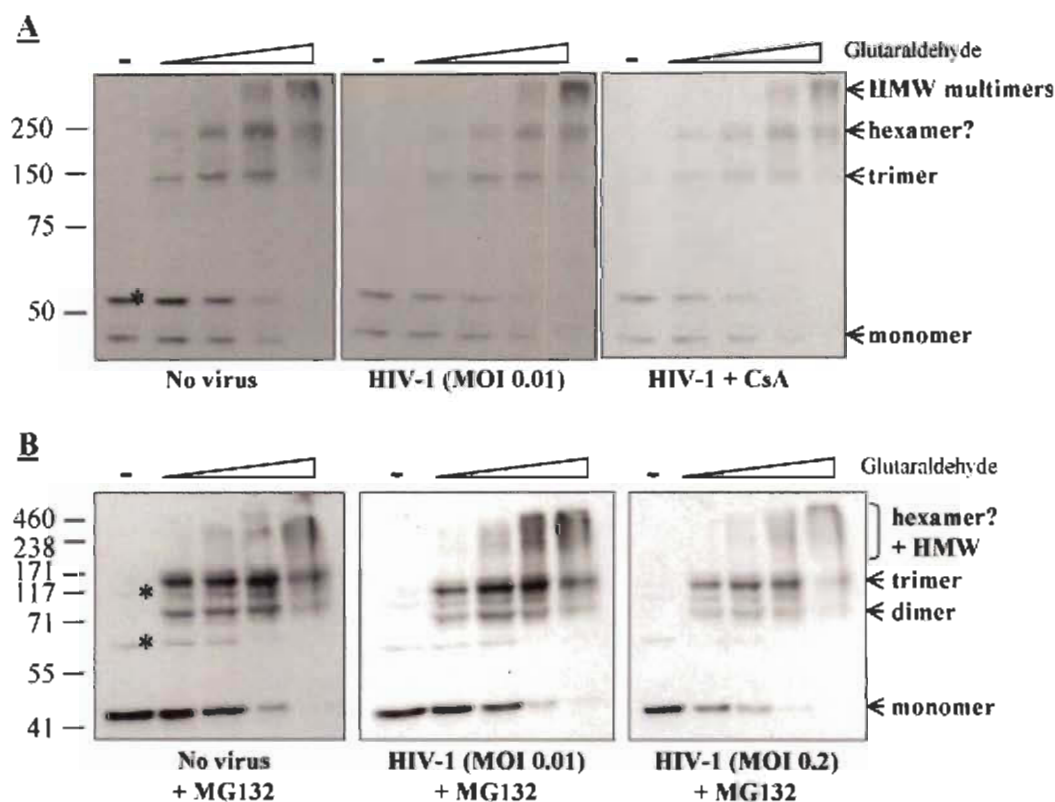


**Figure 1**

**Multimerization profiles of TRIM5 $\alpha$  and TRIMCyp.**

(A) 0.5% NP40 lysates were prepared from *Mus dunni* tail fibroblast cells (MDTFs) stably expressing FLAG-tagged TRIM5 $\alpha$ rh or owl monkey TRIMCyp. The soluble fraction of each lysate was divided in aliquots that were treated for 5 min with the indicated glutaraldehyde concentrations before proteins were denatured by boiling in the presence of SDS. Proteins were then separated on an 8% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a rabbit anti-FLAG antibody (Cell Signaling). The apparent multimeric states are indicated on the right as deduced from the size of the bands. The star indicates an unspecific protein cross-detected by the FLAG antibody. (B) Lysates were prepared from HeLa cells stably transduced with the same constructs as above and in the absence or presence of 100  $\mu$ M  $\beta$ -mercaptoethanol as indicated.

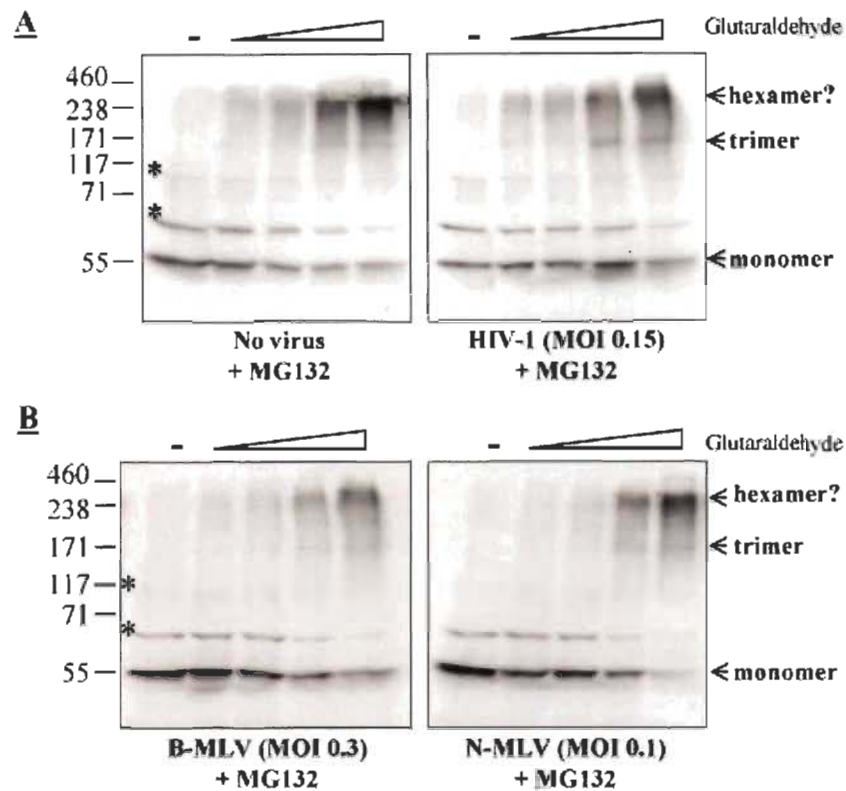




**Figure 2**

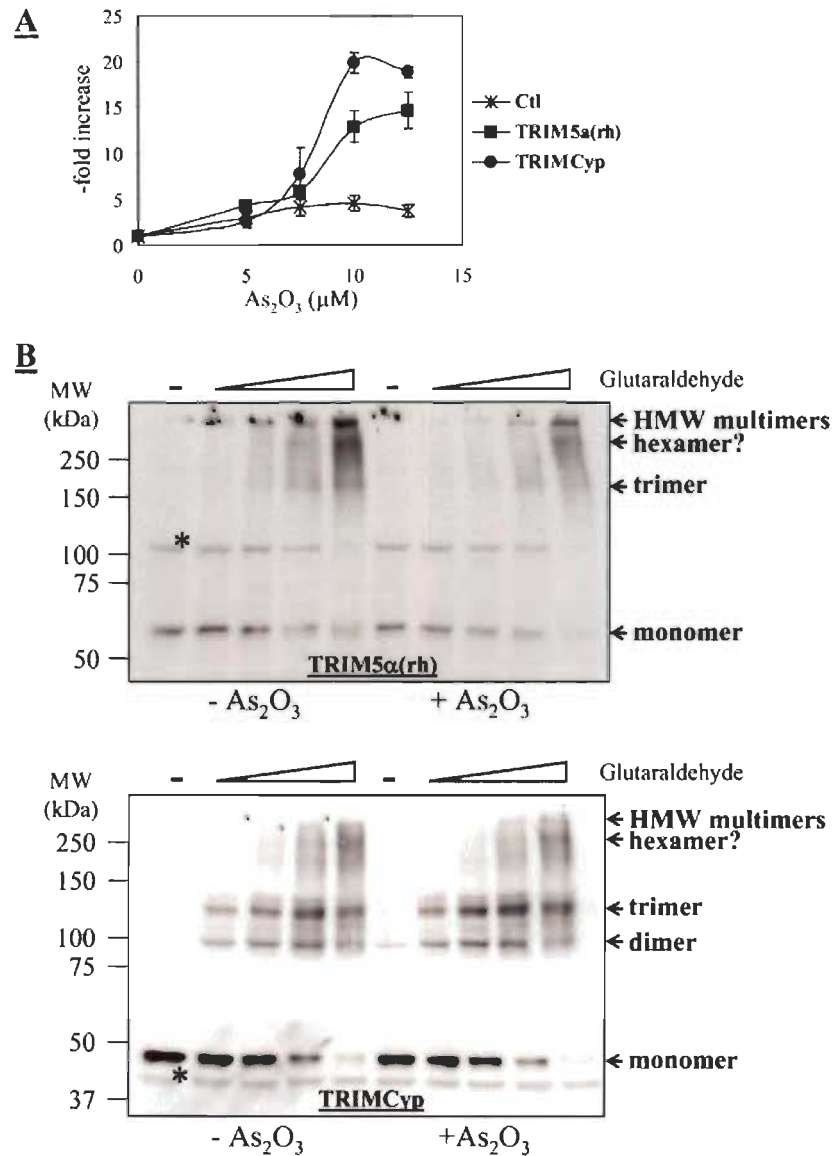
**Multimerization of TRIMCyp in cells infected by HIV-1.**

(A) MDTF-TRIMCyp cells were challenged with an HIV-1 vector expressing GFP, at a dose leading to infection of about 1% of the cells and either in the presence or not in the presence of 5  $\mu$ M cyclosporine A (Sigma). After 6 hours of infection, cells were lysed in presence of increasing glutaraldehyde concentrations as in Fig. 1. Western blot analysis of FLAG-tagged proteins was performed as above. (B) the experiment was repeated in the presence of 1  $\mu$ M MG132 (Sigma) and using two different virus doses. The stars indicate cellular proteins cross-detected by the FLAG antibody as evidenced by analysis of lysates from parental cells (not shown).



**Figure 3** **Multimerization of TRIM5 $\alpha_{AGM}$  is not modulated by infection with restriction-sensitive viruses.**

(A) MDTF cells expressing TRIM5 $\alpha_{AGM}$  were either infected or not infected with HIV-1 for 6 hours, using a virus dose leading to about 15% infected cells and in the presence of 1  $\mu$ M MG132. Crosslinking assays were done as before. (B) MDTF-TRIM5 $\alpha_{AGM}$  cells were infected with identical amounts (as normalized by titration on parental cells) of B-MLV or N-MLV-derived vectors expressing GFP in the presence of MG132. 30% and 10% of the cells were infected (GFP-positive), respectively, by B-MLV-GFP and N-MLV-GFP, as seen by flow cytometry 2 days later.



**Figure 4** **As<sub>2</sub>O<sub>3</sub> does not modify the multimerization of TRIM5α and TRIMCyp.**

(A) human HeLa cells stably expressing TRIM5αrh and TRIMCyp, or control untransduced cells, were challenged with an HIV-1 vector expressing GFP at virus doses leading to about 1% infected (GFP-positive) cells in the absence of drug. Infections were performed in the presence of increasing As<sub>2</sub>O<sub>3</sub> concentration (x-axis) and for 10 hours, after which supernatants were replaced with fresh medium to avoid As<sub>2</sub>O<sub>3</sub>-related toxic effects. Two days after infection, the % of cells expressing GFP were determined by flow cytometry analysis. Results were expressed as -fold increase compared with the untreated control. The averages from triplicate infections with standard deviations are shown. (B) glutaraldehyde assays were performed exactly as before and in the absence or presence of 10 μM As<sub>2</sub>O<sub>3</sub>.

## CHAPITRE III

### THE CONSERVED SUMOYLATION CONSENSUS SITE IN TRIM5 $\alpha$ MODULATES ITS IMMUNE ACTIVATION FUNCTIONS

MARIE-EDITH NEPVEU-TRAVERSY ET LIONEL BERTHOUX

Le contenu de ce chapitre a fait l'objet d'une publication en anglais dans la revue *Virus Research* le 25 février 2014 (doi : 10.1016/j.virusres.2014.02.013; PMID : 24583231).

#### 3.1 Contribution des auteurs

MÉNT et LB ont conçu cette étude. MÉNT a effectué la totalité des expériences. MÉNT et LB ont écrit le manuscrit. Tous les auteurs ont lu et approuvé la version finale du manuscrit.

#### 3.2 Résumé de l'article

TRIM5 $\alpha$  est un facteur de restriction anti-rétroviral induit par les interférons de type I. Il est exprimé chez la majorité des primates et des protéines homologues sont également exprimées chez d'autres mammifères. À l'aide de son domaine C-terminal PRYSPRY (B30.2), TRIM5 $\alpha$  lie la capsid virale intacte (cœur viral) peu de temps après son entrée dans le cytoplasme. Suite à cette interaction spécifique, le cœur rétroviral est déstabilisé et la progression du cycle viral est interrompue. La reconnaissance spécifique de la cible virale par TRIM5 $\alpha$  déclenche aussi l'induction d'une réponse antivirale qui implique l'activation des facteurs de transcription NF- $\kappa$ B- et AP-1. En plus du domaine PRYSPRY, les autres domaines de TRIM5 $\alpha$  sont importants pour sa fonction anti-

rétrovirale, notamment le domaine RING. Ce domaine possède une activité E3 ubiquitine-ligase et il est directement impliqué dans l'inhibition des rétrovirus et dans l'activation de l'immunité innée. Une séquence consensus de SUMOylation hautement conservée chez les orthologues de TRIM5 $\alpha$  est retrouvée entre le domaine RING et l'extrémité N-terminale. Aucun rôle précis dans la restriction n'a été attribué à ce site potentiel de SUMOylation et aucune forme SUMOylée de TRIM5 $\alpha$  n'a été observée. Cette étude a permis de confirmer que la mutation de la lysine (K10) potentiellement SUMOylée de TRIM5 $\alpha$  du singe macaque Rhésus (TRIM5 $\alpha_{Rh}$ ) n'avait qu'un faible effet sur la restriction. Néanmoins, nous avons démontré que la mutation diminuait la capacité de TRIM5 $\alpha$  à générer des chaînes d'ubiquitine liées à K63, un intermédiaire important dans l'activation de la signalisation de l'immunité innée. Conséquemment, la mutation K10R a diminué l'activation de NF- $\kappa$ B et AP-1 médiée par TRIM5 $\alpha$ . De façon concomitante, nous avons découvert que la mutation K10R provoquait une augmentation importante du niveau d'ubiquitination de TRIM5 $\alpha$ . Enfin, le traitement avec un inhibiteur de l'export nucléaire, la leptomyicine B, montre que la mutation K10R augmente la localisation nucléaire de TRIM5 $\alpha_{Rh}$ , tandis qu'au même moment, elle réduit le niveau d'association avec les corps nucléaires de SUMO. En conclusion, le site consensus de SUMOylation de TRIM5 $\alpha$  semble moduler l'activité E3 ubiquitine-ligase du domaine RING adjacent, favorisant la formation de chaînes d'ubiquitines liées à K63 au détriment de l'auto-ubiquitination qui favorise probablement la formation de chaînes d'ubiquitine liées à K48. Mis ensemble, ces résultats suggèrent que le site consensus de SUMOylation est important pour l'activation de l'immunité innée médiée par TRIM5 $\alpha$ . De plus, la lysine 10 régule le transport de TRIM5 $\alpha$  vers le noyau ainsi que sa localisation nucléaire, processus possiblement reliés au rôle de TRIM5 $\alpha$  dans l'activation de l'immunité innée.

### 3.3 Deuxième article scientifique

#### The conserved sumoylation consensus site in TRIM5 $\alpha$ modulates its immune activation functions

##### Abstract

TRIM5 $\alpha$  is a type I interferon-stimulated anti-retroviral restriction factor expressed in most primates and homologous proteins are expressed in other mammals. Through its C-terminal PRYSPRY (B30.2) domain, TRIM5 $\alpha$  binds to incoming and intact post-fusion retroviral cores in the cytoplasm. Following this direct interaction, the retroviral capsid core is destabilized and progression of the virus life cycle is interrupted. Specific recognition of its viral target by TRIM5 $\alpha$  also triggers the induction of an antiviral state involving the activation of transcription factors NF- $\kappa$ B- and AP-1. In addition to PRYSPRY, several other TRIM5 $\alpha$  domains are important for anti-retroviral function, including a RING zinc-binding motif. This domain has “E3” ubiquitin ligase activity and is involved in both the direct inhibition of incoming retroviruses and innate immune activation. A highly conserved sumoylation consensus site is present between the RING motif and the N-terminal extremity of TRIM5 $\alpha$ . No clear role in restriction has been mapped to this sumoylation site, and no sumoylated forms of TRIM5 $\alpha$  have been observed. Here we confirm that mutating the putatively sumoylated lysine (K10) of the Rhesus macaque TRIM5 $\alpha$  (TRIM5 $\alpha_{Rh}$ ) to an arginine has only a small effect on restriction. However, we show that the mutation significantly decreases the TRIM5 $\alpha$ -induced generation of free K63-linked ubiquitin chains, an intermediate in the activation of innate immunity pathways. Accordingly, K10R decreases TRIM5 $\alpha$ -mediated activation of both NF- $\kappa$ B and AP-1. Concomitantly, we find that K10R causes a large increase in the levels of ubiquitylated TRIM5 $\alpha$ . Finally, treatment with the nuclear export inhibitor leptomycin B shows that K10R enhances the nuclear localization of TRIM5 $\alpha_{Rh}$ , while at the same time reducing its level of association with nuclear SUMO bodies. In conclusion, the TRIM5 $\alpha$  sumoylation site appears to modulate the E3 ubiquitin ligase activities of the adjacent RING domain, promoting K63-linked ubiquitin chains at the expense of auto-ubiquitylation which is probably K48-linked. Consistently,

we find this sumoylation site to be important for innate immune activation by TRIM5 $\alpha$ . In addition, lysine 10 regulates TRIM5 $\alpha$  nuclear shuttling and nuclear localization, which may also be related to its role in innate immunity activation.

## Introduction

Proteins belonging to the TRIM5 (tripartite motif 5) family are known for their species-specific anti-retroviral activity (37, 41, 45). Restriction mediated by TRIM5 proteins relies mainly on their ability to recognize incoming intact capsids early after entry of the virus (38). TRIM5 $\alpha$  orthologs differ from each other mostly in the C-terminal hypervariable PRYSPRY domain that confers specificity to retroviral capsid (CA) (17, 30, 40, 50), *e.g.* HIV-1 is inhibited by rhesus macaque TRIM5 $\alpha$  (TRIM5 $\alpha_{RH}$ ), but not by the human version of the protein (50). Interactions between TRIM5 $\alpha$  proteins and CA lead to the inhibition of early steps of the virus life cycle by different mechanisms: disruption of capsid integrity (6, 29, 42), proteasomal degradation (19, 25, 35) and impairment of nuclear import (8). In addition, recent studies have shown that TRIM5 $\alpha$ -CA interactions or the over-expression of TRIM5 $\alpha$  lead to the induction of an antiviral state that includes the activation of NF- $\kappa$ B and AP-1 (31, 44, 46). This pathway also involves the generation of “free” (unconjugated) K63-linked ubiquitin chains (31).

TRIM5 $\alpha$  contains several domains located upstream of the PRYSPRY domain, including RING, B-Box-2 and Coiled-coil (RBCC) domains (34). The RING domain has an intrinsic E3 ubiquitin ligase activity essential for the auto-polyubiquitylation of the protein (11, 21, 22, 26), which is assumed to proceed through K48-linked ubiquitin chains. This process is stimulated by contact with restriction-sensitive viruses and it contributes to the destabilization of retroviral cores (19, 35). Deleting the RING domain or disrupting its zinc-binding properties by mutagenesis abrogates the capacity of TRIM5 $\alpha$  to activate NF- $\kappa$ B and AP-1 and to stimulate K63-linked poly-ubiquitylation (31, 46).

Similar to the the ubiquitylation process, sumoylation is a post-translational modification involved in many cellular mechanisms including cell signaling, transcription and regulation of protein stability (52). Three main isoforms of SUMO are found in mammalian cells, including SUMO-1, which can only achieve mono-sumoylation, and SUMO-2/3 that are able to form SUMO chains (47). SUMO proteins



are localized mostly in the nucleus and modify target proteins through covalent attachment to a lysine present in a specific consensus site, Ψ-K-X-D/E (14, 36). TRIM5 $\alpha$  carries such a sumoylation consensus motif, that is conserved in all known orthologs (not shown), upstream of the RING domain. Despite the fact that TRIM5 $\alpha$  was never identified as a sumoylated protein, recent publications proposed a role for SUMO-1 in TRIM5 $\alpha$ -mediated restriction (2, 24). However, this conclusion was disputed by another report (7).

The main objective of this study was to determine whether the sumoylation consensus site could have a role in TRIM5 $\alpha$ -mediated immune activation. Our results suggest that the putatively sumoylated lysine 10 (K10) of TRIM5 $\alpha_{Rh}$  modulates the activity of the RING domain by decreasing auto-ubiquitylation while increasing the generation of K63-linked ubiquitylation. In conclusion, the consensus sumoylation site of TRIM5 $\alpha_{Rh}$  is involved in the regulation of ubiquitylation pathways that control the activation of innate immunity.

## Materials and Methods

### *Plasmid DNAs and mutagenesis*

pMIP-TRIM5 $\alpha_{Rh}$  expresses a C-terminal FLAG-tagged version of the Rhesus macaque TRIM5 $\alpha$  and has been described before (5, 39). Directed mutagenesis was performed to introduce K10R, using the following mutagenic primers: 5'-TTCCTCGAGATGGCTTCTGGAATCCTGCTTAATGTAAGGGAGGAGGTGACCTGT (forward) and 5'-TCCTGAATTCTTACTTATCGTCGTCATCCTTGTAATC (reverse). The K10R substitution was confirmed by Sanger sequencing. The vector production plasmids pMD-G, p $\Delta$ R8.9, pCL-Eco and pTRIP-CMV-GFP have all been extensively described elsewhere (1, 3, 4, 28, 54). pRK5-HA-Ubiquitin WT and KO (23) were obtained from Ted Dawson (Johns Hopkins University, Baltimore, MD) through Addgene. KO ubiquitin bears the following mutations eliminating all possibilities of ubiquitin chain formation: K6R, K11R, K27R, K29R, K33R, K48R and K63R. pCEP4-

NF- $\kappa$ B-Luc expresses firefly luciferase under the control of an NF- $\kappa$ B-dependent promoter, while pCEP4- $\Delta$ NF- $\kappa$ B-Luc is transcriptionally deficient due to the deletion of the NF- $\kappa$ B binding site (43). Both constructs were kind gifts from M. Emerman (University of Washington, Seattle, WA). pHTS-AP1, a kind gift from J. Luban (University of Massachusetts Medical School, Worcester, MA), expresses firefly luciferase under the control of an AP-1-dependent promoter (31).

### *Cell lines*

Human embryonic kidney (HEK) HEK293T cells, HeLa human adenocarcinoma cervical cells and CRFK feline kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C, 5% CO<sub>2</sub>. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

### *Virus production*

MLV and HIV-1-based vectors were produced through transient transfection of HEK293T cells using polyethylenimine (MW 25,000; Polysciences, Warrington, PA) and collected as previously described (5, 32). To produce the MLV-based MIP vectors, cells were transfected with the relevant pMIP plasmid and co-transfected with pCL-Eco and pMD-G. All stably transduced cell lines were produced as previously described (5, 32). Successfully transduced cells were selected with puromycin, using concentrations of 2  $\mu$ g/ml (HeLa cells) and 4  $\mu$ g/ml (CRFK cells). To produce GFP-expressing HIV-1 vectors, cells were co-transfected with p $\Delta$ R8.9, pMD-G and pTRIP-CMV-GFP as described previously (5).

### *Viral challenges*

HeLa and CRFK cells were plated in 24-well plates at 50,000 cells per well and infected the next day with different amounts of HIV-1<sub>TRIP-CMV-GFP</sub> (nicknamed

“HIV-1-GFP”) or using a defined multiplicity of infection (MOI). Two days post-infection, cells were trypsinized and fixed in 2% formaldehyde in a PBS solution. The % of GFP-positive cells was then determined by analyzing 10,000 cells using a FC500 MPL cytometer with the CXP software (Beckman Coulter).

#### *Western blotting*

Cells were lysed in RIPA buffer (NaCl 150 mM, Triton 1%, SDS 0.1%, TRIS 50 mM (pH 8.0), sodium deoxycholate 0.5% and Complete protease inhibitor cocktail (Roche, Bale, Switzerland)). Whole cell lysates were then boiled in protein sample buffer (60 mM TRIS-Cl pH6.8, 10% glycerol, 0.002% bromophenol blue, 2% SDS, 2% beta-mercaptoethanol) and resolved by SDS-PAGE. After transfer to nitrocellulose membranes, blots were probed with various antibodies as specified throughout the text and visualized using secondary antibodies coupled to horseradish peroxidase (Santa Cruz, Dallas, TX) and a chemiluminescence detection system (SuperSignal West Femto, Thermo scientific, Waltham, MA). The horseradish peroxidase-conjugated anti-actin antibody (Santa Cruz) was used to control for equal loading across lanes. Images were recorded on a UVP (Upland, California) EC3 imaging system, and densitometry analyses were performed using the area density tool of the VisionWorks LS software (UVP).

#### *Immunoprecipitation and auto-ubiquitylation*

HEK293T cells were co-transfected with either WT or mutant FLAG-tagged TRIM5 $\alpha$ , and a plasmid expressing HA-tagged WT ubiquitin (pRK5-HA-Ubiquitin-WT). The “empty” vector pMIP was used as a negative control. Cells were infected with HIV-1<sub>TRIP-CMV-GFP</sub> (HIV-1-GFP; MOI of 0.2) 6 hours before lysis. Two days post-transfection, cells were harvested, lysed with the IP buffer (NaCl 150 mM, Tris-Cl 30 mM (pH 7.5), sodium deoxycholate 0.5%, NP-40 1% and Complete protease inhibitor cocktail (Roche)) and incubated overnight with a polyclonal rabbit anti-FLAG antibody (Cell Signaling, Danvers, MA) used at a 1:50 dilution. Sepharose beads

coupled to protein A (SIGMA, St Louis, MI) were then added and incubated for 10 to 16 hours. Beads were pelleted by centrifugation, washed 4 times in the IP buffer and then resuspended in protein sample buffer. Western blots were performed as described above. Blots were probed with a monoclonal mouse anti-FLAG antibody (M2, SIGMA) and revealed using a goat anti-mouse HRP secondary antibody (Santa Cruz). To detect ubiquitin, a rabbit polyclonal antibody against HA (Santa Cruz) was used.

#### *NF- $\kappa$ B and AP-1 reporter assay*

HEK293T cells were plated in 12-well plates and co-transfected with increasing amounts of either WT or mutant FLAG-tagged TRIM5 $\alpha$  and a fixed concentration of either pCEP4-NF- $\kappa$ B-Luc, pCEP4- $\Delta$ NF- $\kappa$ B-Luc or pHTS-AP1. Cells were lysed with RIPA buffer 48 hours post-transfection and assessed for luciferase activity using the BrightGlow Luciferase kit (Promega). Luminescence was measured with a Synergy HT multi-detection microplate reader (BioTek, Winooski, VT) and analyzed with the Gen5 software (BioTek).

#### *K63 ubiquitin chains formation assay*

HEK293T cells were seeded in 12-well plates and co-transfected with either WT or mutant FLAG-tagged TRIM5 and either WT or KO pRK5-HA-ubiquitin. The “empty” plasmid pMIP was used as a negative control. Cells were lysed in RIPA buffer 48 hours post-transfection and processed for western blotting. A monoclonal human/rabbit chimeric K63-linked ubiquitin chain-specific antibody (clone Apu, Millipore) was used (1:1000 dilution) to detect K63 ubiquitin chains.

#### *Immunofluorescence microscopy*

60,000 CRFK cells were plated on microscope cover glasses (Fisherbrand) in 6-well plates. The next day, cells were exposed to leptomycin B (LMB; 20 ng/ml, Enzo LifeScience) for 12 hours. Cells were washed with PBS, fixed for 10 min in

4% formaldehyde-PBS at 37°C, washed three times in PBS and permeabilized with 0.1% Triton X-100 for 2 min on ice. Cells were then washed twice with PBS and treated with 10% normal goat serum (Vector laboratories) in PBS for 30 min at RT. This saturation step was followed by 4 hour incubation with a rabbit polyclonal FLAG antibody (Cell Signaling) and an antibody against endogenous SUMO-1 (mouse monoclonal antibody, Invitrogen), both at a 1:200 dilution in PBS with 10% normal goat serum. Fluorescent staining was done by incubating with an Alexa594-conjugated goat anti-rabbit antibody and an Alexa488-conjugated goat anti-mouse antibody (each diluted 1:200). Cells were washed 4 times in PBS before mounting in Vectashield (Vector Laboratories). Hoechst-33342 (0.8 µg/ml; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Pictures were generated using a Zeiss AxioObserver microscope equipped with an Apotome module and the Axiovision software. Imaging parameters were set to identical values across samples.

## Results

### *Mutating lysine 10 decreases the capacity of TRIM5 $\alpha_{rh}$ to restrict HIV-1 in HeLa and CRFK cells*

Human HeLa and feline CRFK cells stably expressing WT or K10R TRIM5 $\alpha_{rh}$  were challenged with increasing amounts of a VSV G-pseudotyped HIV-1 vector expressing GFP (HIV-1-GFP) (Fig. 1A and B). Cells transduced with the “empty” vector were used as a non-restrictive control. WT and K10R protein expression levels were comparable in either cell line, as analyzed by western blotting (Fig. 1A and B; see Suppl. data for the uncut version). As expected, WT TRIM5 $\alpha_{rh}$  strongly inhibits HIV-1 infection in both cell lines, by 50- to 100-fold on average. The K10R mutation slightly increased (up to 5-fold and 3-fold in HeLa and CRFK cells, respectively) the infectious power of HIV-1-GFP compared to cells expressing WT TRIM5 $\alpha_{rh}$  (Fig. 1A and B). Thus, K10R causes a significant but small decrease in the restriction of HIV-1.

*The K10R mutation increases TRIM5 $\alpha_{Rh}$  auto-ubiquitylation*

The level of TRIM5 $\alpha_{Rh}$  auto-ubiquitylation was assessed using an IP-based protocol as previously published by others (22, 26). FLAG-tagged WT and K10R TRIM5 $\alpha_{Rh}$  were transiently expressed by transfection of human HEK293T cells with the corresponding pMIP-based plasmids. Simultaneously, cells were co-transfected with a construct expressing HA-ubiquitin. Two days later, cells were lysed and FLAG-tagged proteins were pulled down. Cells transfected with the empty vector were used as a negative IP control (Fig. 2; see Suppl. data for the uncut version). IP pellets were analyzed by western blotting for the presence of FLAG-tagged and HA-tagged proteins. Using a FLAG antibody, we could detect a ~55 kDa band corresponding to TRIM5 $\alpha_{Rh}$  in all lanes except for the cells transfected with the empty vector. When FLAG-TRIM5 $\alpha_{Rh}$  and HA-ubiquitin were co-transfected, ubiquitylated proteins were detected within the ~70 to 300 kDa range using an HA antibody (Fig. 2), including a characteristic “ladder” between ~70 and 100 kDa. No detectable ubiquitylation products were seen in the absence of HA-ubiquitin, and a faint amount were detected in lysates from cells transfected with HA-ubiquitin alone, suggesting low levels of unspecific IP of non-FLAG-tagged ubiquitylated proteins in our experimental conditions (Fig. 2). The amounts of unconjugated TRIM5 $\alpha_{Rh}$  were lower in cells co-transfected with TRIM5 $\alpha_{Rh}$  and HA-ubiquitin, compared with cells transfected with TRIM5 $\alpha_{Rh}$  alone (Fig. 2). Obviously, co-transfecting TRIM5 $\alpha_{Rh}$  and ubiquitin stimulated ubiquitylation of the former by the latter, thus resulting in the observed decrease in unconjugated products. In the presence of TRIM5 $\alpha_{Rh}$ -sensitive HIV-1, poly-ubiquitylation of WT TRIM5 $\alpha_{Rh}$  was markedly increased, confirming that interactions between TRIM5 $\alpha$  and incoming capsids result in an increase in TRIM5 $\alpha$  turnover (35). We found that K10R also caused an increase in the poly-ubiquitylation of TRIM5 $\alpha_{Rh}$  proteins, along with a decrease in the levels of unconjugated protein (Fig. 2), suggesting that the mutation increased RING-dependent auto-ubiquitylation. Interestingly, exposure of cells expressing K10R TRIM5 $\alpha_{Rh}$  to HIV-1 further amplified poly-ubiquitylation (Fig. 2). In summary, the data in Fig. 2 show that mutating the putative sumoylation site in TRIM5 $\alpha_{Rh}$  increases the

auto-ubiquitylation activity of the adjacent RING domain, albeit without reaching saturation levels.

*The K10R mutation affects the ability of TRIM5 $\alpha_{Rh}$  to stimulate the formation of K63-linked poly-ubiquitin chains*

It was previously demonstrated that the RING domain was essential for TRIM5 $\alpha$  to stimulate the formation of K63-linked ubiquitin chains, which contribute to the NF- $\kappa$ B signaling pathway activation through phosphorylation of the TAK1 kinase (31). To analyze the consequence of the K10R mutation on this TRIM5 $\alpha$  function, FLAG-TRIM5 $\alpha_{Rh}$  and HA-ubiquitin were co-transfected in HEK293T cells and the presence of K63-linked poly-ubiquitin was assessed by western blotting using an antibody specific for this type of ubiquitylated chains (Fig. 3A). As a control we used KO-Ub, a version of ubiquitin bearing lysine-to-arginine mutations at all potentially ubiquitylated lysines (23). K63-linked chains were detected between ~70 and 300 kDa, and such poly-ubiquitylation products were present in much smaller amounts in cells transfected with KO HA-Ub (Fig. 3A). A ~130 kDa unspecific band was detected in all lanes at a similar density and thus probably represents a non-ubiquitylated protein recognized by the antibody we used. We performed a densitometry-based quantitative analysis of K63-linked poly-ubiquitylation that removed the artifact caused by the unspecific band at 130 kDa (Fig. 3B), using blots from 3 different experiments. Co-transfection of WT TRIM5 $\alpha_{Rh}$  and WT ubiquitin resulted in a strong stimulation of K63-linked poly-ubiquitylation, compared with cells co-transfected with WT TRIM5 $\alpha_{Rh}$  and KO ubiquitin or compared with cells co-transfected with the empty pMIP and WT ubiquitin (Fig. 3A, 3B). In contrast, K10R-TRIM5 $\alpha_{Rh}$  activated the generation of K63-linked chains only poorly when co-transfected with WT ubiquitin (Fig. 3A, 3B). As exemplified in the experiment shown in Fig. 3A, there was no association between TRIM5 $\alpha_{Rh}$  expression levels and induction of K63-linked chains. Indeed, in this particular experiment K10R was clearly expressed at higher levels than the WT control, yet less K63-linked poly-ubiquitin chains were detected. Therefore, the consensus sumoylation site is important for the capacity of TRIM5 $\alpha_{Rh}$  to stimulate the production of K63-linked ubiquitin chains.

*The K10R mutation decreases the ability of TRIM5 $\alpha_{Rh}$  to activate the NF- $\kappa$ B pathway*

TRIM5 $\alpha_{Rh}$  activates an innate immunity pathway that is NF- $\kappa$ B activation-dependent and that can contribute to increasing the production of interferons and other antiviral factors (31, 43, 46). The lower levels of K63-linked polyubiquitin shown in Fig. 3 indicated that K10R was less efficient at inducing K63-linked ubiquitylation and thus one would expect reduced levels of NF- $\kappa$ B activation with this mutant. To investigate this possibility, we co-transfected HEK293T cells with a mixture of WT or mutant TRIM5 $\alpha_{Rh}$  and a reporter plasmid expressing luciferase under the control of an NF- $\kappa$ B-dependent promoter. As a control, WT TRIM5 $\alpha_{Rh}$  was also co-transfected with an NF- $\kappa$ B binding domain-deleted control (Fig. 4). The C35A RING domain mutant of TRIM5 $\alpha_{Rh}$  was also included, as it is known to be deficient in NF- $\kappa$ B activation (43). Because optimal transactivation of luciferase expression depends upon the relative ratios between the luciferase-expressing reporter plasmid and the plasmid expressing TRIM5 $\alpha$  (43), we co-transfected increasing amounts of the latter plasmid with a fixed amount of the former. We performed a statistical analysis of luciferase activity from triplicate transfections (Fig. 4A) and concomitantly analyzed TRIM5 $\alpha$  expression levels (Fig. 4B). In general, increasing the amount of transfected TRIM5 $\alpha_{Rh}$  resulted in an increase in luciferase expression, but luciferase activity was lower at 3  $\mu$ g of WT or K10R TRIM5 $\alpha_{Rh}$ -expressing plasmid compared with 1  $\mu$ g, probably reflecting a saturation effect as reported by others (43). As expected, C35A TRIM5 $\alpha_{Rh}$  was a weak activator of NF- $\kappa$ B-dependent transactivation (Fig. 4A). The K10R mutation caused a ~20-30% reduction in NF- $\kappa$ B activation compared with the WT control when 1 or 3  $\mu$ g of TRIM5 $\alpha_{Rh}$  were transfected (Fig. 4A). However, this effect was not observed when smaller amounts (0.1 or 0.3  $\mu$ g) of TRIM5 $\alpha_{Rh}$  were transfected (Fig. 4A). The reduced luciferase transactivation observed at the higher concentrations of K10R pMIP-TRIM5 $\alpha_{Rh}$  was not due to reduced protein expression, as evidenced by a western blotting analysis of the protein lysates (Fig. 4B). We repeated this experiment (Fig. 4C, D) and obtain very similar results. Specifically, WT TRIM5 $\alpha_{Rh}$  activated NF- $\kappa$ B more efficiently than K10R TRIM5 $\alpha_{Rh}$  when relatively high amounts of the TRIM5-expressing plasmid were used (in this case, 0.3  $\mu$ g or above) but not when a smaller



amount (0.1  $\mu\text{g}$ ) was transfected. In conclusion, K10R causes a modest but significant decrease in the capacity of TRIM5 $\alpha_{\text{Rh}}$  to activate NF- $\kappa\text{B}$  in certain experimental conditions.

*The K10R mutation decreases the ability of TRIM5 $\alpha_{\text{Rh}}$  to activate AP-1-dependent transcription*

AP-1 is a transcription factor composed of heterodimers of several possible proteins, the most well-known of them being c-Fos and c-Jun (15). AP-1 is activated in response to virus detection by toll-like receptors (53), and also following TRIM5 $\alpha$  over-expression (31, 46). Thus, we examined the impact of the K10R mutation on AP-1 activation by TRIM5 $\alpha$  over-expression, using an AP-1-dependent luciferase expression system (31). Co-transfection of 3  $\mu\text{g}$  of WT TRIM5 $\alpha_{\text{Rh}}$  and 0.6  $\mu\text{g}$  of the AP-1 reporter construct led to luciferase levels that were significantly higher than when the K10R mutant was used (Fig. 5A). Indeed, the capacity of K10R TRIM5 $\alpha_{\text{Rh}}$  to activate AP-1 was intermediate between that of its WT counterpart and that of the RING domain mutant C35A (Fig. 5A). When lower amounts of the TRIM5 $\alpha_{\text{Rh}}$  expressing plasmid were used (1  $\mu\text{g}$  instead of 3  $\mu\text{g}$ ), we saw no significant difference between K10R mutant and the WT control, mirroring our NF- $\kappa\text{B}$  activation data (Fig. 4). K10R TRIM5 $\alpha_{\text{Rh}}$  was expressed at levels slightly higher than its WT counterpart in this experiment (Fig. 5B), ruling out an expression defect as the explanation for the decreased AP-1 activation phenotype.

*K10R TRIM5 $\alpha_{\text{Rh}}$  shows altered nuclear/cytoplasmic distribution and reduced colocalization with SUMO-1 in the nucleus*

It was recently shown that TRIM5 $\alpha$  is able to shuttle between the cytoplasm and nucleus and that TRIM5 $\alpha$  nuclear bodies (NBs) can be observed upon treatment with leptomycin B (LMB), an inhibitor of CRM-1 dependent nuclear export (10). IF microscopy was performed in CRFK cells stably expressing FLAG-tagged TRIM5 $\alpha_{\text{Rh}}$  WT and K10R, in the presence or absence of LMB. As expected, LMB treatment resulted in a fraction of WT and K10R TRIM5 $\alpha_{\text{Rh}}$  being present in the nucleus while no

nuclear TRIM5 $\alpha$  staining was observed in the absence of the drug (Fig. 5A). Nuclear TRIM5 $\alpha$  bodies were recently shown to co-localize with PML bodies (10). PML, also called TRIM19, is the main determinant for the formation of PML bodies, nuclear structures that can harbor multiple different proteins and have known roles in antiviral defense (13). PML is heavily sumoylated, as are many proteins localizing at PML bodies, and SUMO is a major determinant for the formation of PML bodies (27). Thus, we postulated that the TRIM5 $\alpha$  consensus sumoylation site was important for its co-localization with SUMO when present in the nucleus. As shown Fig. 6A, a significant fraction of TRIM5 $\alpha_{Rh}$  NBs co-localized with SUMO-1 in LMB-treated cells, and this was true for both the WT and K10R variants of the protein. We then analyzed multiple randomly selected cells to quantify the amounts of TRIM5 $\alpha_{Rh}$  NBs found in nuclei (Fig. 6B), as well as the percentage of these NBs co-localizing with SUMO-1 (Fig. 6C). Results show that in the presence of LMB, ~5 WT and ~11 K10R TRIM5 $\alpha_{Rh}$  NBs were observed per cell (Fig. 6B), on average. We observed no obvious changes in the apparent number or aspect of the cytoplasmic bodies. In addition, 38.6% of WT TRIM5 $\alpha_{Rh}$  NBs but only 18.3% of K10R TRIM5 $\alpha_{Rh}$  NBs co-localized with SUMO-1 (Fig. 6C). Therefore, the K10R mutation affects the subcellular dynamics of TRIM5 $\alpha_{Rh}$ , specifically its shuttling between cytoplasm and nucleus and its association with SUMO-1 NBs.

## Discussion

Previous studies focusing on the consensus sumoylation site of TRIM5 $\alpha$  concluded that lysine 10 had little or no influence on retroviral restriction, depending on the cellular context (2, 7, 24). Our own data are consistent with these conclusions (Fig. 1). The slight decrease in restriction activity for the K10R mutant could be related to its increased poly-ubiquitylation levels (Fig. 2), which may result in premature TRIM5 $\alpha$  ubiquitylation instead of ubiquitylation following the interception of a restriction-sensitive capsid. Despite it having little involvement in the direct inhibition of incoming retroviruses, the sumoylation site is conserved in all known primate orthologs of TRIM5 $\alpha$  (not shown). Thus, we hypothesized that it was important for a different

function of TRIM5 $\alpha$ , the activation of innate immune responses (44). This function of TRIM5 $\alpha$  is largely dependent upon the ubiquitin ligase activity associated with the RING domain (31, 43, 46), which is adjacent to the consensus sumoylation site. Therefore, we analyzed the effects of mutating lysine 10 on the E3 ubiquitin ligase activity of the TRIM5 $\alpha_{Rh}$  RING domain.

It is established that a single RING domain can recruit different E2s, resulting in different ubiquitylation patterns (9, 18). The RING domain of TRIM5 $\alpha$  functions as a ubiquitin ligase in two different types of ubiquitylation: auto-ubiquitylation that is presumed to be K48-linked (though this has not been demonstrated so far) (11, 21, 22, 26) and the generation of K63-linked ubiquitin chains which may be unconjugated (31). Our results strongly suggest that the putative sumoylation site in TRIM5 $\alpha$  modulates these two ubiquitin ligase activities. Indeed, the K10R mutation leads to an increase in auto-ubiquitylation (Fig. 2) accompanied by a decrease in the formation of K63-linked chains (Fig. 3). The observed phenotype of the TRIM5 $\alpha$  K10R mutant is markedly different from the behavior of mutants affecting the RING finger zinc binding motif, including C15A, H32A and C35A. Indeed, such RING domain mutations abrogate both auto-ubiquitylation (22, 26) and the generation of K63-linked ubiquitin chains (31), and they also abrogate the activation of NF- $\kappa$ B and AP-1 (31, 43, 46). In contrast, the consensus sumoylation site is not required for RING-mediated ubiquitylation but it acts as a regulator. Auto-ubiquitylation of TRIM5 $\alpha$  is accomplished by the E2 ubiquitin-conjugating enzyme UbcH5b (20, 49) while the formation of free K63-conjugated chains is dependent on the E2 heterodimer Ubc13/Uev1a (31). Our data open the possibility that the consensus sumoylation site promotes interactions of the TRIM5 $\alpha$  RING domain with Ubc13/Uev1a rather than UbcH5b, thereby inducing K63-linked ubiquitylation.

The K10R mutation had a more significant effect on K63-linked ubiquitylation than on NF- $\kappa$ B or AP-1 activation. However, a recent study in which endogenous ubiquitin was depleted and cells were concomitantly made to express K63-mutated ubiquitin revealed that NF- $\kappa$ B could be activated by two distinct pathways, one of which

is completely independent of K63-linked ubiquitin chains (48). Hence, it is possible that over-expression of TRIM5 $\alpha$  triggers both pathways and that mutating lysine 10 affects the K63-linked ubiquitylation-dependent pathway but not the second one.

Because K10R decreases both the direct restriction of incoming viruses and the activation of innate immunity pathways, one might suspect that this mutation simply affects the overall structure of TRIM5 $\alpha$ , hence affecting its functions as well. However, two observations point against this conclusion. First, K10R does not affect TRIM5 $\alpha$  expression levels nor its capacity to form cytoplasmic or nuclear bodies (Figs 1, 6). Second, K10R increases the RING-associated E3 activity that leads to self-ubiquitylation, an unlikely observation if K10R was affecting the general functions of the RING domain. In that regard, it is worth noting again the specificity of the K10R phenotype, compared to mutations of the RING zinc finger that decrease all RING-mediated activities including self-ubiquitylation.

What is the role, if any, of the sumoylation pathway in the observed K10R mutation phenotype? Although TRIM5 $\alpha$  has never been found to be sumoylated, it is possible that sumoylation occurs very transiently. In that regard, it is interesting that TRIM5 $\alpha$  is able to shuttle in and out of the nucleus, although its steady-state localization is largely cytoplasmic (10). Nuclear TRIM5 $\alpha$  localizes partly at PML NBs (10) and PML itself, as well as many proteins associating with it, is sumoylated (27). This raises the possibility that TRIM5 $\alpha$  is sumoylated only while transiently present in the nucleus or in SUMO/PML NBs, which would be undetectable by classical analyses. Consistent with a link between SUMO and the nuclear shuttling and localization of TRIM5 $\alpha$ , we find that the K10R mutation doubles the level of nuclear localization in LMB-treated cells, but decreases by half its association with SUMO-1 (Fig. 6). Ubc13, the E2 enzyme for K63-linked ubiquitylation, is present in the nucleus and its activity is sometimes dependent on the modification of its target by SUMO-2/-3 (33). For instance, sumoylation of the PML NB-associated HTLV-1 protein Tax regulates its ubiquitylation, thereby modulating its capacity to activate NF- $\kappa$ B through the involvement of K63-linked ubiquitin chains (12, 16, 51). We speculate that TRIM5 $\alpha$

transiently shuttles to the nucleus where it is sumoylated, localizes at PML/SUMO bodies and recruits Ubc13 to stimulate K63-linked ubiquitylation.

## **Conclusions**

Mutating the putatively sumoylated lysine 10 in TRIM5 $\alpha$  affects the balance between self-ubiquitylation and K63-linked ubiquitylation. It also decreases the capacity of TRIM5 $\alpha$  to activate NF- $\kappa$ B and AP-1. In addition, the consensus sumoylation site seems to be a determinant for the transient co-localization with SUMO in the nucleus, which may be linked to the observed modulation of ubiquitylation.

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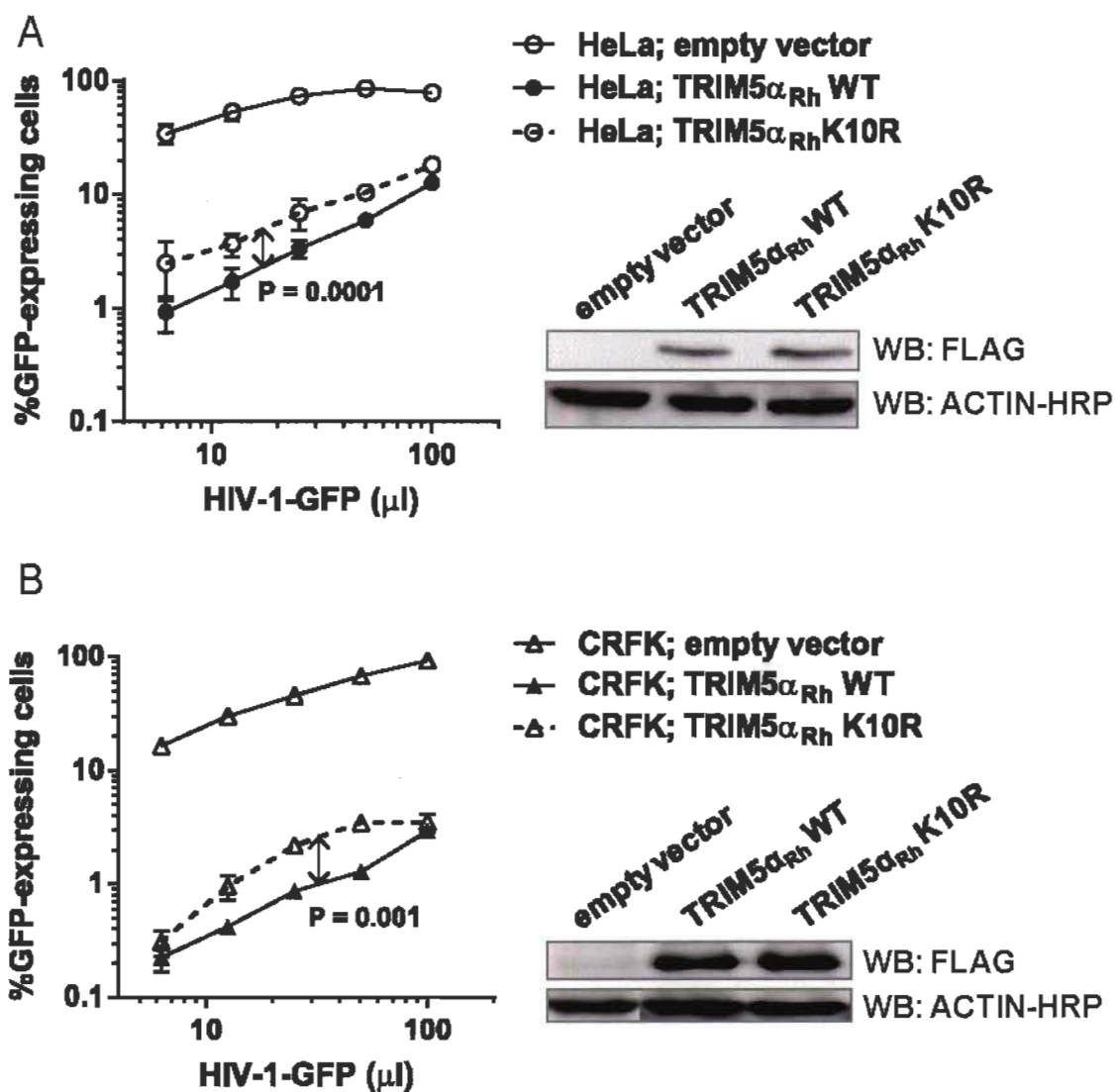
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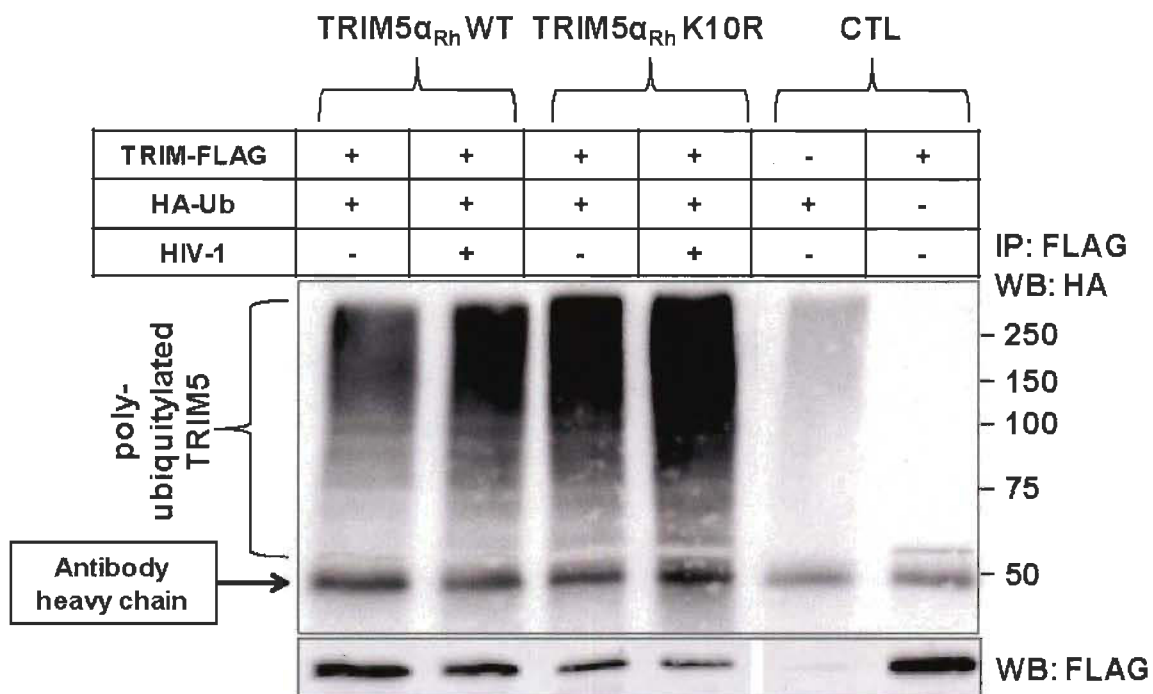
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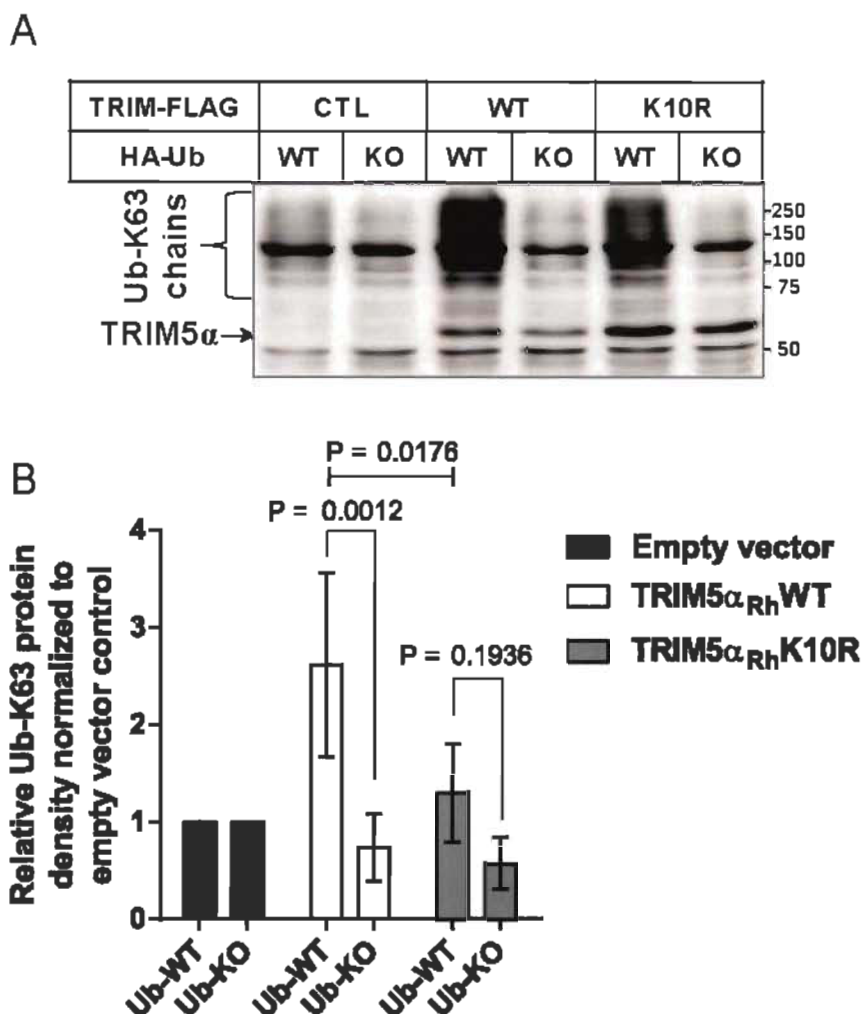


**Figure 1** Contribution of the TRIM5 $\alpha_{Rh}$  sumoylation consensus site to HIV-1 restriction.

(A) HeLa cells or (B) CRFK cells stably expressing WT TRIM5 $\alpha_{Rh}$  or the K10R mutant were challenged with increasing amounts of VSV G-pseudotyped HIV-1<sub>TRIP-CMV-GFP</sub> (“HIV-1-GFP”) in triplicates. The percentage of cells expressing GFP was analyzed by FACS two days later. WT and K10R TRIM5 $\alpha_{Rh}$  protein expression levels were analyzed in both cell lines by western blotting using an anti-FLAG antibody (right panels). Actin expression was analyzed as a loading control. The images show two sections of the same gel that were cut and pasted together to exclude irrelevant lanes.

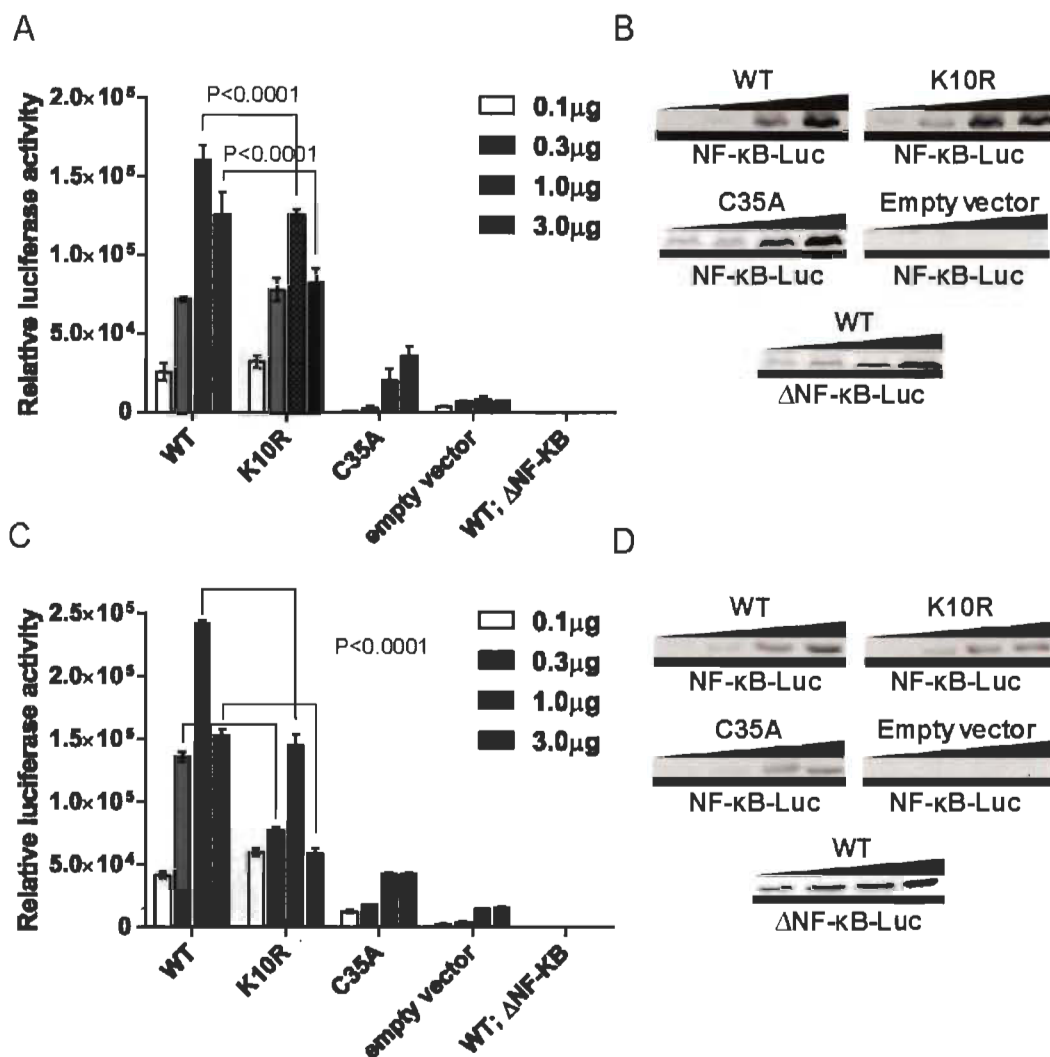


**Figure 2**     **The K10R mutation increases TRIM5 $\alpha_{Rh}$  auto-ubiquitylation levels.**  
 HEK293T cells were co-transfected with WT or K10R FLAG-tagged TRIM5 $\alpha_{Rh}$  and with an HA-tagged version of human ubiquitin. Two days later, transfected cells were infected with HIV-1<sub>TRIP-CMV-GFP</sub> (MOI of 0.2) for 6 hours before lysis, or left uninfected. Controls (CTL) were transfected with WT FLAG-TRIM5 $\alpha_{Rh}$  alone or with HA-ubiquitin alone and were not infected. FLAG-tagged proteins were immunoprecipitated as described in Materials and Methods. The IP products were analyzed by western blotting first using a mouse anti-FLAG antibody and subsequently with a rabbit anti-HA antibody. All lanes shown are from a single western blot experiment and were exposed the same amount of time but some irrelevant lanes were cut out.



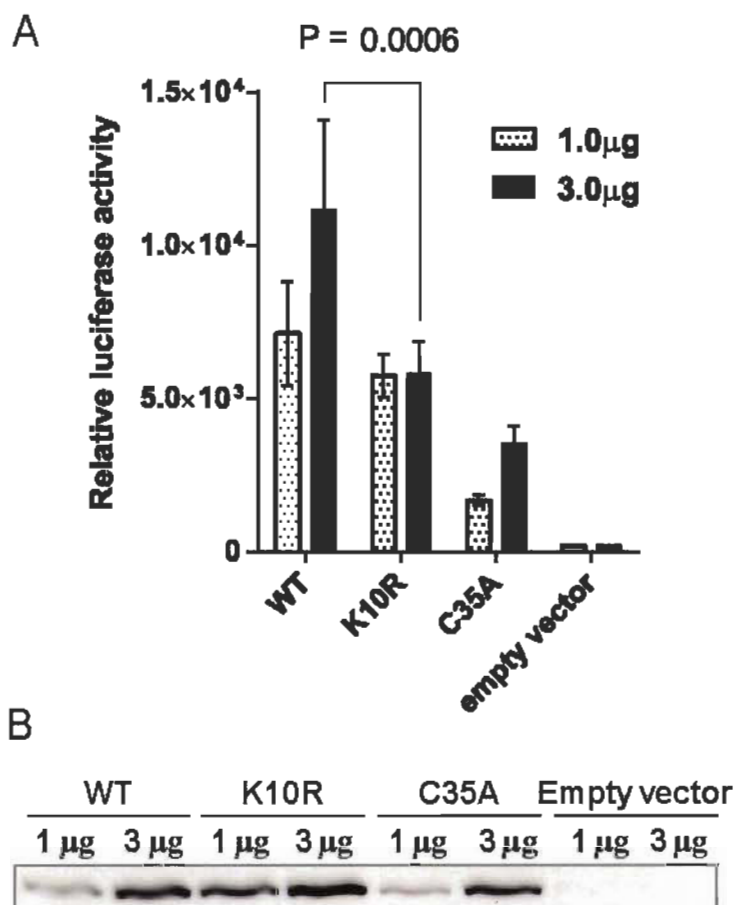
**Figure 3** The K10R mutation decreases the ability of TRIM5 $\alpha_{Rh}$  to form K63-linked ubiquitin chains.

(A) HEK293T cells were transfected with either the “empty” pMIP or with pMIP expressing FLAG-tagged WT or K10R pMIP-TRIM5 $\alpha_{Rh}$ , and co-transfected with the plasmid expressing WT or KO HA-Ubiquitin. KO Ubiquitin has all its lysines mutated to arginines. Two days later, whole protein lysates were analyzed by western blotting using rabbit antibodies directed at K63-linked ubiquitin chain or at FLAG. (B) Density of bands detected using the K63 chains-specific antibody (ranging from 75 to >250 kDa) normalized to the control consisting of cells co-transfected with WT or KO HA-Ub and the “empty” pMIP. Graph shows the average data from 4 independent experiments with standard deviations, and *P*-values were calculated using a two-way ANOVA test.



**Figure 4** The K10R mutation affects TRIM5 $\alpha_{Rh}$ -dependent activation of NF- $\kappa$ B.

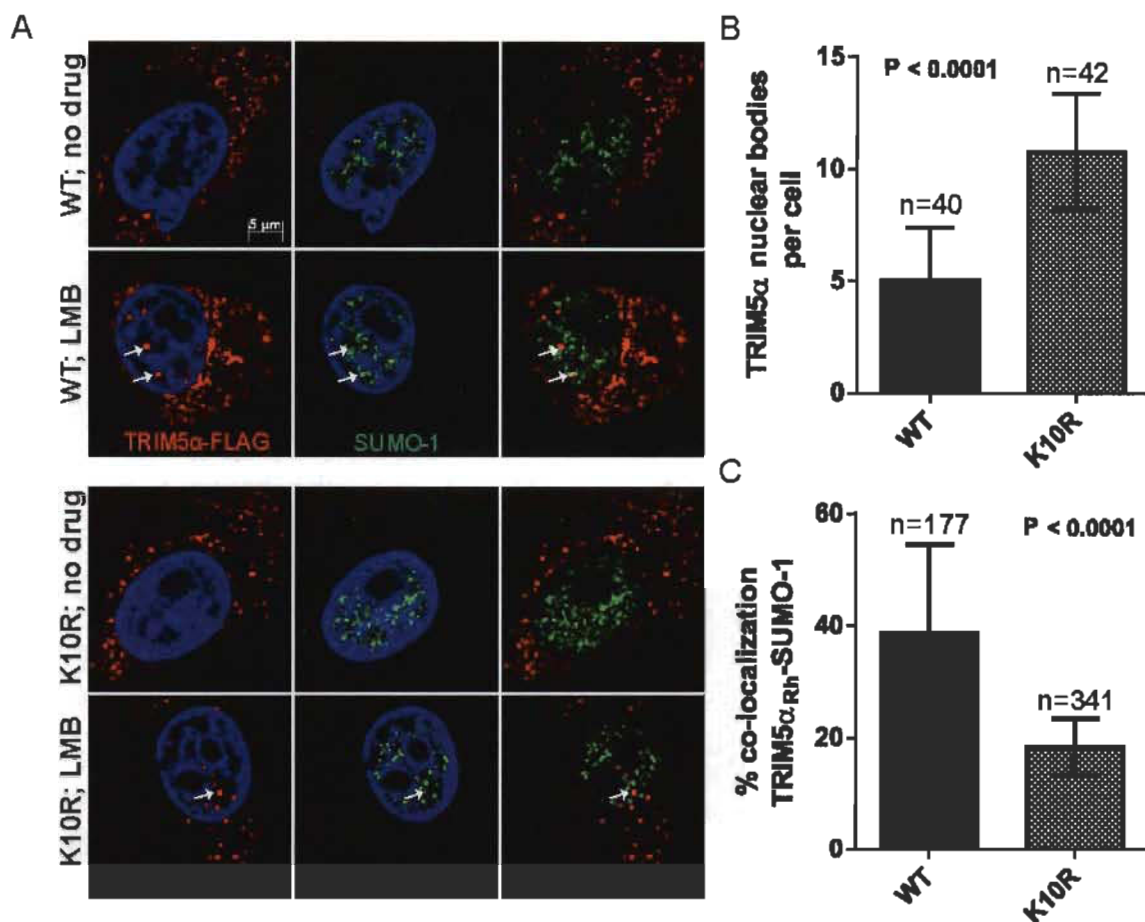
(A) HEK293T cells were transfected with increasing amounts of either the WT or the indicated mutants of pMIP-TRIM5 $\alpha_{Rh}$  and co-transfected with a constant amount (0.6  $\mu$ g) of a reporter plasmid for NF- $\kappa$ B activity expressing luciferase. As controls, cells were co-transfected with the empty pMIP plasmid and the NF- $\kappa$ B reporter construct, or were co-transfected with WT pMIP-TRIM5 $\alpha_{Rh}$  and the activation-deficient mutant of the reporter construct ( $\Delta$ NF- $\kappa$ B-Luc). Two days later, cells were lysed and the luciferase activity was measured. Bars show the average from triplicate transfections with standard deviations. Statistical analysis was done using a 2-way ANOVA test from an experiment done in triplicate. (B) The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5 $\alpha$  expression. For this, we pooled together the 3 lysates from each triplicate transfection and analyzed them by western blotting using an anti-FLAG antibody. (C, D) Repetition of the experiment shown in panels A and B.



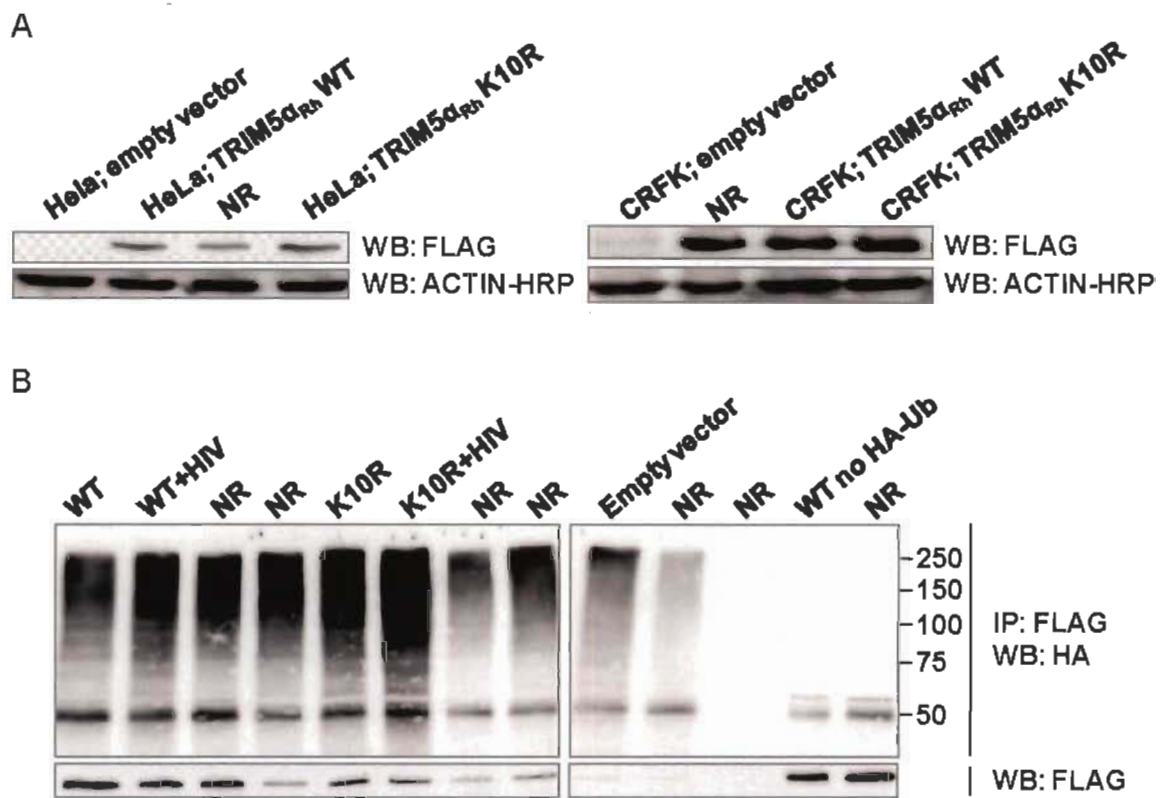
**Figure 5**

**The K10R mutation affects TRIM5 $\alpha_{\text{Rh}}$ -dependent activation of AP-1.** (A) HEK293T cells were transfected with 1 or 3  $\mu\text{g}$  of either the WT or the indicated mutants of pMIP-TRIM5 $\alpha_{\text{Rh}}$  and co-transfected with a constant amount (0.6  $\mu\text{g}$ ) of a reporter plasmid for AP-1 activity expressing luciferase. As a control, cells were co-transfected with the empty pMIP plasmid and the AP-1 reporter construct. Two days later, cells were lysed and the luciferase activity was measured. Bars show the average from triplicate transfections with standard deviations. Statistical analysis was done using a 2-way ANOVA test from an experiment done in triplicate. (B) The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5 $\alpha$  expression. For this, we pooled together the 3 lysates from each triplicate transfection and analyzed them by western blotting using an anti-FLAG antibody.





**Figure 6** **Decreased nuclear co-localization of K10R TRIM5 $\alpha_{Rh}$  and SUMO-1.** (A) CRFK cells stably expressing either WT or K10R FLAG-tagged TRIM5 $\alpha_{Rh}$  and treated or not with LMB were processed for IF microscopy. A rabbit polyclonal anti-FLAG antibody was used for the detection of TRIM5 $\alpha_{Rh}$  (red) and a mouse monoclonal antibody was used for the detection of endogenous SUMO-1 (green). White arrows point to examples of co-localizations of nuclear TRIM5 $\alpha_{Rh}$  and SUMO-1. (B) Average number of TRIM5 $\alpha_{Rh}$  NBs per cell with standard deviation. The total number of nuclei analyzed is shown on top of each bar, and statistical analysis was done using a Student's t-test. (C) Quantitative analysis of the frequency of TRIM5 $\alpha_{Rh}$ -SUMO-1 co-localization in nuclei. The percentage of WT and K10R TRIM5 $\alpha_{Rh}$  NBs co-localizing with SUMO-1 is shown. Each bar represents the % of co-localization observed in 25 randomly chosen nuclei (excluding nuclei that had no TRIM5 $\alpha$  NBs) with standard deviation. Statistical analysis was done using a Student's t-test. The total number of NBs analyzed is shown on top of each bar.



Supplementary data: Full western blots from figure 1 and 2.

## CHAPITRE IV

### IDENTIFICATION OF A PUTATIVE SUMO INTERACTING MOTIF IN RHESUS MACAQUE TRIM5 $\alpha$ IMPORTANT FOR INNATE IMMUNE SIGNALING AND HIV-1 RESTRICTION

MARIE-EDITH NEPVEU-TRAVERSY, ANN DEMOGINES<sup>1</sup>, THOMAS FRICKE<sup>2</sup>,  
MÉLODIE B. PLOURDE, KATHLEEN RIOPEL, MAXIME VEILLETTE, FELIPE  
DIAZ-GRIFFERO<sup>2</sup>, SARA L. SAWYER<sup>1</sup> ET LIONEL BERTHOUX

Le contenu de ce chapitre fait l'objet d'un manuscrit qui sera soumis pour une publication en anglais dans la revue *Journal of Virology*.

#### 4.1 Contribution des auteurs

MÉNT et LB ont conçu cette étude. MÉNT a effectué la majorité des expériences. AD a effectué l'analyse phylogénétique. TF, MBP et KR ont contribué à la réalisation des expériences. MV a contribué à l'analyse bioinformatique et à la modélisation moléculaire. MÉNT, AD, SLS et LB ont écrit le manuscrit. Tous les auteurs ont lu et approuvé la version finale du manuscrit.

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## 4.2 Résumé de l'article

TRIM5 $\alpha$  du singe macaque rhésus (TRIM5 $\alpha_{Rh}$ ) est un facteur de restriction qui inhibe fortement le VIH-1. TRIM5 $\alpha_{Rh}$  se lie spécifiquement à la capsidie du VIH-1 via son domaine B30.2/PRYSPRY et ce, peu de temps après l'entrée du virus dans le cytoplasme. Récemment, trois motifs potentiels d'interaction avec SUMO (SIMs) ont été identifiés dans le domaine PRYSPRY des protéines TRIM5 $\alpha$ . Cependant, la modélisation de la structure du domaine PRYSPRY a révélé que les motifs SIM1 et SIM2 étaient enfouis dans le cœur hydrophobique de la protéine, suggérant ainsi que l'interaction de ces motifs avec SUMO était peu probable. Quant à SIM3, aucune fonction ne lui a été attribuée jusqu'à maintenant. À la lumière de ces résultats, nous avons ré-analysé la séquence PRYSPRY de TRIM5 $\alpha_{Rh}$  et identifié un autre SIM potentiel (<sup>435</sup>VIIC<sup>438</sup>) que nous avons nommé SIM4. Ce motif est exposé à la surface du domaine PRYSPRY favorisant une interaction potentielle avec SUMO ou des protéines SUMOylées. La mutation du motif SIM4 n'a pas affecté la stabilité de TRIM5 $\alpha$  contrairement à SIM1 qui, lorsque muté, diminue significativement la stabilité de TRIM5 $\alpha_{Rh}$ . Fait intéressant, le motif SIM4 a subi une variation significative chez les primates et la substitution du motif SIM4 du macaque rhésus par les motifs SIM4 d'origine naturelle a affecté l'activité de restriction de TRIM5 $\alpha$ , ce qui suggère un rôle pour SIM4 dans la reconnaissance de la capsidie rétrovirale. En outre, le mutant SIM4 (KKIC) a perdu sa capacité à inhiber le VIH-1. Ce mutant a également échoué dans l'activation de NF- $\kappa$ B et AP-1. De plus, la protéine TRIM5 $\alpha_{Rh}$  mutante pour SIM4 a démontré une co-localisation réduite avec les protéines SUMO-1 et PML, une protéine hautement SUMOylée qui dirige la formation de corps nucléaires enrichis en protéines SUMO. En conclusion, SIM4 est essentiel à la fois pour une interaction directe avec les capsides rétrovirales et la signalisation de l'immunité innée, et nous spéculons que ce motif permet d'interagir avec les protéines SUMOylées qui sont impliquées dans l'activation de NF- $\kappa$ B et AP-1.

**Importance de l'étude:** Les mécanismes de l'immunité innée sont importants pour le contrôle de l'infection au VIH-1 *in vivo*. Les protéines TRIM5 $\alpha$  ont été

identifiées depuis des années pour être des effecteurs de l'immunité innée, paralysant les premières étapes de la réplication des rétrovirus sensibles à leur restriction. Plus récemment, TRIM5 $\alpha$  a été identifié comme un récepteur de l'immunité innée capable d'induire une réponse anti-rétrovirale permettant l'activation de NF- $\kappa$ B et AP-1 lors d'infections. Le domaine RING de TRIM5 $\alpha$  possédant une activité E3 ubiquitine-ligase est requis pour déclencher la cascade de signalisation, et nous avons récemment démontré que la lysine potentiellement SUMOylée de TRIM5 $\alpha$  module cette activité E3 ubiquitine-ligase en favorisant l'activation de l'immunité innée. Dans cette étude, nous avons identifié un nouveau motif, cette fois situé à l'intérieur du domaine PRYSPRY, qui est possiblement lié à la SUMOylation. Nous avons démontré que ce motif, SIM4, est essentiel à l'activation de NF- $\kappa$ B et AP-1 et nous présentons des résultats qui suggèrent que SUMO ou des protéines SUMOylées sont impliquées dans cette fonction.

### 4.3 Troisième article scientifique

#### Identification of a putative SUMO interacting motif in rhesus macaque TRIM5 $\alpha$ important for innate immune signaling and HIV-1 restriction

##### Abstract

TRIM5 $\alpha$  from the rhesus macaque (TRIM5 $\alpha_{rh}$ ) is a restriction factor that shows strong activity against HIV-1. TRIM5 $\alpha_{rh}$  binds specifically to HIV-1 capsid through its B30.2/PRYSPRY domain shortly after entry of the virus into the cytoplasm. Recently, three putative SUMO interacting motifs (SIMs) have been identified in the PRYSPRY domain of TRIM5 $\alpha$  proteins. However, structural modeling of this domain revealed that SIM1 and SIM2 were buried in the hydrophobic core of the protein, suggesting that interaction with SUMO was implausible. In light of these results, we re-analyzed the TRIM5 $\alpha_{rh}$  PRYSPRY sequence and identified an additional putative SIM (<sup>435</sup>VIIC<sup>438</sup>) which we named SIM4. This motif is exposed at the surface of the PRYSPRY domain, allowing potential interactions with SUMO or SUMOylated proteins. Mutating SIM4 did not alter stability, unlike mutations in SIM1. Interestingly, SIM4 undergoes significant variation among primates and substituting rhesus macaque SIM4 with naturally occurring SIM4 variants affected the restriction activity of TRIM5 $\alpha$ , suggesting a role in capsid recognition. A SIM4 double substitution mutant (KKIC) lost its ability to restrict HIV-1, and also failed to activate NF- $\kappa$ B and AP-1-mediated transcription. Moreover, SIM4-mutated TRIM5 $\alpha_{rh}$  showed reduced co-localization with both SUMO-1 and PML, a heavily SUMOylated protein that drives the formation of SUMO-enriched nuclear bodies. In conclusion, SIM4 is crucial for both direct interaction with incoming capsids and innate immune signaling, and we speculate that this motif might mediate interactions with SUMOylated proteins which are involved in the AP-1 and NF- $\kappa$ B signaling pathways.

**Importance:** Innate immune mechanisms are important for the control of HIV-1 infection *in vivo*. TRIM5 $\alpha$  has been known for years to be an effector of innate immunity, crippling early stages of restriction-sensitive retroviruses. More recently,

TRIM5 $\alpha$  was found to also be a retrovirus sensor, promoting NF- $\kappa$ B- and AP-1-mediated transcription in response to retroviral infections. The RING domain of TRIM5 $\alpha$ , which has E3 ubiquitin ligase activity, is required for this sensing activity, and we recently showed that a putative SUMOylation site adjacent to the RING domain modulates the TRIM5 $\alpha$  “E3” activity to promote innate immune activation. Here we identify another motif, this time in the PRYSPRY domain, which potentially mediates interactions with SUMO. We find that this motif, SIM4, is a critical determinant for NF- $\kappa$ B and AP-1 activation and we present data suggesting that SUMO-1 or a SUMOylated protein is involved in this function.

## Introduction

Proteins from the TRIM5 $\alpha$  family are known for their restriction activity against retroviruses (66, 73, 78). Restriction mediated by TRIM5 $\alpha$  proteins relies on their ability to recognize the viral capsid (CA) core shortly after entry of the virus in the cell, in a virus-specific and species-specific manner (67). TRIM5 $\alpha$  contains RING, B-Box, coiled-coil (RBCC) domains (47, 63) and the C-terminal PRYSPRY domain (also called B30.2 or SPRY). TRIM5 $\alpha$  orthologs differ mostly in the 3 variable regions (V1, V2 and V3) of PRYSPRY (70) which confer specificity for the retroviral CA (88); for instance, HIV-1 is inhibited by rhesus macaque TRIM5 $\alpha$  (TRIM5 $\alpha_{Rh}$ ), but generally not by the human version of the protein (87). B-Box and coiled-coil domains are important for the dimerization and higher-order multimerization of TRIM5 $\alpha_{Rh}$  that can lead to the formation of cytoplasmic bodies (CBs) observable by light microscopy (13, 24, 34, 49, 63, 73). The RING domain has E3-ubiquitin ligase activity essential for auto-ubiquitylation of the protein and efficient restriction (23).

Interactions between TRIM5 $\alpha$  and the N-terminal domain of retroviral CA proteins that form the outer core destabilize the CA lattice (9, 29, 37, 38, 59, 74). Consequently, TRIM5 $\alpha$  induces core disruption and causes the degradation of some core components as well as its own degradation through the proteasomal machinery (37, 43, 64), and also impairs viral transport to the nucleus (4, 14). Another function has recently been attributed to TRIM5 $\alpha$ : the activation of innate immunity pathways dependent on AP-1 and NF- $\kappa$ B transactivation (60, 76). The RING domain recruits E2-ubiquitin conjugating enzyme Ubc13/Uev1a to generate lysine 63 (K63)-linked polyubiquitin chains, an important step for AP-1 and NF- $\kappa$ B transactivation (60, 80). Activation of NF- $\kappa$ B by TRIM5 $\alpha$  is triggered by contact with a restriction sensitive virus (60) or by over-expression of TRIM5 $\alpha$  (54, 60, 76, 79).

The human genome contains three functional *SUMO* genes encoding SUMO-1/2/3 (31, 65, 75). SUMOylation is a post-translational modification involved in many cellular mechanisms such as cell signaling, notably the NF- $\kappa$ B signaling pathway (44),



transcription (81) and regulation of protein stability (31). SUMO modifies targeted proteins through its covalent attachment to lysines present in a specific consensus sequence:  $\Psi$ -K-X-D/E. SUMO interacting motifs (SIMs) are necessary for the non-covalent interaction of proteins with free SUMO (71) or with SUMOylated proteins (72) and consist of a short domain rich in hydrophobic residues (52). TRIM5 $\alpha$  proteins have a consensus SUMOylation site at a position immediately upstream of the RING domain and three putative SIMs (SIM1, SIM2 and SIM3) located in the PRYSPRY domain (5). The contribution of small-ubiquitin like modifier (SUMO) in TRIM5 $\alpha$  functions has been controversial in part because TRIM5 $\alpha$  has never been revealed to be SUMOylated. However, recent publications proposed a role for SUMO-1 in retroviral TRIM5 $\alpha$ -mediated restriction (5, 42). The predicted SUMOylated lysine present in TRIM5 $\alpha_{RH}$  promotes AP-1 and NF- $\kappa$ B signaling pathways through modulation of the RING domain activity (54). Moreover, it has been recently proposed that SIM1 and SIM2 are important for TRIM5 $\alpha$ -mediated NF- $\kappa$ B activation (42) and retroviral restriction (5, 42), and that mutating SIM3 had no significant effect (5, 12, 42). However, structural data reveal that the phenotypes of SIM1 and SIM2 mutants were probably a consequence of PRYSPRY misfolding. Indeed, SIM1 and SIM2 are buried inside the hydrophobic core of the PRYSPRY domain, making interactions with other proteins unlikely (12). In light of these results, we re-analyzed the PRYSPRY sequence for the presence of other possible SIMs and we uncovered a novel putative SIM that we named SIM4. Unlike SIM1 and SIM2, SIM4 residues are exposed at the surface of the protein, making this motif more suitable for interaction with other proteins. Here we characterize the importance of SIM4 in HIV-1 restriction and in the activation of NF- $\kappa$ B and AP-1. We propose that the SIM4 domain is an important determinant of innate immune signaling by mediating interactions with an unidentified SUMOylated protein.

## Materials and Methods

### *Plasmid DNAs and mutagenesis*

pMIP-TRIM5 $\alpha_{Rh}$  expresses a C-terminal FLAG-tagged version of TRIM5 $\alpha_{Rh}$  and has been described before (8, 68). We designed the following primers to introduce mutations in pMIP-TRIM5 $\alpha_{Rh}$  SIM1 IL376-377KK, 5'-AAGTGCTTGGAAGAAGGGGTATGTGCTGG-3' (forward) and 5'-CATACCCCCTTCTTCCAAGCACTTTCTT-3' (reverse); SIM3 IV430-431KK, 5'-TGCTCCTTTCAAGAAGCCCCTCTCTGTGAT-3' (forward) and 5'-CAGAGAGGGGCTTCTTGAAAGGAGCAAAAG-3' (reverse); SIM4 IV430-431KK, 5'-CTTTCATTGTGCCCTCTCTAAGAAGATTTGTCCTGATCGTGTTG-3' (forward) and 5'-CAACACGATCAGGACAAATCTTCTTAGAGAGGGGCACAATGAAAG-3' (reverse). SIM4 variants were constructed using pMIP-TRIM5 $\alpha_{Rh}$  as template and mutations were introduced with following primers: VKIC, 5'-GTGCCCCTCTCTGTGAAAATTTGTCCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAATTTTCACAGAGAGGGGCACAATGAAAG-3' (reverse); VNIC, 5'-GTGCCCCTCTCTGTGAATATTTGTCCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAATATTCACAGAGAGGGGCACAATGAAAG-3' (reverse); VTIC, 5'-GTGCCCCTCTCTGTGACTATTTGTCCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAATAGTCACAGAGAGGGGCACAATGAAAG-3' (reverse); VIVC, 5'-GTGCCCCTCTCTGTGATTGTTTGTCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAACAATCACAGAGAGGGGCACAATGAAAG-3' (reverse); VIFC, 5'-GTGCCCCTCTCTGTGAAAATTTGTCCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAAATTTTCACAGAGAGGGGCACAATGAAAG-3' (reverse); MTIC, 5'-GTGCCCCTCTCTATGACTATTTGTCCTGATCGTGTTG-3' (forward) and 5'-GATCAGGACAAATAGTCATAGAGAGGGGCACAATGAAAG-3' (reverse). All mutations were confirmed by Sanger sequencing. The vector production plasmids Pmd-G, p $\Delta$ R8.9, pCL-Eco and pTRIP-CMV-GFP have all been extensively described elsewhere (2, 6, 7, 53, 91). pRK5-HA-Ubiquitin WT and KO (40) were obtained from Ted Dawson (Johns Hopkins University, Baltimore, MD) through Addgene. The KO ubiquitin version of this plasmid bears the following mutations eliminating all possibilities of ubiquitin chain formation: K6R, K11R, K27R, K29R, K33R, K48R and

K63R. pCEP4-NF- $\kappa$ B-Luc expresses luciferase under the control of an NF- $\kappa$ B-dependent promoter, while pCEP4- $\Delta$ NF- $\kappa$ B-Luc is transcriptionally deficient due to the deletion of the NF- $\kappa$ B binding site (76). Both constructs were kind gifts from M. Emerman (University of Washington, Seattle, WA). pHTS-AP1-Luc expresses luciferase under the control of an AP1-dependent promoter and was a kind gift from J. Luban (University of Massachusetts Medical School, Worcester, MA).

### *Cell lines*

Human embryonic kidney (HEK) 293T cells and Crandell-Reese feline kidney (CRFK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C, 5% CO<sub>2</sub>. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

### *Virus production*

MLV and HIV-1-based vectors were produced through transient transfection of HEK293T cells using polyethylenimine (MW 25,000; Polysciences, Warrington, PA) and collected as previously described (8, 61). To produce the MLV-based MIP vectors, cells were transfected with the relevant pMIP plasmid and co-transfected with pCL-Eco and pMD-G. All stably transduced cell lines were produced as previously described (8, 61). Successfully transduced CRFK cells were selected with 4  $\mu$ g/ml of puromycin. To produce the GFP-expressing HIV-1 vector HIV-1<sub>TRIP-CMV-GFP</sub> (nicknamed "HIV-1-GFP"), cells were co-transfected with p $\Delta$ R8.9, pMD-G and pTRIP-CMV-GFP.

### *Viral challenges*

CRFK cells were plated in 24-well plates at 30,000 cells per well and infected the next day with different amounts of HIV-1-GFP or using a defined multiplicity of infection (MOI). Two days post-infection, cells were trypsinized and fixed in 2% formaldehyde in a PBS solution. The % of GFP-positive cells was then determined by

analyzing 10,000 cells using a FC500 MPL cytometer with the CXP software (Beckman Coulter).

### *Western blotting*

Cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton, 0.1% SDS, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate and Complete protease inhibitor cocktail (Roche, Bale, Switzerland)). Whole cell lysates were then boiled in protein sample buffer (60 mM Tris-HCl pH 6.8, 10% glycerol, 0.002% bromophenol blue, 2% SDS, 2% beta-mercaptoethanol) and resolved by SDS-PAGE. After transfer to nitrocellulose membranes, blots were probed with rabbit anti-FLAG (1:2000, Cell Signaling, Danvers, Massachusetts), mouse anti-actin HRP (1:20 000, EMD Millipore, Billerica, MA) and visualized using secondary antibodies coupled to horseradish peroxidase (Santa Cruz, Dallas, TX) and a chemiluminescence detection system (SuperSignal West Femto, Thermo scientific, Waltham, MA). Images were recorded on a UVP (Upland, California) EC3 imaging system, and densitometry analyses were performed using the area density tool of the VisionWorks LS software (UVP).

### *Stability assay*

CRFK cells expressing the different TRIM5 $\alpha_{Rh}$  variants were plated in 6-well plates at a cell density leading to 80% confluent cells on the next day. Cells were then treated with 100  $\mu$ M cycloheximide (Sigma, Saint Louis, MO) and harvested at different time points as previously described (23). Cells were lysed in stability lysis buffer (NaCl 100 mM, NP-40 0.5%, SDS 0.1%, Tris 100 mM (pH 8.0) and Complete protease inhibitor cocktail (Roche, Bale, Switzerland)) and further processed for Western blotting. Protein density was measured for each time point and normalized to actin, and then normalized to time zero density.

### *Multimerization assay*

CRFK cells stably expressing either FLAG-tagged WT or SIM4mut TRIM5 $\alpha$ <sub>Rh</sub> were each plated in three 10-cm dishes at a cell density leading to 80% confluent cells on the next day. Cells from the 3 dishes were then pooled together and lysed with NP-40 0.05% on ice for 15 min. After centrifugation, supernatants were divided in 5 aliquots and treated 5 min with different concentrations of glutaraldehyde (J.T. Baker, Phillipsburg, NJ) concentrations (0, 0.25, 0.5, 1.25, 2.5 mM). Tris-HCl 0.1 M pH 7.5 was added to stop reaction and all samples were denaturated and further processed for Western blotting, as described above.

### *NF- $\kappa$ B and AP-1 reporter assays*

HEK293T cells were plated in 12-well plates at a cell density leading to 80% confluent cells at the time of transfection and were then co-transfected with increasing amounts (0.1  $\mu$ g, 0.3  $\mu$ g, 1.0  $\mu$ g and 3.0  $\mu$ g) of TRIM5 $\alpha$  expression plasmid which were completed to a total of 3.0  $\mu$ g with pMIP empty vector, or fixed amount (2  $\mu$ g) of either FLAG-tagged WT or mutant TRIM5 $\alpha$  and a fixed amount (0.6  $\mu$ g) of either pCEP4-NF- $\kappa$ B-Luc, pCEP4- $\Delta$ NF- $\kappa$ B-Luc or pHTS-AP1. Cells were lysed with RIPA buffer 48 h post-transfection and assessed for luciferase activity using the BrightGlow Luciferase kit (Promega). Luminescence was measured with a Synergy HT multi-detection microplate reader (BioTek, Winooski, VT) and analyzed with the Gen5 software (BioTek).

### *K63 ubiquitin chains formation assay*

HEK293T cells were seeded in 12-well plates at a cell density leading to 80% confluent cells and co-transfected with either WT or mutant FLAG-tagged TRIM5 $\alpha$  (2  $\mu$ g) and either WT or KO pRK5-HA-ubiquitin (0.5  $\mu$ g). The “empty” plasmid pMIP was used as a negative control. Cells were lysed in RIPA buffer 48 h post-transfection and processed for Western blotting. A monoclonal human/rabbit chimeric K63- linked ubiquitin chains-specific antibody (clone Apu, Millipore) was used (1:1000 dilution) to detect K63 ubiquitin chains.

### *Immunofluorescence microscopy*

60,000 CRFK cells were plated on microscope cover glasses (Fisherbrand) in 6-well plates. Treatments with the nuclear export inhibitor leptomycin B (LMB, Enzo LifeScience) were done at 20 ng/ml for 12 h prior to fixation. For the experiment involving HIV-1 infection, cells were treated with MG132 (1  $\mu$ g/ml) for 6 h and infected with HIV-1-GFP for 2 h (MOI of 4) prior to fixation. In control experiments, infections were done in presence of ammonium chloride (NH<sub>4</sub>Cl, 20 mM, Sigma). Cells were washed with PBS, fixed for 10 min in 4% formaldehyde-PBS at 37°C, washed three times in PBS and permeabilized with 0.1% Triton X-100 for 2 min on ice. Cells were then washed twice with PBS and treated with 10% normal goat serum (Vector laboratories) in PBS for 30 min at room temperature. This saturation step was followed by a 4 h incubation with a rabbit polyclonal FLAG antibody (Cell Signaling) and an antibody against endogenous SUMO-1 (mouse monoclonal antibody, Invitrogen), both at a 1:200 dilution in PBS with 10% normal goat serum. To stain endogenous promyelocytic leukemia protein (PML), primary mouse monoclonal antibody (Enzo Life Sciences, Farmingdale, NY) was used at a 1:50 dilution. HIV-1 CA was stained using a mouse monoclonal anti CAP24 antibody (purified in our laboratory from the 183 hybridoma clone, AIDS Research and reference Reagent Program, NIAID, NIH). Fluorescent staining was done by incubating with an Alexa594-conjugated goat anti-rabbit antibody and an Alexa488-conjugated goat anti-mouse antibody (each used at 1:200 dilution). Cells were washed 4 times in PBS before mounting in Vectashield (Vector Laboratories). Hoechst33342 (0.8  $\mu$ g/ml; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Pictures were generated using a Zeiss AxioObserver microscope equipped with an Apotome module and the Axiovision software. Imaging parameters were set to identical values across samples.

### *Binding of TRIM5 $\alpha_{Rh}$ to HIV-1 capsid-nucleocapsid (CA-NC) complexes*

The HIV-1 CA-NC protein was expressed, purified and assembled as previously described (12, 39) and further used for the binding assay. HEK293T cells were transfected with plasmids expressing either FLAG-tagged WT or SIM4mut TRIM5 $\alpha_{Rh}$ .

Two days post-transfection, cells were lysed as previously described (12). CA-NC complexes assembled in vitro were incubated with cell lysates for 1 h at room temperature. The mixture was then applied to a 70% sucrose cushion and centrifuged at 100,000 g for 1 h at 4°C. Following centrifugation, the supernatant was removed and pellet was resuspended in protein sample buffer. Western blot was performed to detect TRIM5 $\alpha_{Rh}$  (anti-FLAG antibody, Cell Signaling) and HIV-1 CA-NC (anti-CAP24 antibody, Immunodiagnostics).

### *Phylogenetic analysis*

Primate gene sequences for *TRIM5* were obtained from GenBank. *TRIM5* exon 8 was sequenced from 5 additional primate species (bonobo, pileated gibbon, agile gibbon, yellow-cheeked gibbon and black mangabey). Primary and immortalized primate cell lines were grown and genomic DNA was harvested (detailed description of cell lines can be found in Demogines et al. (19)). *TRIM5* sequence was amplified by PCR using the following primer pairs: 5'-CTCCTTCCAAGACACATAACTTACCC-3' with 5'-AAGAGGTGCTGTACAGAAGGGGC-3'. The PCR fragments were then sequenced using the PCR primers listed above or the following primers: 5'-GCTACTGGGTTATAGGGTTAGAGGAAGG-3' or 5'-CTCCTTATAACTTCTAAACAAGGTTCCCTCCC-3'. Primate *TRIM5* exon 8 gene sequences have been deposited in GenBank (accession numbers: KJ701422-KJ701426).

## **Results**

### *Identification of a new putative SUMO interacting motif in TRIM5 $\alpha_{Rh}$*

New insights in TRIM5 $\alpha_{Rh}$  PRYSPRY structure revealed that the previously proposed motifs SIM1 and SIM2 are located inside the hydrophobic core of this domain, implying that direct interactions with SUMO (or any other protein) were unlikely (12). Re-analysis of the TRIM5 $\alpha_{Rh}$  PRYSPRY domain with the SUMO-binding Motif (SBM) prediction software (GPS-SBM 1.0) (62) yielded several SUMO interacting motifs with

different scores (Fig. 1A). SIM1 (<sup>376</sup>ILGV<sup>379</sup>), SIM2 (<sup>405</sup>VIGL<sup>408</sup>) and SIM3 (<sup>430</sup>IVPL<sup>433</sup>) were confirmed as putative SIMs for TRIM5 $\alpha_{Rh}$ , but the analysis revealed the presence of another putative SIM (<sup>435</sup>VIIC<sup>438</sup>) which we named SIM4. Unlike SIM1 and SIM2, SIM4 residues are exposed at the surface of the protein, next to SIM3 and within the variable region 3 (V3) (Fig. 1B, C). All three variable regions of the PRYSPRY domain are involved, to a certain extent, in recognition of and interaction with the retroviral CA (57).

To investigate the potential role of SIM4 in TRIM5 $\alpha_{Rh}$ -mediated HIV-1 restriction, this motif was mutated to <sup>435</sup>KKIC<sup>438</sup> (Fig. 1D). The mutants of SIM1 and SIM3 (SIM1mut and SIM3mut) described by others (5, 12, 42) were also included in this study. SIM2mut was not included since its phenotype was identical to that of SIM1mut (5, 12, 42).

#### *SIM4mut TRIM5 $\alpha_{Rh}$ does not bind HIV-1 CA and does not restrict HIV-1*

To analyze the ability of SIM4-mutated TRIM5 $\alpha_{Rh}$  to restrict HIV-1, CRFK cells stably expressing WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{Rh}$  were challenged with increasing amounts of VSV G-pseudotyped HIV-1-GFP. Cells transduced with the empty vector were used as a non-restrictive control. As expected (5, 12, 42), both WT and SIM3mut TRIM5 $\alpha_{Rh}$  strongly restricted HIV-1, by up to ~100-fold, while cells expressing SIM1mut TRIM5 $\alpha_{Rh}$  were permissive to HIV-1 infection at levels similar to cells expressing the empty vector (Fig. 2A). Expression of SIM4mut TRIM5 $\alpha_{Rh}$  resulted in a decrease in HIV-1 permissiveness of less than 2-fold, showing that the ability of this TRIM5 $\alpha_{Rh}$  mutant to restrict HIV-1 was almost completely lost (Fig. 2A). Levels of TRIM5 $\alpha$  expression were similar for all mutants, except for SIM1mut whose expression level was significantly decreased (Fig. 2B), as previously seen by others (12, 42). Therefore, mutating SIM4 affects restriction of incoming HIV-1 seemingly without altering protein stability, unlike SIM1mut which reduces both.



We investigated the ability of WT and SIM4mut TRIM5 $\alpha_{Rh}$  to physically interact with HIV-1 CA-NC complexes in a CA-binding assay *in vitro* (39). Briefly, purified and assembled HIV-1 CA-NC complexes were mixed with TRIM5 $\alpha_{Rh}$  proteins, which were over-expressed and isolated from HEK293T cells. The mixture was then applied to a 70% sucrose cushion and following centrifugation, pellets and supernatants were analyzed by Western blotting. In this assay, binding of TRIM5 $\alpha_{Rh}$  to HIV-1 CA is evidenced when both are present in the pellet fraction. This analysis had previously been done for SIM1mut and SIM3mut and results were consistent with functional restriction results since SIM3mut was found to bind HIV-1 CA-NC complexes while SIM1mut did not (12). Similarly to SIM1mut, SIM4mut TRIM5 $\alpha_{Rh}$  failed to bind HIV-1 CA-NC complexes (Fig. 2C), hence explaining its inability to restrict this virus.

HIV-1 restriction by TRIM5 $\alpha$  correlates with the co-localization of both proteins following infection, suggesting the existence of a “sequestration” mechanism of restriction (14). To further investigate TRIM5 $\alpha$ -CA interactions in a cellular context, we assessed the co-localization of FLAG-tagged WT, SIM1mut or SIM4mut TRIM5 $\alpha_{Rh}$  with incoming HIV-1 CA. Cells were infected with VSV G-pseudotyped HIV-1 GFP for 2 h and then stained with antibodies against FLAG and CA. MG132 was used as a proteasome inhibitor to prevent the degradation of TRIM5 $\alpha_{Rh}$  (64). Fig. 2D shows representative photographs obtained using our protocol. In this assay, each CA foci observed is likely a single virus (45), but it does not distinguish between fused and unfused particles. WT, SIM1mut and SIM4mut TRIM5 $\alpha_{Rh}$  were all able to form cytoplasmic bodies (CBs). Ammonium chloride (NH<sub>4</sub>Cl) treatment was used as a no-entry control as it inhibits the acidification of endosomes, thus preventing the release of VSV G-pseudotyped viruses in the cytoplasm and making TRIM5 $\alpha$ -CA co-localization improbable. CA foci were bigger when cells were treated with NH<sub>4</sub>Cl (Fig. 2D), probably a consequence of CA accumulation in endosomes. As expected (14), a significant fraction (14%) of HIV-1 CA foci co-localized with WT TRIM5 $\alpha_{Rh}$  CBs (Fig. 2D). In contrast, SIM1mut and SIM4mut TRIM5 $\alpha_{Rh}$  showed very little co-localization with HIV-1 CA (0.6% and 0.7%, respectively). CA-TRIM5 $\alpha_{Rh}$  co-localization dropped to 0.1% when cells were treated with NH<sub>4</sub>Cl, suggesting that the

co-localization events observed in absence of this drug were specific and involved post-entry virus cores. Taken together, these results show that mutating SIM4 can disrupt physical interactions of TRIM5 $\alpha_{Rh}$  with its viral CA target, hence preventing restriction.

*SIM4mut TRIM5 $\alpha_{Rh}$  is stable and able to shuttle to the nucleus*

A previous study proposed that phenotypes observed with SIM1mut and SIM2mut were a consequence of substituting buried residues which may result in misfolding of the PRYSPRY domain (12). As shown in Fig. 3A, SIM1mut but not SIM4mut altered steady-state levels of TRIM5 $\alpha_{Rh}$ , suggesting that protein folding was affected by the former but not the latter mutation. To further investigate protein stability, CRFK cells stably expressing WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{Rh}$  were treated with the translation inhibitor cycloheximide (CHX) and reduction in protein levels was then monitored over time by Western blotting coupled with densitometry analysis. As shown in Fig. 3A, the SIM1mut TRIM5 $\alpha_{Rh}$  half-life was <1.5 h, compared to >5 h for WT, SIM3mut and SIM4mut TRIM5 $\alpha_{Rh}$ . Of note, other studies reported a shorter half-life for WT TRIM5 $\alpha_{Rh}$  than in our assay (23, 64), but they used different cell lines; the rate of decay of a given protein can vary depending on the cell type used and growth conditions (26, 28). Using a two-way ANOVA followed by a Dunnett's multiple comparison test, we found no statistically significant differences for SIM3mut and SIM4mut when compared to WT in this assay ( $P$  values = 0.8832 and 0.3038 respectively). On the other hand, SIM1mut stability was significantly decreased ( $p < 0.0001$ ), supporting the misfolding hypothesis.

TRIM5 $\alpha$  has the capacity to shuttle between the nucleus and cytoplasm, and the fraction of the protein present in the nucleus can be enriched by treatment with the CRM-1/exportin 1 inhibitor leptomycin B (LMB) (12, 22, 36). In the presence of this drug, TRIM5 $\alpha_{Rh}$  forms nuclear bodies (NBs) that co-localize with promyelocytic leukemia (PML) bodies (22). CRFK cells transduced with SIM1mut, SIM4mut or WT TRIM5 $\alpha_{Rh}$  were treated with LMB for 12 h and the presence of TRIM5 $\alpha_{Rh}$  NBs was analyzed (Fig. 3B). In absence of the drug, WT, SIM4mut and SIM1mut TRIM5 $\alpha_{Rh}$

formed CBs but not NBs, as expected (73). The appearance of SIM1mut TRIM5 $\alpha_{RH}$  CBs was similar to that of the WT control, unlike SIM4mut TRIM5 $\alpha_{RH}$  CBs that were noticeably bigger. In presence of LMB, WT TRIM5 $\alpha_{RH}$  could be found in both cytoplasmic and nuclear compartments as expected (Fig. 3B). SIM1mut TRIM5 $\alpha_{RH}$  was not observed in the nucleus as previously reported (12). Interestingly, the distribution of SIM4mut was similar to that of the WT protein, showing that SIM4mut did not affect nuclear translocation (Fig. 3B).

The relatively large size of SIM4mut TRIM5 $\alpha_{RH}$  CBs (Fig. 3B) prompted us to analyze the potential of this mutant to multimerize. CRFK cells stably expressing WT or SIM4mut TRIM5 $\alpha_{RH}$  were lysed and the lysates were treated with increasing concentrations of glutaraldehyde, a crosslinking agent previously used in similar experiments (49, 56). As shown in Fig. 3C, in the absence of glutaraldehyde we observed only monomers of TRIM5 $\alpha_{RH}$ -FLAG (approximately 54 kDa). Multimers of SIM4mut TRIM5 $\alpha_{RH}$  were present in greater relative amounts regardless of the glutaraldehyde concentration used, compared to the WT (Fig. 3C), suggesting that this mutation increased TRIM5 $\alpha_{RH}$  multimerization potential, thus explaining the enlarged CBs. Altogether, the results shown in Fig. 2 and Fig. 3 suggest that SIM4mut disrupts restriction by affecting surface determinants of interaction with CA, although we cannot exclude that the effect on multimerization patterns also plays a role. On the other hand, SIM1mut probably caused major misfolding of the PRYSPRY domain as also proposed by others (12).

#### *SIM4mut abolishes the ability of TRIM5 $\alpha_{RH}$ to activate AP-1 and NF- $\kappa$ B*

TRIM5 $\alpha$  is a pattern recognition receptor that promotes the activation of innate immune signaling pathways dependent on transcription factors AP-1 and NF- $\kappa$ B (54, 60, 76). These innate immune pathways result in the production of various inflammatory cytokines and chemokines (3). A previous study proposed that specific interactions between TRIM5 proteins and HIV-1 CA lead to the activation of AP-1 and NF- $\kappa$ B (60). However, TRIM5 $\alpha_{RH}$  can also activate both NF- $\kappa$ B and AP-1 when it is over-expressed

and in the absence of infection (42, 76, 79). To determine whether the different SIM mutations could interfere with this TRIM5 $\alpha_{Rh}$  function, HEK293T cells were co-transfected with increasing amounts of WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{Rh}$ , and a constant concentration of a plasmid expressing luciferase under the control of the NF- $\kappa$ B response element (76). The TRIM5 $\alpha_{Rh}$  RING domain mutant C35A, which has no ubiquitin ligase activity and is unable to activate NF- $\kappa$ B, was used as a negative control (76). Another control consisted of cells co-transfected with WT TRIM5 $\alpha_{Rh}$  and a version of the luciferase expression plasmid bearing a deletion in the NF- $\kappa$ B response element. As shown in Fig. 4A, transfection of 3  $\mu$ g of WT and SIM3mut TRIM5 $\alpha_{Rh}$  resulted in strong NF- $\kappa$ B activation ( $\sim$ 17-fold and  $\sim$ 16-fold compared to the empty vector control respectively;  $p < 0.0001$ ). Transfection of 1  $\mu$ g of these plasmids had similar effects, but using smaller amounts (0.1-0.3  $\mu$ g) activated NF- $\kappa$ B only weakly (Fig. 4A), as seen previously (54). C35A and SIM1mut TRIM5 $\alpha_{Rh}$  were unable to activate NF- $\kappa$ B as previously described by others (42). Interestingly, SIM4mut TRIM5 $\alpha_{Rh}$  did not induce significant NF- $\kappa$ B activation since levels of luciferase expression were similar to that of the C35A control and the empty vector control (Fig. 4A). Levels of luciferase expression in cells transfected with 3  $\mu$ g of SIM1mut and SIM4mut TRIM5 $\alpha_{Rh}$  were significantly decreased compared to cells transfected with the WT plasmid ( $\sim$ 65-fold for SIM1mut and  $\sim$ 11-fold for SIM4mut TRIM5 $\alpha_{Rh}$ ;  $p < 0.0001$ ). In this experiment, levels of TRIM5 $\alpha$  expression were similar for the WT and the various mutants, with the exception of SIM1mut which showed reduced expression levels (Fig. 4B). Therefore, the inability of SIM4mut TRIM5 $\alpha_{Rh}$  to activate NF- $\kappa$ B was not caused by decreased expression levels. On the other hand, SIM1mut TRIM5 $\alpha_{Rh}$  low expression levels partly explain the very low levels of NF- $\kappa$ B induction for this mutant (Fig. 4A). However, higher protein amounts were observed when 3  $\mu$ g of SIM1mut TRIM5 $\alpha_{Rh}$  were transfected than when 0.3  $\mu$ g of either WT or SIM3mut TRIM5 $\alpha_{Rh}$  were used (Fig. 4B), while NF- $\kappa$ B activation was readily detected for WT and SIM3mut but not for SIM1mut TRIM5 $\alpha_{Rh}$  at these same conditions (Fig. 4A). Thus, we conclude that both SIM1 and SIM4 mutants are deficient for NF- $\kappa$ B activation.

To evaluate the effects of mutating the TRIM5 $\alpha_{RH}$  SIMs on AP-1-dependent transactivation, HEK293T cells were co-transfected with 1 or 3  $\mu$ g of WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{RH}$  and with 0.6  $\mu$ g of a reporter plasmid expressing luciferase under the control of the AP-1 response element (60). WT and SIM3mut TRIM5 $\alpha_{RH}$  significantly activated AP-1 at both DNA concentrations compared to the empty vector control (Fig. 4C). On the other hand, SIM1mut and SIM4mut TRIM5 $\alpha_{RH}$  were very weak activators of AP-1, as evidenced by low luciferase signal in both cases. Specifically, when 3  $\mu$ g of the TRIM5 $\alpha_{RH}$ -expressing plasmid were used, WT and SIM3 TRIM5 $\alpha_{RH}$  increased luciferase activity by  $\sim$ 21-fold and  $\sim$ 9-fold relative to the empty vector control, respectively ( $p < 0.0001$ ) while SIM1mut and SIM4mut TRIM5 $\alpha_{RH}$  increased it by only  $\sim$ 2.3-fold ( $p = 0.1957$ ) and  $\sim$ 2.2-fold ( $p = 0.2304$ ), respectively. Thus, these two mutants did not activate AP-1 to significant levels, while they resulted in a very significant ( $p < 0.0001$ ) decrease in activation levels when compared to the WT (Fig. 4C). Levels of protein expression were determined in the transfected cells by Western blotting (Fig. 4D) and we found that SIM4mut TRIM5 $\alpha_{RH}$  was expressed at WT-like levels while SIM3mut TRIM5 $\alpha_{RH}$  was expressed at smaller levels (a 2- to 3-fold decrease). This explains that in the experiment shown in Fig. 4C, AP-1 activation by SIM3mut TRIM5 $\alpha_{RH}$  is similarly less efficient than for the WT control. On the other hand, the inability of SIM1mut and SIM4mut to activate AP-1 does not result from poor expression levels. Thus, we conclude that SIM4mut TRIM5 $\alpha_{RH}$  is unable to activate both AP-1 and NF- $\kappa$ B signaling pathways, suggesting that SIM4 is a determinant for innate immune signaling.

*All SIM mutants are able to trigger K63-linked polyubiquitin chains formation*

The E3 ubiquitin ligase activity associated with the RING domain is essential for the ability of TRIM5 $\alpha$  to promote formation of K63-linked ubiquitin chains (20, 60) that can then mediate the activation of AP-1 and NF- $\kappa$ B signaling through phosphorylation of TAK1 (1, 3, 35). To study the ability of SIM1, SIM3 and SIM4 mutants to generate Ub-K63 chains, FLAG-tagged TRIM5 $\alpha_{RH}$ , WT or mutated, were transfected in HEK293T cells along with a construct expressing ubiquitin. To detect K63-linked

polyubiquitin, Western blots were performed using an antibody specific for this type of ubiquitylated chains (Fig. 5A) (82). As controls, cells were instead co-transfected with KO-Ub, a polyubiquitylation-deficient version of ubiquitin in which all potentially ubiquitylated lysines have been substituted to arginines (40). K63-linked ubiquitin chains were detected between ~70 and 300 kDa, consistent with previous data (54, 60), and such polyubiquitylation products were almost undetectable in cells transfected with the KO ubiquitin mutant (Fig. 5A). The C35A TRIM5 $\alpha_{Rh}$  RING domain mutant was used as a negative control. As expected, K63-linked ubiquitin chains were almost undetectable in cells transfected with this mutant (Fig. 5A). Co-transfection of WT, SIM1mut, SIM3mut and SIM4mut TRIM5 $\alpha_{Rh}$  with WT ubiquitin resulted in strong stimulation of K63-linked polyubiquitylation, compared to cells co-transfected with TRIM5 $\alpha_{Rh}$  and KO ubiquitin or compared to cells co-transfected with the empty vector and WT ubiquitin (Fig. 5A, 5B). A densitometry-based quantitative analysis was performed using Western blots from 3 independent experiments (Fig. 5B). Values were obtained by normalizing relative K63-linked ubiquitin chains densities from the indicated TRIM5 $\alpha_{Rh}$  to the one obtained with the empty vector control (CTL) when co-transfected with WT ubiquitin. The mean levels of TRIM5 $\alpha$  protein expression in the 3 experiments were similar for all FLAG-tagged proteins utilized (data not shown). Results show that only C35A TRIM5 $\alpha_{Rh}$  was unable to significantly generate K63-linked ubiquitin chains ( $p = 0.916$  when compared to the empty vector). In contrast, WT, SIM1mut, SIM3mut and SIM4mut TRIM5 $\alpha_{Rh}$  all promoted the formation of K63-linked ubiquitin chains at similar levels (Fig. 5B). Specifically, all three SIM mutants showed statistically significant induction of K63-linked polyubiquitin when compared to the empty control while none of them showed statistically significant differences with the WT (Fig. 5B). Therefore, SIM1 and SIM4 are dispensable for the formation of K63-linked ubiquitin chains even though they are required for NF- $\kappa$ B and AP-1 activation.

*SIM4mut TRIM5 $\alpha_{Rh}$  shows reduced co-localization with PML and SUMO-1 in the nucleus*

PML/TRIM19, a member of the TRIM family of proteins, is the main determinant for the formation of PML bodies, nuclear structures that can harbor multiple proteins and have known roles in antiviral defense (30). PML is heavily SUMOylated and SUMO is a major contributing factor to the formation of PML NBs (51). TRIM5 $\alpha_{Rh}$  is able to shuttle to the nucleus and co-localize with PML NBs (22). We hypothesized that putative interactions between the SIM4 motif and either SUMO or a SUMOylated protein may determine the localization of TRIM5 $\alpha$  at PML NBs. Thus, we reasoned that mutating SIM4 would affect the co-localization of TRIM5 $\alpha$  with PML or SUMO or both. To test this possibility, IF microscopy was performed on CRFK cells stably expressing WT or SIM4mut TRIM5 $\alpha_{Rh}$  and in the presence or absence of LMB. As shown in Fig. 6A, LMB treatment resulted in a fraction of WT and SIM4mut TRIM5 $\alpha_{Rh}$  being present in the nucleus, while no nuclear TRIM5 $\alpha$  staining was observed in the absence of the drug as expected. A significant fraction of TRIM5 $\alpha_{Rh}$  NBs co-localized with SUMO-1 (left panel) and PML (right panel) in LMB-treated cells, and this was observed for both WT and SIM4mut TRIM5 $\alpha_{Rh}$ . However, co-localization between SIM4mut TRIM5 $\alpha_{Rh}$  NBs and both SUMO-1 and PML was less frequent than for WT TRIM5 $\alpha_{Rh}$  NBs. Specifically, an analysis of multiple randomly selected fields (Fig. 6B) showed that in presence of LMB, 38.6% of WT TRIM5 $\alpha_{Rh}$  NBs but only 12.9% of SIM4mut TRIM5 $\alpha_{Rh}$  NBs co-localized with SUMO-1 ( $p < 0.0001$ ). Similarly, 17.6% of WT TRIM5 $\alpha_{Rh}$  NBs but only 6.7% of SIM4mut TRIM5 $\alpha_{Rh}$  NBs co-localized with PML ( $p < 0.0001$ ). Therefore, the SIM4 mutation affects TRIM5 $\alpha_{Rh}$  association with SUMO-1 and PML NBs, suggesting that this motif is a determinant for the interaction with SUMO or a SUMOylated protein.

*Natural simian variants of SIM4 activate both NF-KB and AP-1 signaling pathways and exhibit partial HIV-1 restriction*

Our results suggest that SIM4 (VIIC) in rhesus macaque is an important motif for the ability of TRIM5 $\alpha_{Rh}$  to inhibit HIV-1 and activate innate immune signaling. We

wondered whether this motif was well conserved among primates given that *TRIM5 $\alpha$*  is known to have evolved under positive selection in order to restrict different retroviruses (32). For this purpose, we aligned the *TRIM5 $\alpha$*  coding sequence from 36 primate species. The relationship between these species is shown in Fig. 7. None of the New World monkeys (NWM) or the prosimians encode an intact VIIC motif. On the other hand, all representatives of the great apes (human, chimpanzee, West African chimpanzee, bonobo, gorilla, Sumatran orangutan and Bornean orangutan) and a majority of the Old World monkeys (OWM) have conserved SIM4 VIIC motifs. Based on these observations, we propose that SIM4 VIIC did not appear independently in these two groups but, instead, arose before the split of Old World monkeys and hominoids which occurred approximately 29 million years ago. Within the OWM and hominoids lineages, SIM4 has been mutated at 5 independent times throughout evolution (stars on branches in Fig. 7). Analysis of *TRIM5 $\alpha$*  exon 8 sequences from 34 individual crab-eating macaques was done using *BLAST* and revealed 2 individuals from the Mauritian population which encode VIVC. For rhesus macaque, sequences from 15 individuals were analyzed and one individual from Guangxi was found to encode VTIC. These data demonstrate that SIM4 is quite variable both between and within primate species.

All natural SIM4 variants were tested for their ability to activate innate immune signaling and restrict HIV-1. Using mutagenesis, we substituted *TRIM5 $\alpha$ <sub>Rh</sub>*-SIM4 (VIIC) with all the natural variants (VKIC, VNIC, VTIC, VIVC, VIFC and MTIC), resulting in the production of 6 new proteins. First, their capacity to activate NF- $\kappa$ B and AP-1 signaling pathways was tested, as described above, with co-transfections in HEK293T cells. All the SIM4 variants were able to activate NF- $\kappa$ B (Fig. 8A) and AP-1 (Fig. 8B) since significant luciferase activity was observed in both experiments. The SIM4 variants seemed to show some level of reduction (~30% to 60%) in their capacity to activate both pathways, compared to the WT, but this could largely be attributed to decreased expression levels (Fig. 8C, 8D). In contrast, SIM4mut *TRIM5 $\alpha$ <sub>Rh</sub>* was unable to activate both signaling pathways, as expected. Taken together, these results suggest that, despite the emergence of variations within the SIM4 sequence among primates, its capacity of activating NF- $\kappa$ B and AP-1 is conserved.



The second function to be analyzed was TRIM5 $\alpha$  capacity to inhibit HIV-1 infection. CRFK cells stably expressing all the SIM4 variants, SIM4mut or WT TRIM5 $\alpha_{Rh}$  were challenged with increasing amounts of HIV-1 GFP and further analyzed by flow cytometry. As expected, WT TRIM5 $\alpha_{Rh}$  strongly inhibited HIV-1 (Fig. 8E) and SIM4mut TRIM5 $\alpha_{Rh}$  failed to restrict HIV-1 and was only slightly less permissive than cells expressing the empty vector control. All the SIM4 variants restricted HIV-1 at an intermediate level: ~8-fold more permissive than WT in average and ~6-fold more restrictive than empty vector control in average. Levels of protein expression were determined by Western blotting and we found TRIM5 $\alpha$  expression levels to be comparable in all transduced cells (Fig. 8E). An upper unspecific band was apparent with the anti-FLAG antibody, as previously observed in CRFK cells (Fig. 3C). Thus, mutating VIIC into naturally occurring SIM4 in other species is affecting TRIM5 $\alpha_{Rh}$  ability to optimally restrict HIV-1, consistent with this motif being involved in interactions with CA.

## Discussion

In this study, we identified a new putative SIM in TRIM5 $\alpha_{Rh}$  PRYSPRY which is, on one hand, involved in specific interactions with the retroviral capsid, and on the other hand, is also essential for the activation of innate immunity. Unlike the previously described SIM1 and SIM2 (5, 12, 42), SIM4 (<sup>435</sup>VIIC<sup>438</sup>) is not buried inside the hydrophobic core of TRIM5 $\alpha_{Rh}$  (Fig. 1) and, interestingly, mutating this motif to <sup>435</sup>KKIC<sup>438</sup> affected restriction (Fig. 2) and innate immune signaling (Fig. 4), while a similar mutation in the adjacent SIM3 affected neither function. As also proposed by Brandariz-Nuñez et al. (12), our results support the hypothesis that mutating SIM1 causes gross misfolding of the PRYSPRY domain, preventing the nuclear translocation of TRIM5 $\alpha_{Rh}$  (Fig. 3) and resulting in its proteasomal degradation(55). Misfolded proteins are generally degraded by the ubiquitin-proteasome system to ensure cellular homeostasis (26). In contrast to SIM1mut, SIM4mut TRIM5 $\alpha_{Rh}$  was stable and able to shuttle to the nucleus (Fig. 3) indicating that this mutant was probably not misfolded, consistent with SIM4 being a surface motif.

Unlike HIV-1 CA, which has never been shown to be SUMOylated, the CA protein of the murine leukemia virus (N-MLV), which is restricted by human TRIM5 $\alpha$  (TRIM5 $\alpha_{Hu}$ ) (59), is likely modified by SUMO-1 (90). It has been proposed that TRIM5 $\alpha_{Hu}$  restricts N-MLV efficiently through the binding of TRIM5 $\alpha_{Hu}$  SIM1 and/or SIM2 to the incoming SUMOylated N-MLV CA (5). Recent structural data showing that SIM1 and SIM2 are internal motifs (12) invalidate this theory, but the surface SIM4 identified here, which is present in the human ortholog (Fig. 7) could potentially have this function. Is the SUMOylation pathway important for the direct restriction of incoming retroviruses? Arriagada and colleagues showed that over-expression of SUMO-1 in HEK 293T cells restricts N-MLV but only in conditions in which this virus is also restricted by TRIM5 $\alpha_{Hu}$  (5), suggesting that SUMO is a co-factor for TRIM5 $\alpha$ . However, over-expression of SUMO-1 had much smaller effects on other human cell lines permissiveness to N-MLV, and a follow-up study focusing this time on TRIM5 $\alpha_{Rh}$  did not show that over-expression of SUMO-1 inhibited HIV-1 in restrictive conditions (42). Instead, the authors reported that SUMO-1 depletion canceled HIV-1 restriction by TRIM5 $\alpha_{Rh}$ , but this conclusion was weakened by inefficient SUMO-1 knockdown and abnormally low levels of HIV-1 restriction by TRIM5 $\alpha_{Rh}$  in the control cells (42). In addition, a more recent study reported that perturbing the SUMOylation pathway by over-expression of Gam1 (10, 16) had no impact on the restriction of N-MLV in human cells (12), and we found that Gam1 does not influence the restriction of HIV-1 by TRIM5 $\alpha_{Rh}$  expressed in human cells (55). Altogether, there is presently no strong or consensual evidence that the SUMOylation pathway is important for direct restriction of incoming retroviruses, especially for HIV-1. On the other hand, the presence of a conserved putative SUMOylation site and of SIMs indicates a role for SUMO in TRIM5 $\alpha$  functions, and we propose that SUMO and the SUMOylation pathway are involved in the activation of innate immunity pathways by TRIM5 $\alpha$  rather than the direct restriction of incoming retroviruses. Recently, we have shown that mutating the putatively SUMOylated lysine (lysine 10) reduces the capacity of TRIM5 $\alpha_{Rh}$  to generate K63-linked polyubiquitin and to activate NF- $\kappa$ B and AP-1 (54). Here we searched for a SIM motif that could also be a determinant of these TRIM5 $\alpha$  function. SIM3, being a surface motif, was a good candidate, but we searched for additional potential SIM motifs

and identified SIM4 which is adjacent to SIM3 (Fig. 1). We show that a double substitution in SIM4 completely disrupts the NF- $\kappa$ B/AP-1 activation potential of TRIM5 $\alpha_{Rh}$ , while mutating SIM3 has no effect (Fig. 4).

It has been previously suggested that activation of NF- $\kappa$ B and AP-1 by TRIM5 $\alpha$  is a consequence of a signaling cascade that includes the formation of K63-linked ubiquitin chains. Similarly to TRAF6, a RING domain-containing protein, TRIM5 $\alpha$  recruits the E2 heterodimer Ubc13/Uev1A to catalyze the formation of K63-linked ubiquitin chains (60) which are important for the activation of TAK1 (20), a key factor for the downstream activation of both NF- $\kappa$ B and AP-1 (1). Interestingly, all SIM mutants tested in this study, including SIM1mut, were able to trigger significant formation of K63-linked ubiquitin chains (fig. 5). This result suggests that the various SIMs, and possibly the PRYSPRY domain itself, are irrelevant to the formation of K63-linked ubiquitin chains. Further, these data indicate that the presence of K63-linked polyubiquitin chains is not the sole requirement for the activation of NF- $\kappa$ B and AP-1. Thus, SIM4 seems to be a determinant for an as yet unidentified activation step that is required for the activation of NF- $\kappa$ B and AP-1. We cannot exclude, however, that the K63-linked ubiquitin chains generated by SIM4mut TRIM5 $\alpha$  are defective in a way that could not be revealed by our simple Western blot analysis, or that they are not properly located in the cell to activate NF- $\kappa$ B and AP-1. A direct role for SIM4 in activating NF- $\kappa$ B and AP-1 appears more likely, however, and we propose that SIM4 interacts with SUMO or an unidentified SUMOylated protein and that this interaction contributes to the activation of the innate immunity pathways. TAK-1 binding protein 2 (TAB2) was recently proposed to be SUMOylated. In fact, mutating its SUMOylated lysine enhanced the activity of TAB2 and resulted in the increased activation of AP-1. Thus, SUMOylation is regulating the activity of TAB2 (83). Also, NF- $\kappa$ B essential modulator (NEMO/IKK $\gamma$ ) is SUMOylated in response to various signals of stress signals and this allows the activation of NF- $\kappa$ B by translocating the SUMOylated NEMO to the nucleus (33, 69, 85). The inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ ), which hides the nuclear localization signal, is usually degraded by the proteasome to allow translocation of NF- $\kappa$ B to the nucleus, but SUMOylation of I $\kappa$ B $\alpha$  stabilizes it and consequently inhibits NF- $\kappa$ B activation (21).

In addition to having an important role in nuclear transport of many proteins (27, 84), SUMO is also involved in regulating many transcription factors (50, 81). SUMO-1 modifies AP-1 and down-regulates its transcriptional activity in the nucleus (11, 77). RelA, a subunit of NF- $\kappa$ B, is SUMOylated resulting in the down-regulation of NF- $\kappa$ B activity (41). Altogether, activation of NF- $\kappa$ B and AP-1 is heavily controlled by SUMOylation, providing several speculative models for TRIM5 $\alpha$  interacting with a SUMOylated factor in order to promote innate immune activation. For instance, TRIM5 $\alpha$  could interact with I $\kappa$ B $\alpha$  to disrupt its interaction with NF- $\kappa$ B and thus promotes NF- $\kappa$ B translocation to the nucleus. We observe that the frequency of colocalization between SIM4mut TRIM5 $\alpha_{Rh}$  NBs and SUMO-1 or PML was significantly decreased compared with WT TRIM5 $\alpha_{Rh}$  (Fig. 6), supporting the hypothesis that SIM4 directly interacts with a SUMOylated protein, although this is of course indirect evidence.

The involvement of the PRYSPRY/B30.2 domains belonging to different TRIM proteins in innate immune signaling was previously described and found to involve interactions with other proteins (17, 18, 58). For example, TRIM25 is known to activate innate immune signaling, in response to viral infections, via the interaction between its B30.2/SPRY domain and retinoic acid-inducible gene-1 (RIG-I), resulting in activation of the latter (18). TRIM25 also catalyzes the formation of both anchored and unanchored K63-linked ubiquitin chains through its RING domain, and in some cases, the activation of RIG-I is independent of the anchored K63-linked chain formation (48), thus mirroring our observations. Of note, RIG-I is known to be SUMOylated and this modification is involved in the activation of type 1 interferon signaling (15, 46). Thus, TRIM5 $\alpha$  could activate NF- $\kappa$ B and AP-1 through two distinct mechanisms, one dependent on the RING domain and lysine 10 (54) and involving the generation of K63-linked polyubiquitin, the other one mapping to the PRYSPRY domain and involving SIM4.

A phylogenetic analysis of SIM4 revealed some significant changes in some of the 36 primates analyzed (Fig. 7). Six naturally occurring SIM4 variants were tested for their ability to restrict HIV-1 when placed in a TRIM5 $\alpha_{Rh}$  context and all were less

efficient than their WT counterpart in this assay (Fig. 6E), confirming that SIM4 is an important motif for CA interactions in addition to being important for the activation of innate immunity. Interestingly, SIM4 showed intra-species polymorphism in rhesus and crab-eating macaques. The analysis of NCBI TRIM5 $\alpha$  sequences from 34 crab-eating macaques revealed 2 isolates bearing the motif VIVC, and 1 of 15 rhesus macaques had the motif VTIC. These species are already known for their polymorphism due to the occasional presence of a supplementary allele coding for TRIMCyp (25, 89). Interestingly, individuals expressing the modified SIM4 (VIVC and VTIC) did not have the additional TRIMCyp allele. It is possible that TRIMCyp and SIM4 variant emerged in response to different viruses. The high level of polymorphism in SIM4 also supports a role for this motif in interacting with incoming retroviral CA cores. Of note, the VIIC motif is found in great apes and in OWM, the two primate groups living in Africa where one finds the most cases of SIV infections. It is possible that the primates in other parts of the world do not undergo a strong selective pressure in order to make TRIM5 $\alpha$  ultra-responsive towards lentiviruses. Interestingly, the ability to activate innate immune signaling pathways was maintained for the six naturally occurring SIM4 variants tested here (Fig. 8), suggesting that this function is essential and is evolutionarily conserved despite variations in the sequence.

In conclusion, we characterized a new putative SIM that is important for TRIM5 $\alpha$ -dependent innate immune signaling, possibly through its capacity to interact with a SUMOylated protein, and also has a role in the retroviral tropism of TRIM5 $\alpha$ .

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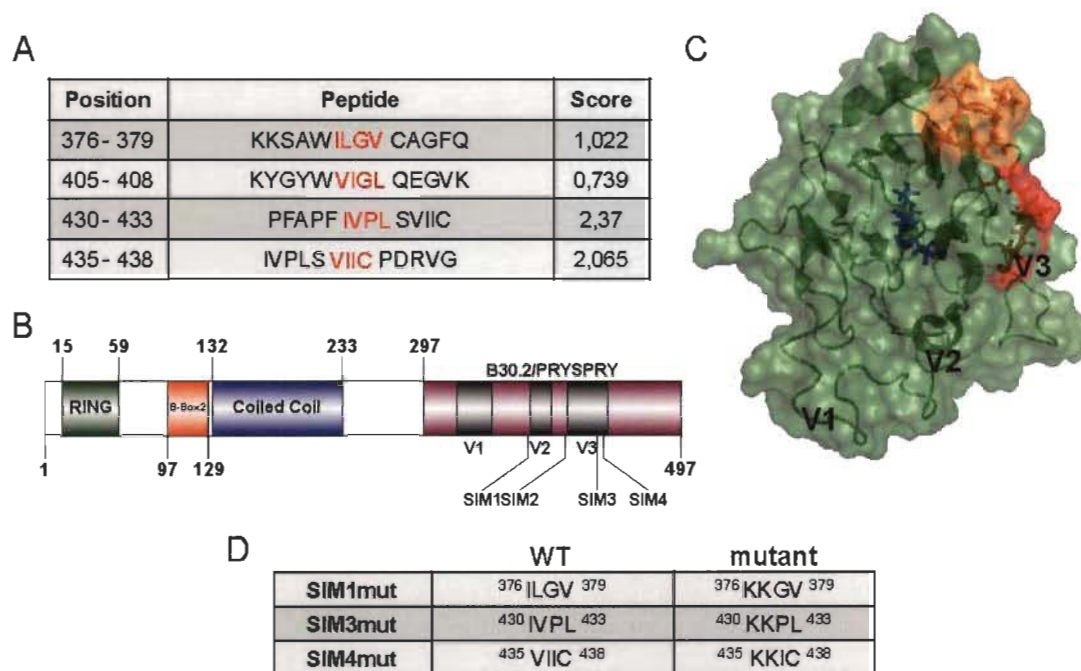
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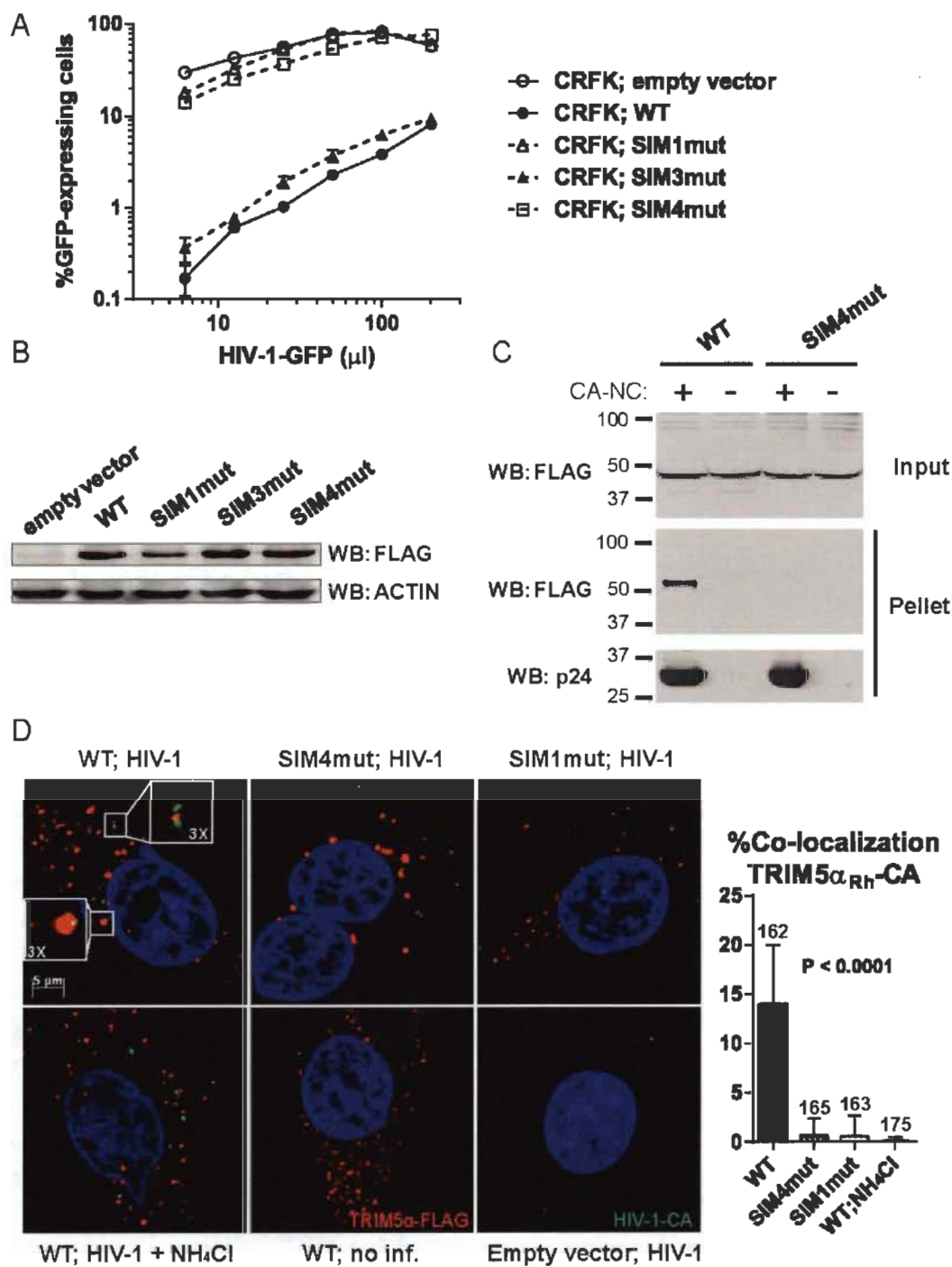
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**Figure 1**

**Putative SUMO interacting motifs in TRIM5 $\alpha_{Rh}$ .**

(A) Table showing the different scores of the predicted SIMs (red letters) and their current location within the TRIM5 $\alpha_{Rh}$  PRYSPRY. (B) Schematic of the full-length TRIM5 $\alpha_{Rh}$  protein depicting its main domains and the positions of the various SIMs. (C) Structural model of the TRIM5 $\alpha_{Rh}$  PRYSPRY domain mapping the position of SIM1 (blue), SIM3 (red) and SIM4 (orange). The localization of variable regions V1, V2 and V3 is shown. (D) Table showing the mutations introduced in TRIM5 $\alpha_{Rh}$  with their respective amino acid position.

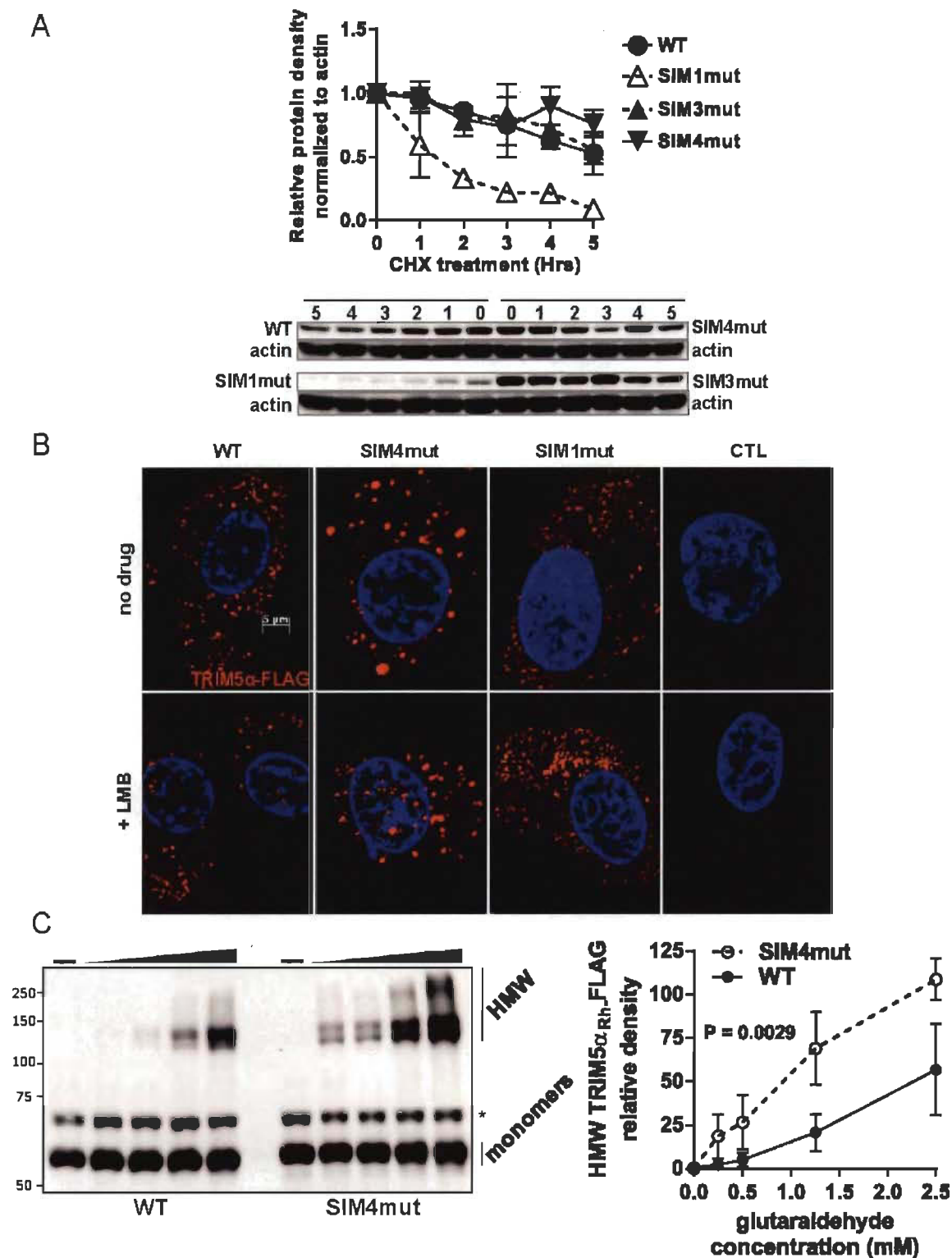


**Figure 2** The VI→KK mutation in SIM4 (SIM4mut) suppresses the interaction of TRIM5 $\alpha_{Rh}$  with HIV-1 CA and abolishes restriction of HIV-1.

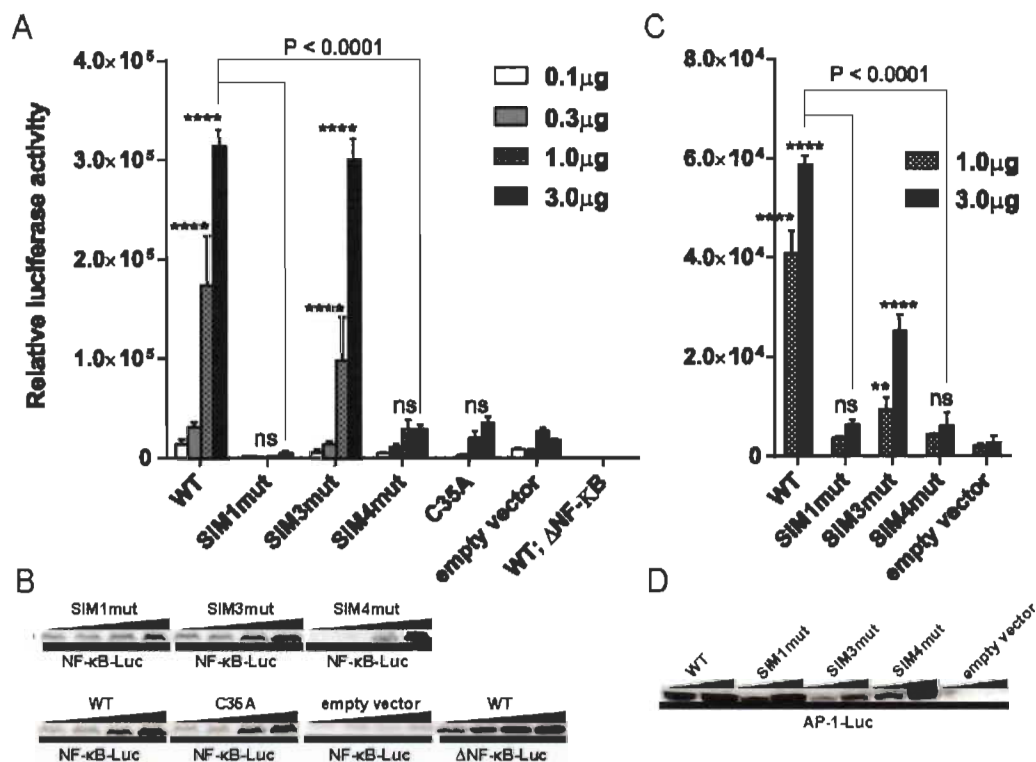
(A) Functional restriction assay. CRFK cells stably expressing WT TRIM5 $\alpha_{Rh}$  or the various SIM mutants were challenged with increasing amounts of VSV G-pseudotyped HIV-1-GFP. Infections were performed in triplicates and the percentage of cells expressing GFP was analyzed by



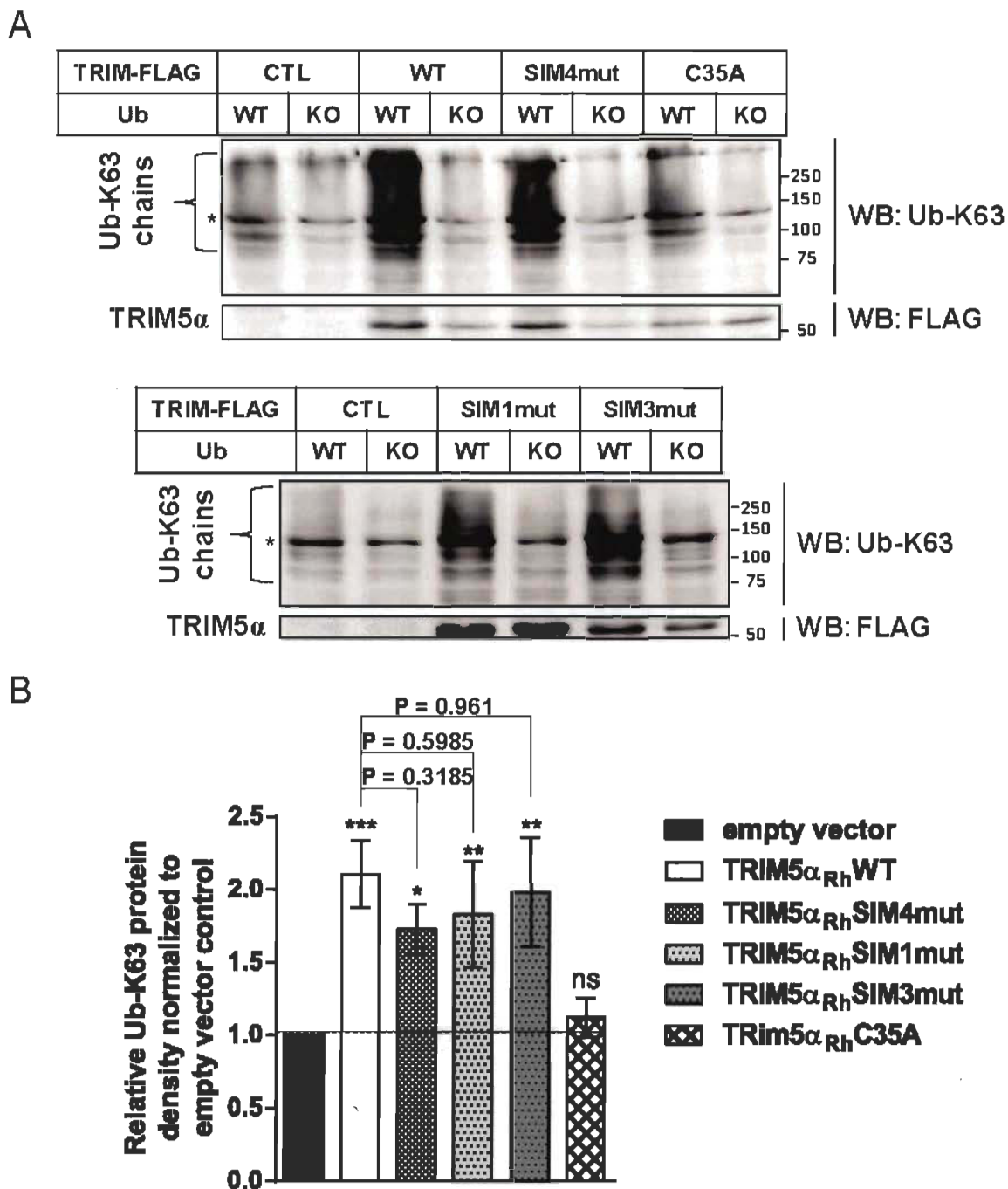
FACS two days later. Standard deviation bars are shown. (B) TRIM5 $\alpha_{Rh}$  expression levels. WT and mutant TRIM5 $\alpha_{Rh}$  proteins expression levels in stably transduced CRFK cells were determined by western blotting with an anti-FLAG antibody. Actin expression was analyzed as a loading control. (C) CA binding assay. HIV-1 CA-NC complexes were assembled *in vitro* and a binding assay was then performed using FLAG-tagged WT or SIM4mut TRIM5 $\alpha_{Rh}$  expressed in HEK293T cells. Lysates from the transfected HEK293T cells were incubated with HIV-1 CA-NC complexes. The mixtures were applied to a 70% sucrose cushion and centrifuged. “Input” shows a western blotting analysis of pre-centrifugation lysates using a FLAG antibody, while “Pellet” shows post-centrifugation pellets analyzed using antibodies against FLAG or CA. A single experiment representative of three independent experiments is presented. (D) TRIM5 $\alpha$  association with incoming HIV-1 CA in cells. CRFK cells stably expressing WT, SIM1mut or SIM4mut TRIM5 $\alpha_{Rh}$ -FLAG were infected 2 h with HIV-1-GFP. A rabbit polyclonal anti-FLAG antibody was used for the detection of TRIM5 $\alpha_{Rh}$  (red) while HIV-1 CA was stained using a mouse monoclonal antibody (green). DNA was stained using Hoeschst33342 (blue). Examples of co-localizations between WT TRIM5 $\alpha_{Rh}$  cytoplasmic bodies and HIV-1 CA foci are magnified in the white boxes. A quantitative analysis of co-localization frequency was performed and is shown in the graph on the right. Each bar represents the mean % of co-localization observed in 15 randomly chosen fields, each field considered as a replicate for statistical analysis. *P*-values were calculated using the one-way ANOVA test. The total number of CA foci in the 15 fields is shown on top of each bar.



prepared at the indicated time points. The graph shows the mean values of relative protein densities from two independent experiments normalized to actin at different time points following cycloheximide treatment, and then normalized to the value obtained at  $T_0$ . Error bars are shown. Western blots from one experiment are presented below the graph and show the expression levels of the different TRIM5 $\alpha$ -FLAG tagged proteins following cycloheximide treatment. (B) Subcellular distribution as analyzed by IF microscopy. CRFK cells stably expressing FLAG-tagged WT, SIM1mut, SIM4mut TRIM5 $\alpha_{Rh}$  and “empty vector” transduced cells were treated with leptomycin B (LMB) for 12 hours or left untreated prior to fixation and staining with an anti-FLAG antibody (red). DNA was stained using Hoeschst33342 (blue). Representative images from multiple fields analyzed are shown. (C) Multimerization assay. Lysates were prepared from CRFK stably expressing FLAG-tagged WT or SIM4mut TRIM5 $\alpha_{Rh}$ . The soluble fraction of each lysate was divided in aliquots that were treated with different glutaraldehyde concentrations (0, 0.25, 0.5, 1.25 and 2.5 mM) before performing western blots using an anti-FLAG antibody. A single experiment representative of three independent experiments is presented. High molecular weight (HMW) bands correspond to apparent TRIM5 $\alpha_{Rh}$  multimers according to the size of the bands. The graph on the right shows the average (from three experiments) of relative HMW protein density (ranging from 120 to >250 kDa) calculated at each glutaraldehyde concentration. Standard deviation bars are shown on the graph and the *P*-value was calculated using two-way ANOVA statistical test. \*points to a non-relevant band detected by the antibody used.



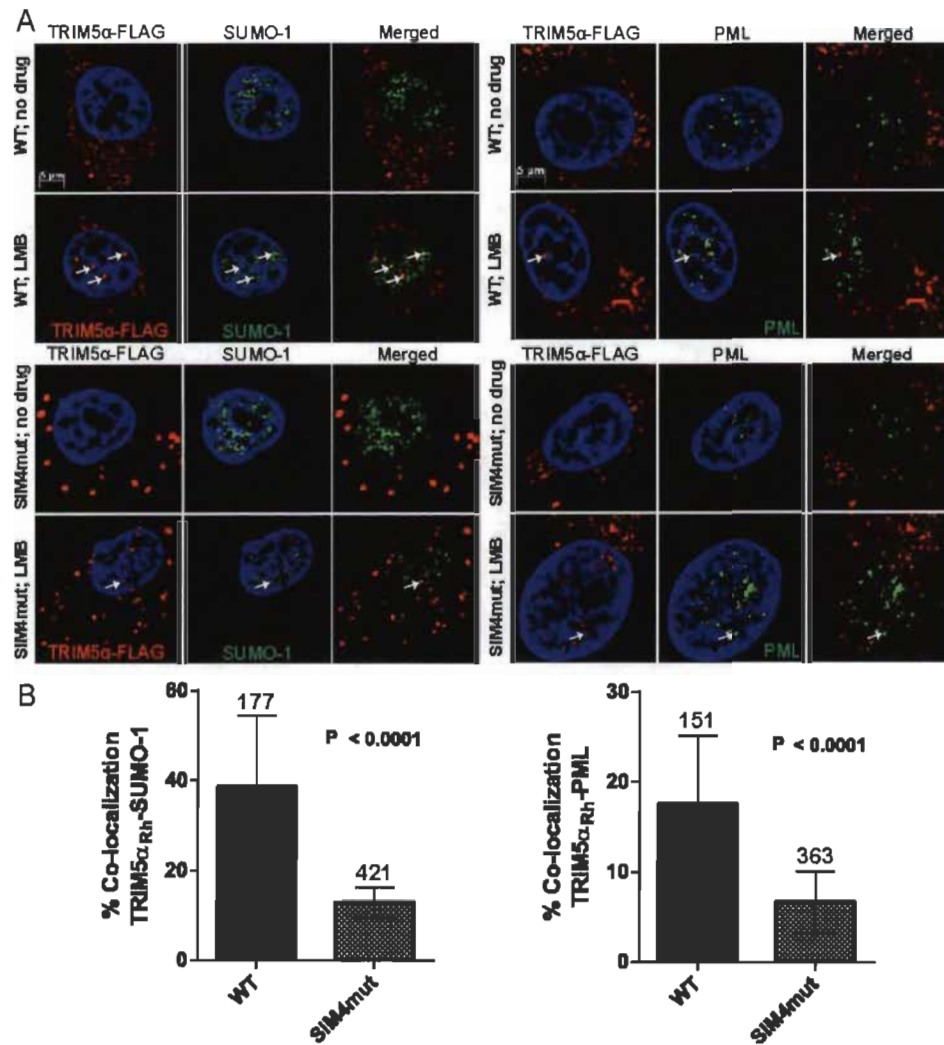
**Figure 4** **SIM4mut affects TRIM5 $\alpha_{Rh}$ -mediated activation of NF- $\kappa$ B and AP-1.** (A) HEK293T cells were transfected with increasing amounts of either the WT or the indicated mutants of pMIP-TRIM5 $\alpha_{Rh}$  and co-transfected with a constant amount (0.6  $\mu$ g) of a reporter plasmid expressing luciferase from a NF- $\kappa$ B-dependant promoter. As controls, cells were co-transfected with the empty pMIP plasmid and the NF- $\kappa$ B reporter construct, or were co-transfected with WT TRIM5 $\alpha_{Rh}$  and the activation-deficient mutant of the reporter construct ( $\Delta$ NF- $\kappa$ B-Luc). Two days later, cells were lysed and luciferase activity was measured. Bars show averages from triplicate transfections with standard deviations. P-values were calculated using a two-way ANOVA test. (B) The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5 $\alpha$  expression. Specifically, the three lysates from each triplicate transfection were pooled and analyzed by western blotting using an anti-FLAG antibody. (C) Same as (A), using a reporter plasmid expressing luciferase from an AP-1-dependant promoter. Two different DNA concentrations (1.0 and 3.0  $\mu$ g) of the different pMIP plasmids expressing WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{Rh}$  were co-transfected with a constant concentration of AP-1-Luc (0.6  $\mu$ g). The empty vector was used as a negative control. The graph shows relative luciferase activity 48 h post-transfection. The experiment was performed in triplicate and standard deviations are shown. P-values were calculated with a two-way ANOVA test: \* $<0.05$ , \*\* $<0.01$ , \*\*\* $<0.001$ , \*\*\*\* $<0.0001$ . (D) TRIM5 $\alpha$  protein expression was analyzed as described in (B).



**Figure 5** Mutating putative SIM domains in the PRYSPRY domain does not affect TRIM5 $\alpha_{Rh}$  ability to trigger K63-linked ubiquitin chains formation.

(A) HEK293T cells were transfected with either the empty pMIP or with pMIP expressing FLAG-tagged WT, SIM1mut, SIM3mut or SIM4mut TRIM5 $\alpha_{Rh}$ , and co-transfected with WT or KO HA-Ubiquitin. KO Ubiquitin has all its lysines mutated to arginines to prevent polyubiquitylation. C35A TRIM5 $\alpha_{Rh}$  was used as a negative control for its

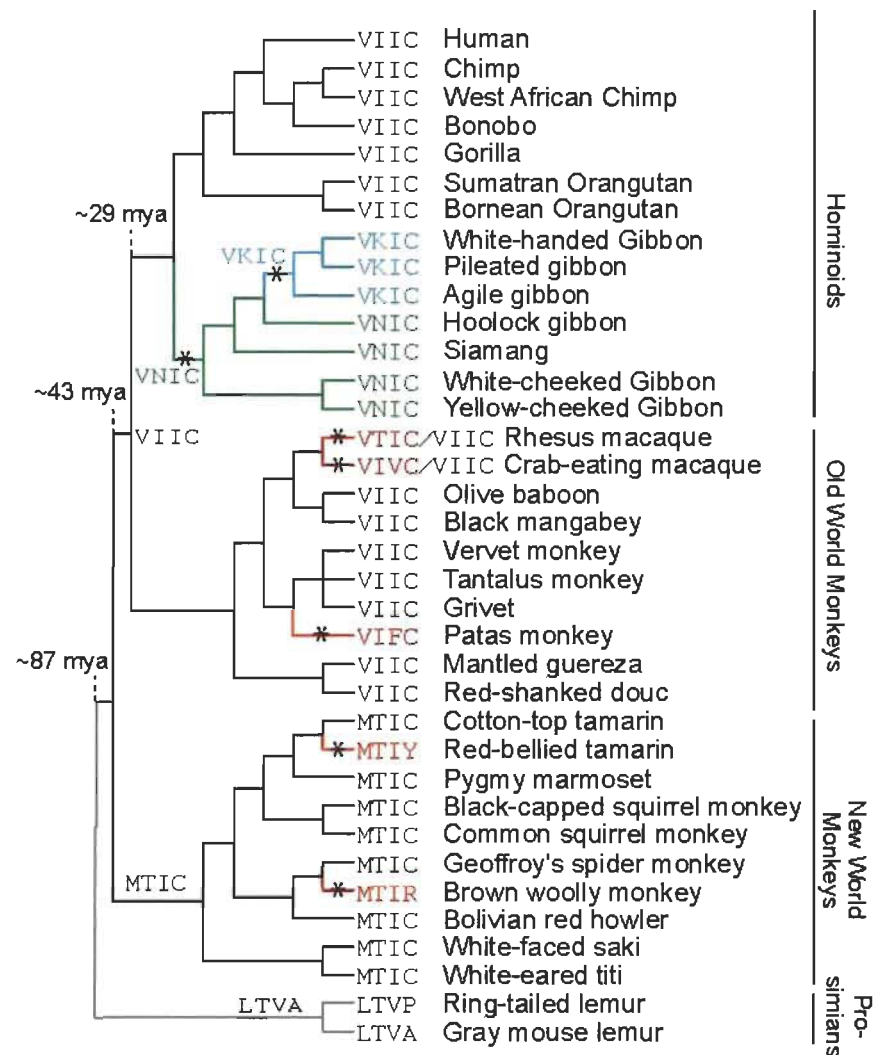
incapacity to significantly trigger Ub-K63 chains formation. Two days later, whole protein lysates were analyzed by Western blotting using rabbit antibodies directed at K63-linked ubiquitin chain or at FLAG. A single experiment representative of three independent experiments is presented. \* points to a non-relevant band detected by the antibody used. (B) The graph represents the mean densities of bands detected using the K63 chains-specific antibody (ranging from 75 to >250 kDa) normalized to the control (CTL) consisting of cells co-transfected with WT-Ub and the empty pMIP. Data were obtained in three independent experiments and standard deviations are shown on the graph. *P*-values were calculated using a one-way ANOVA test: \* $<0.05$ , \*\* $<0.01$ , \*\*\* $<0.001$ .



**Figure 6** Decreased nuclear co-localization of SIM4mut TRIM5 $\alpha_{Rh}$  with endogenous SUMO-1 and PML.

(A) CRFK cells stably expressing either FLAG-tagged WT or SIM4mut TRIM5 $\alpha_{Rh}$  were treated or not with LMB for 12 h and then processed for IF microscopy. A rabbit anti-FLAG antibody was used for the detection of TRIM5 $\alpha_{Rh}$  (red) and mouse antibodies were used for the detection of endogenous SUMO-1 and PML (green). DNA was stained with Hoechst33342 (blue). White arrows point to examples of co-localizations of nuclear TRIM5 $\alpha_{Rh}$  with SUMO-1 (left panels) or PML (right panels). (B) Quantitative analysis of the frequency of WT or SIM4mut TRIM5 $\alpha_{Rh}$  nuclear bodies (NBs) co-localization with either SUMO-1 (left panel) or PML (right panel). The percentage of WT and SIM4mut TRIM5 $\alpha_{Rh}$  NBs co-localizing with SUMO-1 or PML is shown. Each bar represents the mean % of co-localization observed in 15 randomly chosen fields considered as replicates. *P*-values were calculated using Student T-test and standard deviations are shown. The total number of TRIM5 $\alpha_{Rh}$  NBs in the 15 fields is shown on top of each bar.





**Figure 7 Divergence of the TRIM5 $\alpha$  SIM4 motif during simian primate speciation.**

The SIM4 motif was analyzed in 36 primate species (bases 1291-1302 of the *TRIM5* gene, encoding residues 431-434 in the human TRIM5 $\alpha$  protein). These primate species represent ~87 million years of primate evolution and the four major primate groups are shown. Amino acid residues encoded in this motif by each primate genome are indicated. Ancestral forms of the SIM4 motif were predicted with the Program PAML (86). Asterisks indicate positions where mutations in this motif are predicted to have occurred. SNPs within the SIM4 domain have been reported in both rhesus and crab-eating macaque populations (*M. mulatta* from Guangxi province and individuals from the Mauritian *M. fascicularis* population, respectively) (25, 89). These polymorphisms are indicated on the tree with hyphens.



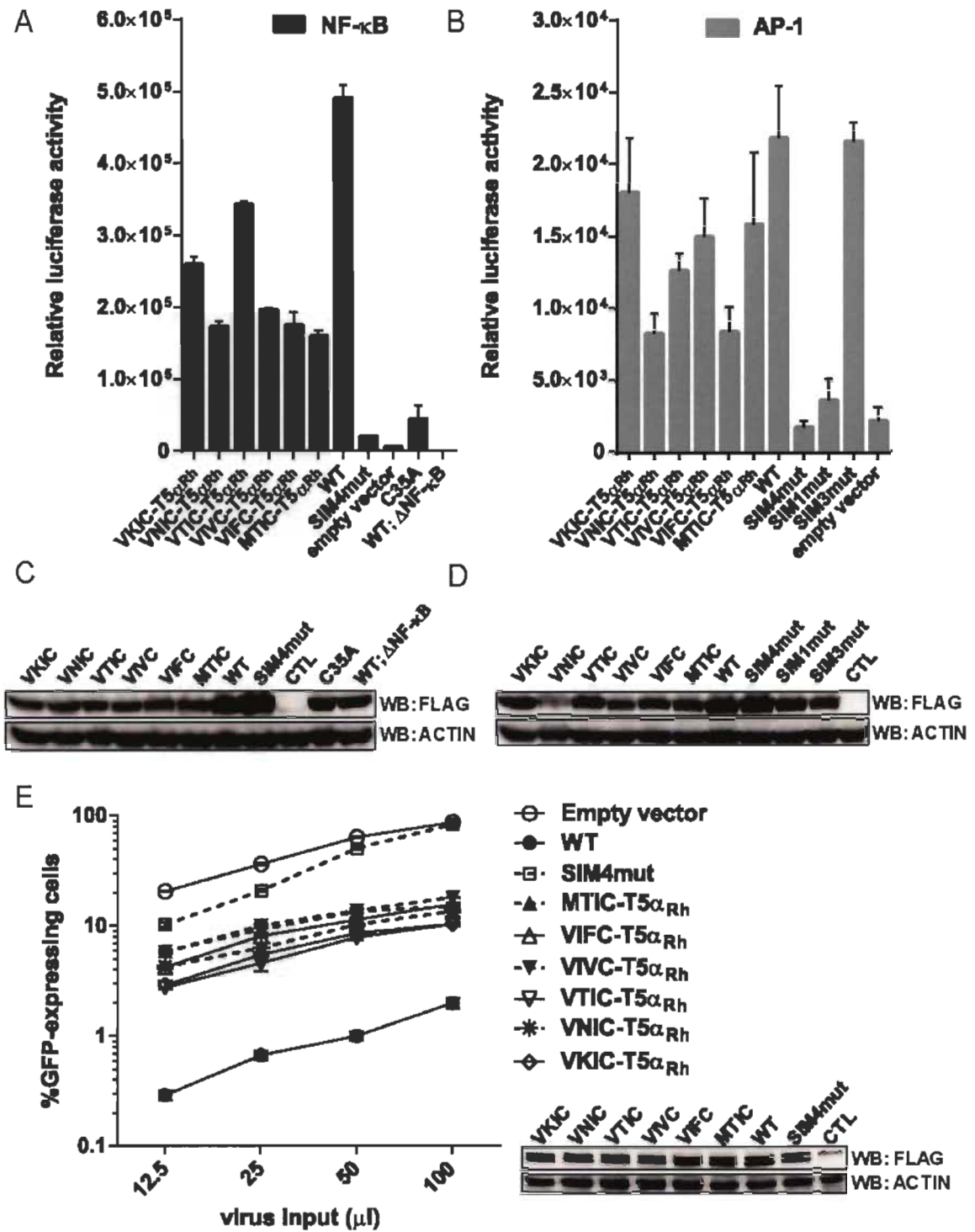


Figure 8

### Natural simian variants of SIM4 activate both NF-κB and AP-1.

(A) HEK293T cells were co-transfected with either WT or the indicated mutants of pMIP-TRIM5 $\alpha_{Rh}$  (2.0  $\mu$ g) and a reporter plasmid expressing luciferase from a NF- $\kappa$ B-dependent promoter (0.6  $\mu$ g). As controls, cells were co-transfected with the empty pMIP plasmid and the NF- $\kappa$ B reporter construct, or were co-transfected with WT TRIM5 $\alpha_{Rh}$  and the activation-

deficient mutant of the reporter construct ( $\Delta$ NF- $\kappa$ B-Luc). Cells were lysed 48 h post-transfection and luciferase activity was measured. Bars show averages from triplicate transfections with standard deviations. (B) imilar experiment using a reporter plasmid expressing luciferase from an AP-1-dependent promoter. The empty vector was used as a negative control. The graph shows relative luciferase activity 48 h post-transfection. The experiment was performed in triplicate and standard deviations are shown. (C) The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5 $\alpha_{Rh}$  expression. Specifically, the three lysates from each triplicate transfection were pooled and analyzed by western blotting using an anti-FLAG antibody. (D) TRIM5 $\alpha_{Rh}$  expression levels in cells from the experiment shown in (B). The analysis was done as in (C). (E) The HIV-1 restriction activity of all TRIM5 $\alpha_{Rh}$  SIM4 variants construct was assessed by infecting CRFK cells stably expressing the indicated TRIM5 $\alpha_{Rh}$  with increasing amount of HIV-1-GFP. Percentages of GFP-positive cells were measured by FACS two days later. The experiment was performed in triplicate and standard deviation bars are shown on the graph. WT and mutant TRIM5 $\alpha_{Rh}$  proteins expression levels in stably transduced CRFK cells were determined by western blotting with an anti-FLAG antibody. Actin expression was analyzed as a loading control.

## CHAPITRE V

### DISCUSSION GÉNÉRALE

#### 5.1 La mutlimérisation de TRIM5 $\alpha$

Au moment de la publication de l'article (se référer au chapitre II) portant sur la multimérisation de TRIM5 $\alpha$  et TRIMCyp (157), les techniques fréquemment utilisées pour permettre une caractérisation au niveau structurel et mettre en évidence les interactions protéine-protéine se limitaient à l'utilisation d'agents de réticulation communément appelés agents de « cross-linking ». Il y a plusieurs agents chimiques disponibles (68, 90), mais le glutaraldéhyde (123, 124, 151, 157) et l'EGS (éthylène glycol bis-(succinimidylsuccinate)) (97, 102, 116) ont été les plus utilisés dans la caractérisation de TRIM5 $\alpha$ . Ces deux agents de « cross-linking » sont homobifonctionnels étant donné la présence de deux groupements réactifs identiques situés à chaque bout de la molécule, qui elle agit à titre de séparateur. De plus, les deux agents chimiques agissent de façon similaire soit en attaquant les groupements amines primaires (-NH<sub>2</sub>) de la partie N-terminale, retrouvée chez toutes les protéines, ou de la chaîne latérale d'une lysine qui permet ainsi la formation d'une liaison covalente entre deux ou plusieurs protéines qui sont à une distance égale ou plus courte à la longueur de la molécule séparatrice (68, 73). Par contre, cette technique s'avère peu spécifique dans un contexte cellulaire puisque l'ajout d'un tel agent (homobifonctionnel ciblant les groupements amines) se traduira par la conjugaison aléatoire de sous-unités protéiques, de protéines qui interagissent et de tous les autres polypeptides dont la chaîne latérale de la lysine se trouve à proximité d'une autre. En résumé, cette fonction est idéale pour capturer un « instantané » de toutes les interactions entre protéines, mais elle ne peut pas fournir la précision nécessaire pour caractériser, par exemple, la structure quaternaire exacte d'une protéine comme TRIM5 $\alpha$ .

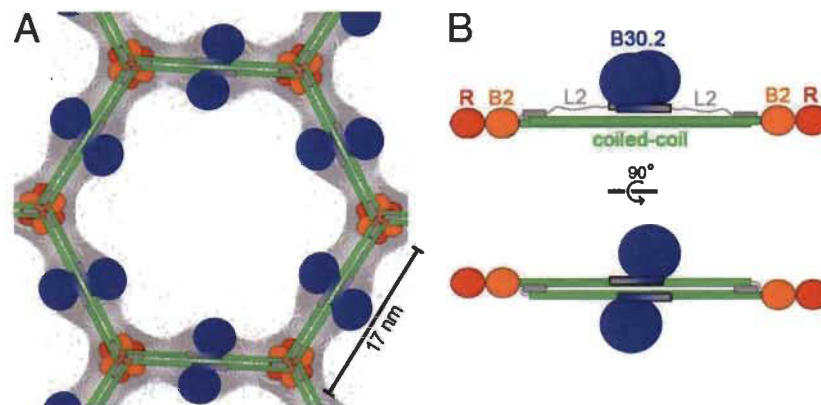
La construction d'une protéine chimère entre TRIM5 $\alpha_{Rh}$  et le domaine RING de la protéine humaine TRIM21, qui possède une demi-vie plus longue que TRIM5 $\alpha$ , a permis l'expression et la purification, à l'aide d'une technique utilisant les baculovirus, d'une protéine chimérique TRIM5-21R. Cette dernière est capable d'interagir avec la capsid du VIH-1 de façon à inhiber ce dernier de manière comparable à TRIM5 $\alpha_{Rh}$  (102, 116). Des études biochimiques ont permis de mettre en évidence la présence de dimères de TRIM5-21R. La formation de dimères de TRIM5 $\alpha$  serait un pré-requis pour interagir avec la capsid rétrovirale et permettre ainsi son activité de restriction (97, 102). Fait intéressant, le patron d'oligomérisation de la protéine purifiée TRIM5-21R était similaire au patron de TRIM5 $\alpha_{Rh}$  provenant d'un lysat cellulaire (116), ce qui suggère que la technique utilisant des agents de « cross-linking » dans un contexte cellulaire serait un modèle plausible pour la caractérisation de l'oligomérisation. Cependant, nos résultats proposaient la formation de dimères et de trimères, tels que proposés par d'autres équipes (97, 151).



**Figure 5.1** Schéma représentant un modèle possible de l'interaction des hexamères de TRIM5 $\alpha$  avec le treillis hexamérique de la capsid virale  
(L'illustration provient de cette publication : (61).)

Une étude a proposé que la migration des produits obtenus après un « cross-linking » migre de façon anormalement lente, ce qui fausse le poids moléculaire réel des

produits obtenus (102). Par contre, il se pourrait que la formation de dimères s'effectue de plusieurs façons et que les produits issus migrent différemment vu leur conformation. D'ailleurs, une étude récente propose que le domaine coiled-coil de TRIM5 $\alpha$  forme plutôt des dimères de type épingles anti-parallèles au lieu du modèle impliquant des dimères parallèles tels qu'illustrés à la figure 5.1. Sanchez *et al.* ont proposé un modèle qui permettrait la formation de dimères anti-parallèles de TRIM5 $\alpha$  de deux manières différentes (Figure 5.2). Le dimère le plus plausible (modèle du bas) semble plus encombré spatialement comparativement à l'autre modèle qui semble plus compact (modèle du haut) (191), ce qui pourrait expliquer la différence dans la vitesse de migration des produits dimérisés. Nos résultats ont également proposé la formation d'hexamères de TRIM5 $\alpha$  et TRIMCyp et ceci concorde avec les modèles proposés qui stipulent que TRIM5 $\alpha$  devrait se dimériser pour interagir avec la capsid virale et l'interaction serait ensuite stabilisée par la formation d'un treillis composé d'hexamères de dimères autour de la capsid virale, qui est, elle aussi formée d'hexamères (Figure 5.1) (61, 191).



**Figure 5.2** Modèle illustrant les structures quaternaires possibles de TRIM5 $\alpha$ .  
**A.** Configuration possible du treillis hexamérique **B.** Conformations possible d'un dimère de TRIM5 $\alpha$  (L'illustration provient de cette publication : (191).)

La formation de structures de second niveau ou de structures dites quaternaires (dimères, hexamères, multimères) est un processus indispensable à la restriction efficace des rétrovirus par les protéines TRIM5 $\alpha$  (38, 42, 61, 123), mais selon nos résultats,

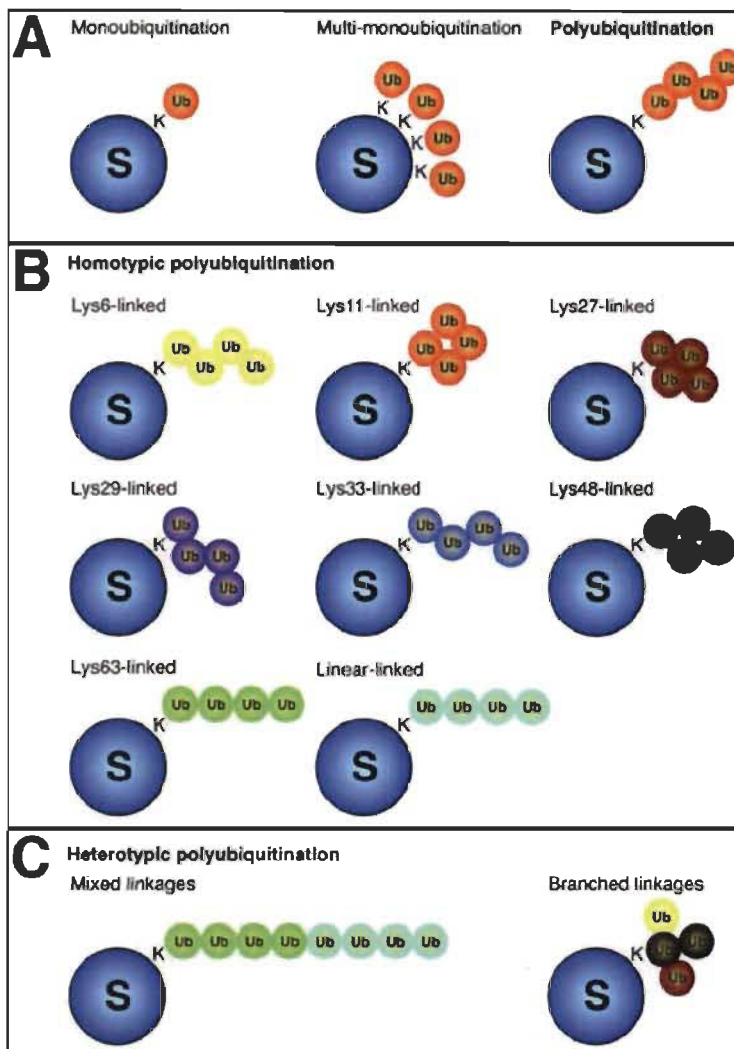
l'opposé ne s'appliquerait pas puisque la capacité de TRIM5 $\alpha$  à former des structures de second niveau n'est pas influencée par la présence de virus sensibles à la restriction ou de drogues inhibant la restriction (157). Dans un contexte cellulaire où TRIM5 $\alpha$  n'est pas sollicitée par une réponse antivirale, il y a dans le cytoplasme de la cellule des monomères, mais également plusieurs dimères de TRIM5 $\alpha$  qui seraient d'ailleurs la forme la plus communément retrouvée (102, 116). De plus, l'apparence générale des corps cytoplasmiques de TRIM5 $\alpha$  (20) ou TRIMCyp (se référer à l'annexe B, figure 2 pour un exemple) en microscopie à fluorescence demeure semblable que ce soit dans des conditions dites restrictives ou non-restrictives. La formation de dimères, d'hexamères et de multimères est essentielle à la fonction de restriction de TRIM5 $\alpha$  et TRIMCyp, mais cette capacité à multimériser est un phénomène qui a lieu indépendamment de l'interaction avec la capsid et se produit autant dans des conditions non-restrictives.

Pour conclure sur le sujet de la multimérisation de TRIM5 $\alpha$ , l'utilisation d'agents de « cross-linking » a permis une première caractérisation de la multimérisation de TRIM5 $\alpha$ , notamment la mise en évidence d'hexamères, forme proposée pour la première fois par notre équipe (157). Ensuite, la purification de chimères (61, 102, 116) ou de domaines isolés de TRIM5 $\alpha$  (14, 191) a permis de proposer des modèles plausibles d'arrangement spatial de la protéine. Néanmoins, il est important de mentionner que malgré les nombreuses données sur la capacité de TRIM5 $\alpha$  à multimériser, l'impossibilité de purifier la protéine sous sa « pleine longueur » constitue un obstacle majeur pour une caractérisation exacte de la structure quaternaire de TRIM5 $\alpha$ . De plus, les structures quaternaires de protéines sont souvent influencées par des modifications post-traductionnelles comme la phosphorylation de résidus sérine, thréonine ou tyrosine (178). Jusqu'à maintenant, la phosphorylation de TRIM5 $\alpha$  n'a fait l'objet d'aucune étude, mais plusieurs protéines membres de la famille TRIM sont reconnues pour être phosphorylées (26, 57, 130, 206, 212). Selon le prédicteur PhosphoSitePlus<sup>®</sup>, des sites potentiels de phosphorylation sont prédits pour TRIM5 $\alpha$  : la sérine en position 86 (S86) et les tyrosines en positions 176 et 329 (Y176 et Y329) (85). Une étude du rôle de la phosphorylation possible de TRIM5 $\alpha$  pourrait nous informer énormément sur ses propriétés physiques et chimiques tels que le repliement, la conformation, la stabilité et

l'activité même de la protéine (11, 15), notamment le rôle de la phosphorylation dans la restriction des rétrovirus.

## 5.2 Rôle de la lysine 10 dans l'ubiquitination de TRIM5 $\alpha$

Le contenu du chapitre III porte sur la caractérisation de la lysine en position 10 (K10) de TRIM5 $\alpha_{Rh}$  et son rôle possible dans la restriction des rétrovirus et l'activation de l'immunité innée (156). Cette lysine se situe dans une séquence consensus de SUMOylation. Cependant, TRIM5 $\alpha$  n'a jamais été démontrée pour être SUMOylée et la présence d'un tel site consensus ne garantit pas sa SUMOylation (234). Selon nos résultats, la mutation de cette lysine spécifique affecte significativement l'activité de restriction de TRIM5 $\alpha_{Rh}$  et sa capacité à activer la signalisation de l'immunité innée. La lysine 10 modulerait l'activité E3 ubiquitine-ligase du domaine RING adjacent en favorisant le recrutement de l'enzyme de conjugaison Ubc13/Uev1a qui est nécessaire à la formation de chaînes d'ubiquitines liées à K63, étape importante dans l'activation de NF- $\kappa$ B et AP-1 (1). De plus, la mutation de la lysine 10 provoque une augmentation du niveau d'ubiquitination de TRIM5 $\alpha$ , mais le type d'ubiquitination demeure inconnu. Il est possible que ce soit une augmentation de l'ubiquitination liée à K48. En effet, le domaine RING de TRIM5 $\alpha$  permet de recruter l'enzyme de conjugaison UbcH5b pour s'auto-ubiquitiner et conséquemment diriger la protéine vers une dégradation par le protéasome (41, 109, 187), processus qui est normalement dépendant de la formation de chaînes d'ubiquitines liées à K48 (216). Par contre, la majorité des études sur l'ubiquitination de TRIM5 $\alpha$ , notamment *in vitro*, ont utilisé l'ubiquitine non mutée et son utilisation ne permet pas de spécifier le type d'ubiquitination que la protéine subit. Il existe plusieurs types de modifications par l'ubiquitine et elles sont toutes représentées dans la figure 5.3. Néanmoins, une étude suggère que TRIM5 $\alpha$  formerait des chaînes d'ubiquitines liées à K48 puisque celles-ci seraient observées dans les corps cytoplasmiques de TRIM5 $\alpha$  (34) qui eux sont reconnus pour co-localiser avec des sous-unités du protéasome (132). Il n'est toutefois pas exclu que l'augmentation du niveau d'ubiquitination du mutant K10R TRIM5 $\alpha$  puisse être la conséquence d'un autre type d'ubiquitination qui n'est pas lié à K48.

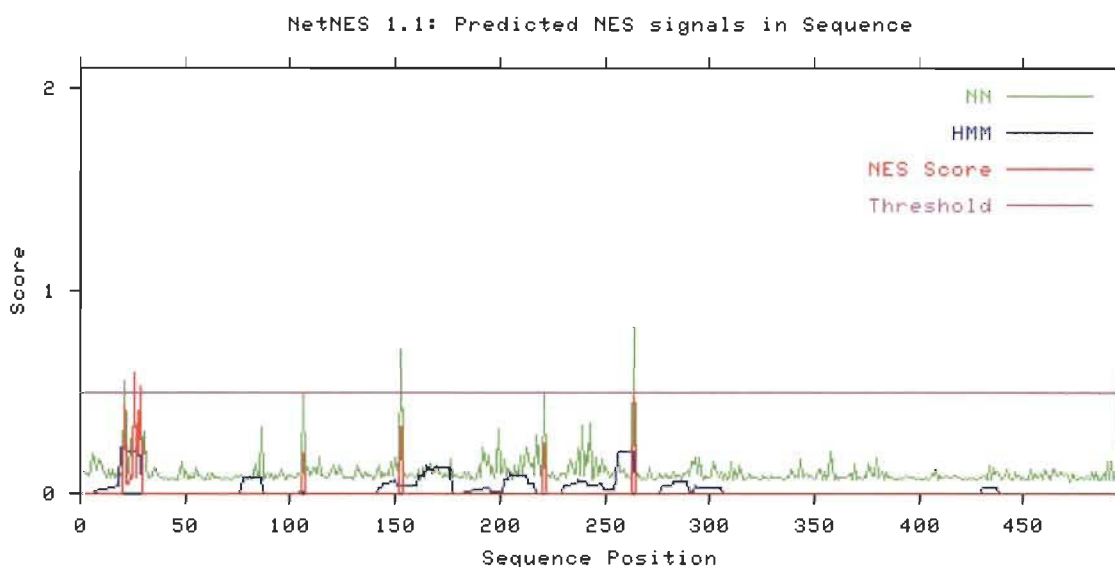


**Figure 5.3** Types d'ubiquitination. **A.** Les différents types d'ubiquitination. **B.** Les différents types de polyubiquitination homotypique (chaînes formées à partir d'un seul type de lysine). **C.** Les différents types de polyubiquitination hétérotypique (chaînes formées à partir de différentes lysines) (l'illustration provient de cette publication (112)).

Une équipe a déjà proposé que TRIM5 $\alpha$  était monoubiquitiné (237). La monoubiquitination est souvent impliquée dans la signalisation cellulaire, notamment dans l'import/export nucléaire (120, 127, 217, 223). À ce sujet, le mutant K10R TRIM5 $\alpha_{Rh}$  s'accumulait dans le noyau comparativement à la protéine sauvage ou WT, ce qui suppose que la protéine mutante est soit anormalement surdirigée vers le noyau ou emprisonnée dans le noyau parce qu'un autre mécanisme d'export nucléaire indépendant de CRM-1 (221, 225) est affecté par cette mutation. La seconde option



pourrait être plausible puisque TRIM5 $\alpha_{Rh}$  possède deux présumés signaux d'export nucléaire (NES) riche en leucines (114) juste au tout début du domaine RING (L26 et L28), donc juste à côté de la lysine 10 (Figure 5.4). En effet, le signal des ondes rouges représentant les NES prédits est supérieur au seuil limite de détection (ligne horizontale rose) aux positions L26 et L28. La SUMOylation ou l'ubiquitination d'une lysine près du NES peut cacher le signal d'export nucléaire (223) et dans ce cas-ci, la mutation K10R a effectivement provoqué une augmentation importante du niveau d'ubiquitination de TRIM5 $\alpha_{Rh}$  qui cache possiblement le NES.



**Figure 5.4** Signaux NES prédits (L26 et L28) pour la séquence de TRIM5 $\alpha_{Rh}$ . Résultats obtenus avec le logiciel NetNES 1.1. La ligne rose représente le seuil limite de détection. Les ondes vertes indiquent les NN (Neural Network algorithm), les ondes bleues indiquent les HMM (Hidden Markov Model algorithm) et les ondes rouges prédisent les NES (114).

L'accumulation des corps nucléaires de K10R TRIM5 $\alpha_{Rh}$  a eu pour conséquence de diminuer de moitié le pourcentage de co-localisation avec SUMO-1. La co-localisation n'implique pas nécessairement une interaction fonctionnelle (discuté en détail dans l'article en préparation de l'annexe B) et il est difficile de conclure que la lysine 10 a un rôle à jouer dans la SUMOylation, quoiqu'il n'est pas exclu que ce soit le cas. De ce fait, la SUMOylation de TRIM5 $\alpha$  pourrait avoir lieu de façon transitoire dans le noyau seulement. Toutefois, nos résultats suggèrent que la lysine 10 module l'activité

du domaine RING et il est possible que cette lysine soit impliquée dans plusieurs types d'ubiquitination, notamment la monoubiquitination de TRIM5 $\alpha$ . La lysine 10 est possiblement elle-même ubiquitinée et sa mutation affecterait la capacité de TRIM5 $\alpha$  à s'auto-ubiquitiner convenablement. TRIM5 $\alpha_{Rh}$  possède 33 lysines et aucune étude n'a porté sur l'ubiquitination possible de celles-ci; elles sont donc toutes des sites potentiels d'ubiquitination. Si l'hypothèse de SUMOylation de cette lysine est maintenue, il se pourrait que cette lysine soit à la fois SUMOylée et ubiquitinée ce qui se traduirait en différents signaux, phénomène préalablement observé chez d'autres protéines, entre autres chez les protéines I $\kappa$ B $\alpha$  (37) et NEMO (87) qui sont toutes deux impliquées dans l'activation de NF- $\kappa$ B (223). Les résultats obtenus avec la formation de chaînes d'ubiquitines liées à K63 suggèrent que la lysine 10 favoriserait leur formation et activerait conséquemment AP-1 et NF- $\kappa$ B (156). La possibilité que cette lysine 10 soit également impliquée à différents niveaux de la cascade de signalisation n'est pas écartée. Par ailleurs, différents types d'ubiquitination sont impliqués dans l'activation ou l'inhibition de NF- $\kappa$ B, dont la monoubiquitination (71). La sous-unité RelA de NF- $\kappa$ B peut être monoubiquitinée et cette modification peut réguler négativement son activité de transcription en inhibant sa liaison avec le co-activateur *CREB-binding protein* (CBP) (80).

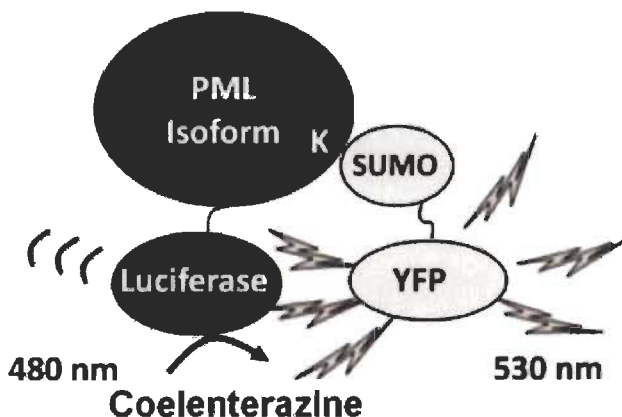
En conclusion, la lysine 10 de TRIM5 $\alpha_{Rh}$  agit de concert avec le domaine RING pour recruter l'enzyme de conjugaison E2 nécessaire à son auto-ubiquitination, qui selon les signaux environnants va se traduire, entre autres, en la formation de chaînes d'ubiquitines liées à K48 ou K63. Il y aura conséquemment dégradation de TRIM5 $\alpha$  par le protéasome ou activation de la signalisation de l'immunité innée, respectivement. La lysine 10 serait également importante dans la localisation nucléaire de TRIM5 $\alpha$  qui pourrait être dépendante, par exemple, d'un autre type d'ubiquitination qui favoriserait l'accessibilité du signal NES. L'identification des lysines ubiquitinées et la caractérisation du type d'ubiquitination pourraient grandement contribuer à la compréhension du rôle spécifique de cette modification post-traductionnelle dans les fonctions de TRIM5 $\alpha$  (112). Il existe plusieurs logiciels permettant de prédire les lysines potentiellement ubiquitinées (85, 179, 230). La mutation des lysines potentiellement

ubiquitinées pourrait permettre de déterminer quelle lysine est modifiée. De plus, il existe plusieurs anticorps spécifiques aux différents types d'ubiquitination et l'utilisation de plasmides exprimant différents types d'ubiquitines pourrait également contribuer à la caractérisation de l'ubiquitination de TRIM5 $\alpha$ .

### **5.3 Rôle des motifs d'interaction avec SUMO (SIMs) de TRIM5 $\alpha$ dans la restriction et l'immunité innée**

Les résultats présentés dans le chapitre IV suggèrent qu'une séquence spécifique de TRIM5 $\alpha_{Rh}$  nommée SIM4 serait importante 1) pour l'interaction avec la capsid virale et ce, possiblement indépendamment d'une interaction directe avec SUMO ou des protéines SUMOylées, et 2) pour l'activation de l'immunité innée en permettant l'interaction de TRIM5 $\alpha_{Rh}$  avec SUMO ou des protéines SUMOylées impliquées dans la signalisation de l'immunité innée. En effet, il s'agit d'un site potentiel d'interaction avec SUMO qui, contrairement aux motifs SIM1 et SIM2 de TRIM5 $\alpha_{Rh}$ , est exposé à la surface externe de la protéine, ce qui suggère que l'interaction avec d'autres protéines serait plausible. TRIM5 $\alpha$  est un facteur de restriction qui inhibe la réplication virale à différentes étapes, ainsi, il est plausible de suggérer que le motif SIM4 de TRIM5 $\alpha$  est possiblement impliqué dans l'inhibition de certaines étapes du cycle de réplication virale du VIH-1 et ce, indépendamment ou non d'une interaction directe avec SUMO ou des protéines SUMOylées, puisqu'en interagissant spécifiquement avec la capsid virale, la protéine TRIM5 $\alpha$  inhibe, entre autres, la transcription inverse (208) et le transport du cœur viral vers le noyau (163, 209), quoique ces fonctions n'ont pas été directement testées dans un contexte où le motif SIM4 de TRIM5 $\alpha$  est muté. Toutefois, il est important de mentionner que la capacité du mutant SIM4 TRIM5 $\alpha_{Rh}$  à co-localiser avec SUMO-1 et PML a été diminuée de moitié comparativement à la protéine WT. Similairement à ce qui avait été observé avec le mutant K10R TRIM5 $\alpha_{Rh}$ , le mutant SIM4 s'accumule davantage dans le noyau, comparativement à la protéine WT, lorsque l'export nucléaire dépendant de CRM-1 est inhibé. Si nous considérons l'hypothèse selon laquelle TRIM5 $\alpha_{Rh}$  peut être exportée de façon dépendante et indépendante à CRM-1, il se peut qu'en plus du NES en N-terminal, le transport de TRIM5 $\alpha$  vers le

cytoplasme requière une interaction soit avec SUMO ou des protéines SUMOylées qui sont reconnues pour être sollicitées dans la fonction de l'export nucléaire (186, 223). Malgré la diminution de co-localisation du mutant SIM4 TRIM5 $\alpha_{Rh}$  avec SUMO-1 et PML, il nous est impossible de confirmer avec ces expériences de microscopie à fluorescence qu'il s'agit d'une véritable interaction. Pour ce faire, il existe d'autres techniques plus spécifiques capables de déterminer s'il y a des interactions et des relations dynamiques entre deux ou plusieurs protéines : BRET (*Bioluminescence resonance energy transfer*). D'ailleurs, cette technique (Figure 5.5) a été utilisée pour démontrer la SUMOylation de PML et la liaison non-covalente de PML à SUMO-1 via un motif SIM (140, 165). La mise au point de cette technique dans un contexte impliquant TRIM5 $\alpha$  et SUMO-1 pourrait nous confirmer si les phénotypes observés en microscopie sont caractéristiques d'une interaction covalente ou non-covalente avec SUMO.



**Figure 5.5 Mécanisme de la technique BRET.**

La protéine PML est couplée à la luciférase (bioluminescente) qui agit à titre de donneur d'énergie et lorsque PML se lie à SUMO qui est couplé à une protéine fluorescente (YFP), il y a transfert d'énergie et émission de fluorescence. La coelenterazine est le substrat que la luciférase utilise pour émettre de la bioluminescence. (L'illustration provient de cette publication (165)).

Le rôle de SUMO-1 dans l'activation de NF- $\kappa$ B dépendante de TRIM5 $\alpha_{Rh}$  a été précédemment proposé par une équipe (131) et nos résultats suggèrent que le motif SIM4 est essentiel à l'activation de NF- $\kappa$ B et AP-1. Comme mentionné dans la discussion de l'article présenté au chapitre IV, plusieurs protéines impliquées dans l'activation de NF- $\kappa$ B et AP-1 sont SUMOylées. Des expériences d'immunoprécipitation pourraient nous permettre d'identifier les protéines qui sont capables d'interagir avec TRIM5 $\alpha$  et l'utilisation du mutant pour SIM4 TRIM5 $\alpha_{Rh}$  pourrait nous informer si ces interactions sont dépendantes de ce motif. SUMO est également très impliqué dans la régulation de la transcription, notamment celle dépendante de NF- $\kappa$ B et AP-1. De toute évidence, une caractérisation plus poussée est nécessaire à une meilleure compréhension des mécanismes impliquant SUMO et le motif SIM4 de TRIM5 $\alpha$ . L'utilisation de techniques telles que BRET pourrait nous informer sur le type d'interactions que TRIM5 $\alpha$  établit avec SUMO.

L'étude sur SIM4 comprend une analyse phylogénétique de 36 espèces de primates qui a permis de suggérer que ce motif a subi des variations significatives dans sa séquence, possiblement pour permettre aux espèces exprimant ces variants de SIM4 l'interaction avec différents rétrovirus. Fait intéressant, les primates humains et non-humains ayant conservé le motif VIIC intact se situent principalement en Afrique, endroit où il y a le plus d'individus infectés par le virus de l'immunodéficience simienne (VIS) ou le VIH (198). C'est également l'endroit où l'on retrouve la plus grande variabilité chez le VIS et le VIH (158, 192, 199). Cette pression sélective est possiblement plus importante pour les primates vivant en Afrique que ceux vivant ailleurs dans le monde, notamment les singes du nouveau monde (NWM). La substitution du motif VIIC par les variants naturels, notamment exprimés par les singes du nouveau monde et les gibbons, a affecté la capacité de TRIM5 $\alpha_{Rh}$  à inhiber le VIH-1 efficacement. En plus de son rôle possible dans l'interaction avec les protéines SUMOylées, SIM4 semble important pour l'interaction spécifique et le tropisme de TRIM5 $\alpha$  pour la capsidie rétrovirale. Cependant, les variations de séquence n'ont pas affecté la capacité de TRIM5 $\alpha_{Rh}$  à activer les voies de signalisations NF- $\kappa$ B et AP-1,

supportant l'importance de ce motif dans la signalisation de l'immunité innée dépendante de TRIM5 $\alpha$  dans la réponse anti-rétrovirale.

#### 5.4 Répercussion de nos recherches sur le développement de la thérapie génique

Puisque l'objectif ultime de ces recherches est d'utiliser TRIM5 $\alpha$  comme outil thérapeutique dans une stratégie impliquant la thérapie génique, sa caractérisation à tous les niveaux est primordiale. L'orthologue humain de TRIM5 $\alpha$  est incapable d'inhiber efficacement le VIH-1 donc il est inconcevable de penser que l'on pourrait utiliser la forme WT de la protéine. Si l'on veut construire une protéine capable d'inhiber complètement le VIH-1, il faudra insérer des mutations dans la protéine exprimée de façon endogène, et ce, sans changer la nature de la protéine au niveau structurel et fonctionnel. La majorité de nos résultats ont démontré l'importance de certains motifs de TRIM5 $\alpha_{Rh}$  dans sa capacité à inhiber les rétrovirus et à activer la signalisation de l'immunité innée. À ce sujet, l'expression stable de la protéine TRIM5 $\alpha_{Hu}$ , préalablement modifiée pour lui permettre d'inhiber le VIH-1, ne devrait pas activer constitutivement l'immunité innée; cette activation devrait plutôt être inductible lors de l'infection par un rétrovirus sensible à la restriction par TRIM5 $\alpha$ . Un mutant de TRIM5 $\alpha_{Hu}$  (R332G-R335G) a précédemment été décrit comme étant capable d'inhiber efficacement le VIH-1 (172). Comme souhaité, l'activation de l'immunité innée, notamment NF- $\kappa$ B, n'est pas déclenchée lorsque cette protéine mutante est exprimée stablement dans des cellules (Annexe A). Néanmoins, nos conclusions suggèrent qu'une caractérisation approfondie des modifications post-traductionnelles, telles que la phosphorylation, l'ubiquitination, et la SUMOylation de TRIM5 $\alpha$ , est essentielle pour définir sa structure spatiale native, c'est-à-dire sa conformation à l'état naturel *in vivo*, et conséquemment les fonctions de cette protéine, spécialement sa capacité à agir avec d'autres protéines.

La thérapie génique impliquant TRIM5 $\alpha$  est toujours d'actualité et tout comme la trithérapie, la combinaison de plusieurs molécules anti-rétrovirale pourrait constituer une cure potentielle (5, 24, 25). Le défi se résume à trouver une technique idéale qui

permettrait d'insérer de façon stable un ou plusieurs gènes d'intérêt permettant l'expression de la ou des protéines à un niveau suffisant, comme dans le contexte où TRIM5 $\alpha$  serait exprimé, ou inversement, permettrait d'introduire une mutation dans le gène inhibant ainsi l'expression d'une protéine nuisible, comme dans le cas où l'expression du co-récepteur CCR5 serait inhibée. De nouvelles technologies potentielles ont été identifiées chez les bactéries et les plantes, et elles permettraient la modification de gènes que l'on appelle communément « *gene editing* ». En effet, la reconnaissance spécifique de séquences cibles d'ADN par des nucléases tels que les zinc-finger (ZFN), les TALEN (*transcription activator-like effector nucleases*) et le système CRISPR/Cas9 (47) permettrait soit d'interférer avec l'expression de certains gènes ou d'insérer des gènes qui expriment en continu une protéine d'intérêt (134). La technique impliquant les nucléases ZFN demeure la plus caractérisée jusqu'à maintenant et a fait l'objet de plusieurs études préliminaires sur l'utilisation de la thérapie génique dans le développement d'une cure pour l'infection au VIH, notamment pour inhiber l'expression du co-récepteur CCR5 empêchant ainsi l'entrée du VIH dans les cellules (83, 93, 215). Néanmoins, il n'existe, à ce jour, aucune thérapie génique efficace et sécuritaire chez l'homme capable d'éliminer le VIH-1 (215).

## 5.5 Conclusion générale

Pour conclure cette étude qui a porté sur la caractérisation de TRIM5 $\alpha$ , nous pouvons affirmer que nos expériences ont, premièrement, permis d'identifier pour la première fois la formation d'hexamères de TRIM5 $\alpha$ , structure quaternaire maintenant reconnue comme composante essentielle dans le modèle proposé actuel d'interaction avec la capsid rétrovirale. L'étude du rôle des motifs spécifiques de SUMOylation de la protéine TRIM5 $\alpha$  a permis de révéler plusieurs fonctions possibles de TRIM5 $\alpha$ . Pour commencer, la lysine potentiellement SUMOylée en position 10 joue un rôle important dans l'ubiquitination de TRIM5 $\alpha$ ; précisément, elle module l'activité du domaine RING en permettant à celui-ci de recruter les enzymes de conjugaisons nécessaires aux différents types d'ubiquitination. D'ailleurs, l'ubiquitination de TRIM5 $\alpha$  s'avère particulièrement plus complexe que ce qui a été proposé jusqu'à maintenant. En second

lieu, SIM4 est un motif essentiel à l'activation de l'immunité innée médiée par les protéines TRIM5 $\alpha$ , et nos résultats proposent que cette fonction soit dépendante de l'interaction avec SUMO ou des protéines SUMOylées. De plus, une étude phylogénétique suggère que les variations de séquences de ce motif seraient impliquées dans le tropisme de TRIM5 $\alpha$  pour les différentes capsides rétrovirales sans toutefois affecter son rôle dans la signalisation de l'immunité innée. Les modifications post-traductionnelles de TRIM5 $\alpha$  déterminent sa spécificité d'action et sa capacité à interagir avec d'autres protéines telles que SUMO ou même d'autres protéines TRIM5 $\alpha$ . Cependant, je crois qu'il reste encore beaucoup de caractérisation à faire pour bien comprendre tous ces mécanismes et leur rôle potentiel dans la restriction des rétrovirus par les protéines TRIM5 $\alpha$ .



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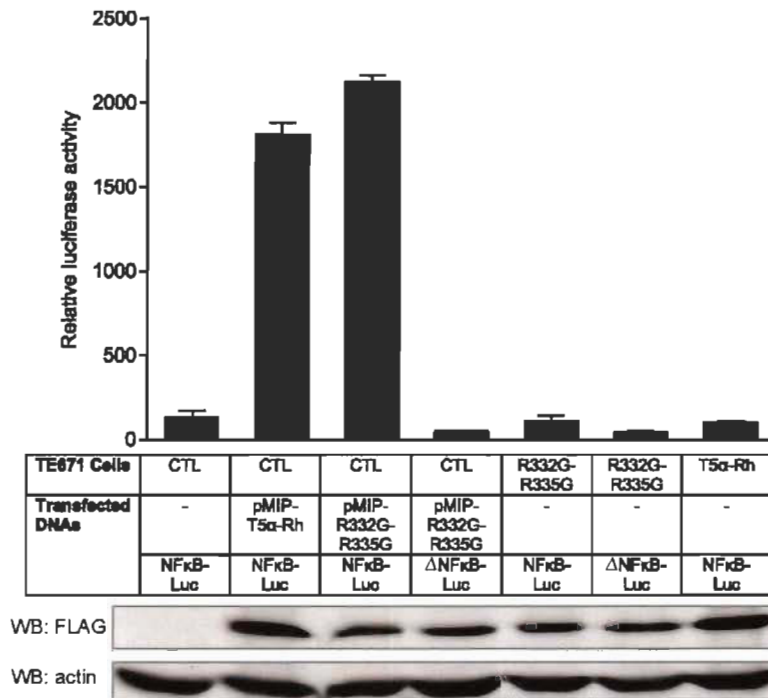
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## ANNEXE A

Le résultat présenté dans cette annexe fera l'objet d'un article en préparation qui porte sur un mutant de TRIM5 $\alpha_{Hu}$  (R332G-R335G) qui est capable d'inhiber le VIH-1 à un niveau similaire à TRIM5 $\alpha_{Rh}$ . Ce mutant est un candidat intéressant pour une éventuelle thérapie génique. Pour s'assurer que, dans un contexte où il n'y a pas d'infection, l'activation de NF- $\kappa$ B n'est pas initiée lorsque la cellule exprime de façon stable le mutant R332G-R335G TRIM5 $\alpha_{Hu}$ , une expérience utilisant des cellules TE671 transduites a été effectuée. Le graphique présente le signal luciférase relatif émis suite à l'activation de NF- $\kappa$ B, et il y a activation seulement dans un contexte où les cellules sont transfectées avec le mutant R332G-R335G TRIM5 $\alpha_{Hu}$  ou WT TRIM5 $\alpha_{Rh}$ . Les résultats suggèrent que l'activation de NF- $\kappa$ B dans les cellules exprimant stablement TRIM5 $\alpha$  se produit probablement dans un contexte où il y a une réponse à l'infection dépendante de TRIM5 $\alpha$ , quoique cela n'ait pas encore été testé dans un contexte où il y a infection.



**Figure 1** NF- $\kappa$ B activation in TE671 cells stably expressing the mutant R332G-R335G TRIM5 $\alpha_{Hu}$ .

TE671 transduced with empty vector control (CTL) were co-transfected with R332G-R335G TRIM5 $\alpha_{Hu}$  or WT TRIM5 $\alpha_{Rh}$  and the NF- $\kappa$ B luciferase reporter construct. As a control, TE671 were co-transfected with R332G-R335G TRIM5 $\alpha_{Hu}$  and the activation-deficient mutant of the reporter construct ( $\Delta$ NF- $\kappa$ B-Luc). TE671 stably expressing R332G-R335G TRIM5 $\alpha_{Hu}$  or WT TRIM5 $\alpha_{Rh}$  were transfected with NF- $\kappa$ B luciferase reporter construct or  $\Delta$ NF- $\kappa$ B-Luc. Cells were lysed 48 h post-transfection and luciferase activity was measured. Bars show averages from triplicate transfections with standard deviations. The cellular lysates prepared to quantify luciferase activity were also used to analyze TRIM5 $\alpha_{Rh}$  expression. Specifically, the three lysates from each triplicate transfection were pooled and analyzed by western blotting using an anti-FLAG antibody. Actin was used as a loading control.



## ANNEXE B

### CO-LOCALIZATION OF OWL MONKEY TRIMCYP AND INCOMING HIV-1 CAPSID IN RESTRICTIVE AND NON-RESTRICTIVE CONDITIONS

MARIE-EDITH NEPVEU-TRAVERSY, JULIE BÉRUBÉ ET LIONEL BERTHOUX

Le contenu de cette annexe présente un manuscrit en préparation.

#### **B1 Contribution des auteurs**

MÉNT et LB ont conçu cette étude. MÉNT et JB ont effectué les expériences. MÉNT et LB ont écrit le manuscrit.

#### **B2 Résumé en français**

**Mise en contexte :** La protéine TRIMCyp du singe-hibou (omkTRIMCyp) appartient à la famille des protéines cytoplasmiques TRIM5 capables d'inhiber certains rétrovirus dans des cellules de mammifères, et ce, à des stades précoces de la réplication virale. Le domaine de la cyclophiline A de TRIMCyp peut se lier à une boucle spécifique située à l'intérieur de la capsidie lentivirale. Cette interaction inhibe fortement plusieurs étapes subséquentes telles que la décapsidation, la transcription inverse et l'import nucléaire. Les analyses précédentes de microscopie à fluorescence ont montré que les corps cytoplasmiques de TRIM5 $\alpha$ , un autre membre de la famille TRIM5, co-localisaient avec les capsides rétrovirales sensibles à la restriction de TRIM5 $\alpha$ , suggérant ainsi que la co-localisation est un marqueur phénotypique d'une restriction fonctionnelle. L'objectif de cette étude était d'analyser (i) si TRIMCyp co-localisait

avec la capsid virale à des étapes précoces de l'infection, et (ii) si cette association est dépendante d'une interaction moléculaire directe entre TRIMCyp et la capsid virale.

**Résultats :** Dans les premières heures suivant l'infection, la capsid du VIH-1 se retrouvait principalement dans de petits points cytoplasmiques représentant possiblement des virus individuels. 0,5 % à 2 % de ces « points de capsides » se sont associés avec les corps cytoplasmiques de TRIMCyp et, tel que rapporté par d'autres, le nombre de co-localisations augmentait suite à un traitement avec le MG132, un inhibiteur du protéasome. Nos résultats ont révélé que la co-localisation entre TRIMCyp et la capsid virale a considérablement augmenté lorsque les cellules étaient traitées avec du trioxide d'arsenic, suggérant que cette drogue interfère avec des mécanismes qui sont également inhibés avec le MG132. Nous avons inhibé les interactions entre TRIMCyp et la capsid, et interféré avec la restriction en introduisant des mutations dans l'une des deux protéines ou en traitant les cellules avec de la cyclosporine A. Étonnement, aucune de ces interventions n'a inhibé la co-localisation de la capsid du VIH-1 et des corps cytoplasmiques de TRIMCyp.

**Conclusions :** Nos résultats supportent l'existence de co-localisations spécifiques et détectables entre TRIMCyp et sa cible, la capsid du VIH-1. Cependant, ils suggèrent également que, contrairement à ce qui a été observé précédemment avec TRIM5 $\alpha$ , la co-localisation entre TRIMCyp et la capsid virale peut avoir lieu sans impliquer une interaction physique directe entre les deux protéines. Nous spéculons que TRIMCyp et la capsid virale interagissent probablement avec d'autres facteurs, possiblement liés au cytosquelette, ce qui résulterait en une co-localisation dans des conditions dites restrictives et non-restrictives.

### B3 Article scientifique en préparation

#### Co-localization of owl monkey TRIMCyp and incoming HIV-1 capsid in restrictive and non-restrictive conditions

##### Abstract

**Background:** The Owl monkey protein TRIMCyp (omkTRIMCyp) belongs to the TRIM5 family of cytoplasmic proteins that can sense and counteract retroviral infections of mammalian cells at the early stages of replication. The cyclophilin A domain of omkTRIMCyp can bind to a specific loop within the capsid proteins of incoming restriction-sensitive lentiviruses. This interaction results in a strong inhibition of subsequent replication steps, such as capsid disassembly, reverse transcription and nuclear import. Previous fluorescent microscopy analyses showed that cytoplasmic bodies of TRIM5 $\alpha$ , another member of the TRIM5 family, co-localized with restriction-sensitive retroviral cores, hence suggesting that co-localization is a phenotypic marker for functional restriction. The objective of our study was to analyze (i) whether omkTRIMCyp similarly co-localizes with HIV-1 capsid cores at early stages of infection, and (ii) whether this association is dependent on direct omkTRIMCyp-capsid molecular interactions.

**Results:** In the first hours of infection, HIV-1 capsids were found mostly within cytoplasmic foci that were probably individual viruses. 0.5 to 2% of such “capsid foci” associated with omkTRIMCyp cytoplasmic bodies and, as noted before by others, this co-localization increased upon inhibition of the proteasome by MG132 treatment. Our data reveal that omkTRIMCyp-capsid co-localization also increases markedly when cells are treated with arsenic trioxide, suggesting that this drug may inhibit omkTRIMCyp function by interfering with the same mechanism that is disrupted by MG132. We disrupted omkTRIMCyp-capsid interactions and abrogated restriction by introducing mutations in either of the two proteins or by cyclosporin A treatment. Surprisingly, none of these interventions abrogated the localization of HIV-1 capsid cores at omkTRIMCyp cytoplasmic bodies.

**Conclusions:** Our data support the existence of detectable, specific co-localization of omkTRIMCyp and its target, the HIV-1 capsid core. However, they also suggest that contrary to what was previously observed with TRIM5 $\alpha$ , co-localization of omkTRIMCyp and capsid can occur without direct physical interactions between the two proteins. We speculate that omkTRIMCyp and capsid interact with other factors, perhaps related to the cytoskeleton, leading to co-localization in the presence or absence of restriction.

## Background

The replication of retroviruses can be restricted shortly after entry into host cells by the cytoplasmic protein TRIM5 $\alpha$  or the related TRIMCyp, also called TRIM5CypA (23, 27, 40, 43, 45, 48). Different versions of TRIMCyp are found in both Old World and New World monkeys, resulting from several independent events of retrotransposition of the cyclophilin A (CypA) cDNA into the *TRIM5* gene, yielding different hybrid proteins (12, 28, 34, 35, 40, 50, 51). Restriction depends on the initial binding of TRIM5 $\alpha$  and TRIMCyp to their targets, the correctly matured capsid (CA) proteins that form the retroviral core (20, 42, 46). The HIV-1 CA core is recognized by TRIM5 $\alpha$  from the Rhesus macaque (rhTRIM5 $\alpha$ ) but not by its human ortholog (46). Restriction causes instability of the retroviral CA core and a block to nuclear import of the viral DNA (2, 8, 14, 37, 46, 53).

The Owl monkey TRIMCyp (omkTRIMCyp) inhibits HIV-1 through effector mechanisms similar to those of rhTRIM5 $\alpha$  (19, 36). The main distinction between the two restriction factors is that target recognition and binding are achieved by the CypA domain of TRIMCyp, while they involve a PRYSPRY/B30.2 domain for TRIM5 $\alpha$  (32, 44, 48). Some differences have been reported in the behavior and mechanism of restriction of omkTRIMCyp compared with TRIM5 $\alpha$ . For instance, TRIM5 $\alpha$  can shuttle between the nucleus and the cytoplasm while omkTRIMCyp cannot (17). CypA has peptidyl-prolyl cis-trans isomerase activity and is able to catalyze the cis/trans isomerization of the G89-P90 bound in HIV-1 capsid (9, 52).

TRIM5 $\alpha$  was reported to form *de novo* cytoplasmic bodies (CBs) that seem to interact with incoming restriction-sensitive viruses. Specifically, the co-localization of rhTRIM5 $\alpha$  CBs and HIV-1 cores post-entry was several times more frequent than the co-localization of HIV-1 cores and a PRYSPRY-mutated version of rhTRIM5 $\alpha$  (14). In addition, inhibition of proteasomal degradation by MG132 treatment seemed to increase the co-localization of rhTRIM5 $\alpha$  CBs and incoming HIV-1 cores, suggesting that complexes comprising HIV-1 and TRIM5 $\alpha$  undergo proteasomal degradation (14). Indeed, TRIM5 $\alpha$  CBs were found to co-localize with ubiquitin (14) and proteasomal

subunits (16, 27). omkTRIMCyp is able to form CBs in the absence of infection (3, 7, 18, 36) but it was not known whether omkTRIMCyp would also specifically co-localize with incoming HIV-1 cores.

Pharmacological and genetic interventions can prevent the binding of HIV-1 CA to CypA and to omkTRIMCyp. Introduction of the G89V mutation in the CypA-binding loop of HIV-1 CA was described to abrogate CypA-CA interactions *in vitro*, as analyzed by surface plasmon resonance spectroscopy (22, 52). As a consequence of disrupted CA-CypA interactions, omkTRIMCyp was found to be unable to restrict HIV-1 bearing the G89V mutation in HIV-1 CA (24, 35, 40, 49). Similarly, the H436Q mutation in the enzymatic pocket of omkTRIMCyp, corresponding to H126Q in CypA, was found to abrogate interactions with CA *in vitro* (11) and to cause a loss in HIV-1 restriction when cloned into a version of TRIMCyp made from human components (31). Finally, treatment with CsA, a drug that competes with HIV-1 CA for binding to CypA, also prevents the CA-CypA interaction as analyzed by co-immunoprecipitation (10) and abrogates the encapsidation of CypA into HIV-1 particles (10, 15, 21). As expected, interactions between omkTRIMCyp and CA were found to be completely disrupted by CsA treatment as analyzed by co-immunoprecipitation (26). Consequently, restriction of HIV-1 by omkTRIMCyp is prevented by treatment with CsA in the low micromolar range (15, 26, 35, 40, 49). Using these investigatory tools, the aim of the present study was to investigate whether omkTRIMCyp CBs co-localized with incoming HIV-1 cores in restrictive and non-restrictive conditions.

## Results

### *Genetic and pharmacological disruption of omkTRIMCyp-mediated restriction of HIV-1*

We first verified that the restriction of HIV-1 by omkTRIMCyp could be abrogated in our experimental system by previously described pharmacological or genetic interventions. For this, we performed a virus dose-dependent analysis of wild-type (WT) or mutant HIV-1 restriction by WT or mutant omkTRIMCyp in *Mus dunni* tail fibroblasts (MDTF) cells (Figure 1A). The use of murine cells is desirable as they do not express any endogenous TRIM5. As expected, WT HIV-1 was strongly restricted (100- to 200-fold) by WT omkTRIMCyp in these cells (Figure 1A). Introduction of the G89V mutation in CA had no effect on the infectivity of HIV-1, but it almost completely abrogated restriction by omkTRIMCyp (Figure 1A). As expected from published results with a humanized TRIMCyp (31), we found that the H436Q mutant of omkTRIMCyp had very little restriction activity against the HIV-1 vector used. Compared with the mock-transduced control cells, infectivity in cells expressing H436Q omkTRIMCyp was reduced by only 2- to 4-fold, depending on the viral dose used (Figure 1A). Because the H436Q mutation in omkTRIMCyp and the G89V mutation in HIV-1 CA each greatly affected CA-CypA interactions and restriction, one would expect no detectable restriction of G89V HIV-1 by H436Q omkTRIMCyp. However, we observed that G89V HIV-1 infectivity in cells expressing H436Q omkTRIMCyp was the same as in cells expressing WT omkTRIMCyp, i.e. it was reduced 2- to 4-fold compared to control cells infected with WT HIV-1. These results suggest that the residual restriction observed in cells expressing WT omkTRIMCyp and infected with G89V HIV-1, or in cells expressing H436Q omkTRIMCyp and infected with WT HIV-1, is not dependent on CA-TRIMCyp interactions. Most probably, the exogenous over-expression of omkTRIMCyp (WT or mutant) in these cells creates an antiviral state, perhaps through the activation of NF- $\kappa$ B-dependent pathways (38, 47).

Unlike TRIM5 $\alpha$ , omkTRIMCyp-mediated restriction can be pharmacologically abrogated by treatment with cyclosporine A (CsA), which competes with HIV-1 CA for binding to the CypA enzymatic pocket (40, 48, 49). We analyzed the effect of CsA

treatment on the restriction mediated by omkTRIMCyp in MDTF cells infected with the HIV-1 vector at a MOI of ~0.2 (Figure 1B). Like before, WT HIV-1 replication was greatly reduced in the presence of WT omkTRIMCyp (200-fold) while it was only slightly reduced in cells expressing H436Q omkTRIMCyp (2.5-fold). G89V HIV-1 replicated as efficiently as its WT counterpart in cells expressing WT or mutant omkTRIMCyp (Figure 1B). CsA slightly increased (2-fold) the infectivity of WT HIV-1 in these cells (Figure 1B), but it had the same effect on G89V HIV-1, showing that this effect was not due to interactions with endogenous CypA. As expected, CsA completely disrupted HIV-1 restriction by WT omkTRIMCyp, but only slightly increased HIV-1 replication in cells expressing H436Q omkTRIMCyp (Figure 1B). In summary, and as expected, CsA completely abrogated omkTRIMCyp-mediated restriction of HIV-1 but had no effect when restriction was already prevented by genetic interventions, i.e. mutations in omkTRIMCyp or in CA.

Ammonium chloride (NH<sub>4</sub>Cl) inhibits the entry of VSV-G-pseudotyped HIV-1 by disrupting endosomal acidification (1), and was used as a control in subsequent microscopy experiments. To verify that NH<sub>4</sub>Cl indeed abrogates infectivity, MDTF cells stably expressing omkTRIMCyp, H436Q and control cells were infected with either WT or G89V HIV-1 in the presence or absence of ammonium chloride for the duration of the infection. Treatment with ammonium chloride decreased replication of the HIV-1 vectors used (WT or G89V) by 100- to 200-fold in all conditions tested (Figure 1C).

#### *Co-detection of HIV-1 CA cores and omkTRIMCyp CBs in acutely infected MDTF cells*

The Hope laboratory has pioneered the visualization of incoming HIV-1 cores using a variety of viral markers, including Vpr, CA and the viral DNA (14, 29). We first wished to determine (i) whether we could visualize WT and G89V HIV-1 cores post-entry using a CA monoclonal antibody, (ii) whether H436Q omkTRIMCyp forms CBs, and (iii) whether we could detect occasional events of co-localization resembling those described by McDonald *et al.* (29). For this, we infected MDTF cells stably expressing either WT or H436Q omkTRIMCyp with WT or G89V HIV-1 and then processed them



for immunofluorescence (IF) microscopy as detailed before (7). We used conditions for which there was no detectable CA signal in the absence of infection and no FLAG signal in control cells (Figure 2 and data not shown). We observed CA foci (Figure 2) with sizes typically ranging from 0.1 to 0.5  $\mu\text{m}$ , consistent with what was found by others using a different method to detect CA (14, 29). Thus, it is likely that most of these foci are individual viruses as characterized previously (14, 29). It should be noted, however, that our staining procedure does not differentiate post-entry cytoplasmic particles from the unfused viruses still present at the surface of cells or within endocytic vesicles. The G89V mutation in HIV-1 CA had no effect on the presence of CA foci (Figure 2 and data not shown), consistent with this mutation having no detrimental effect on the viral fitness (Figure 1). As expected (3, 18), omkTRIMCyp was found to form CBs of diverse sizes and shapes. Additionally, the H436Q mutation did not have any apparent effect on the size or abundance of omkTRIMCyp CBs (Figure 2 and data not shown). This was expected considering that the determinants for the formation of omkTRIMCyp CBs are present in the RBCC segment of the fusion protein rather than in the CypA domain (3, 18). Treatment with CsA slightly affected the shapes of both omkTRIMCyp CBs and CA foci, but in different ways: it decreased the average size of omkTRIMCyp CBs, while it caused the CA foci to sometimes appear larger and less bright (compare the two top panels in Figure 2). The latter effect may be explained by CsA-dependent CA core disassembly (25). A minor fraction of the CA signal co-localized with omkTRIMCyp, and when it was the case, CA often seemed to be present within omkTRIMCyp bodies. Examples are shown in boxed areas of the 4 upper panels of Figure 2. Despite abrogating infectivity, treatment with the VSV-G-mediated entry inhibitor  $\text{NH}_4\text{Cl}$  had no effect on the aspect and distribution pattern of CA foci (Figure 2, bottom-right panel) nor did it affect the omkTRIMCyp staining. This indicates that as found by others (14), unfused, endosomal VSV-G-pseudotyped HIV-1 virions cannot be distinguished from their fused, cytoplasmic counterparts in CA staining experiments. In summary, we were able to visualize individual WT or G89V HIV-1 cores and WT or H436Q omkTRIMCyp, in the absence or presence of CsA or  $\text{NH}_4\text{Cl}$ .

*Pharmacological or genetic inhibition of omkTRIMCyp-mediated restriction does not prevent the co-localization of HIV-1 cores and omkTRIMCyp CBs*

Next we performed large-scale IF microscopy experiments to study the importance of functional restriction, and of omkTRIMCyp-CA interactions, on the co-localization of omkTRIMCyp and CA in acutely infected cells. To provide statistical power to our analysis, we compiled data from 20 randomly chosen fields, counting hundreds to thousands of CA foci for each experimental condition. We found that approximately 1 to 2% of WT CA foci co-localized with WT omkTRIMCyp in the absence of drugs (Figures 3A and 3B). We did not count as positive the CA foci that appeared to be merely in contact or in close proximity with a omkTRIMCyp CB, since images from NH<sub>4</sub>Cl-treated cells had shown that omkTRIMCyp CBs were frequently found to be very close to unfused HIV-1 particles present in endocytic vesicles (Figure 2). To validate our protocol for quantifying omkTRIMCyp-CA co-localization, we decided to first analyze the effect of the proteasome inhibitor MG132 on the levels of CA-TRIMCyp co-localization. MG132 counteracts proteasome-dependent TRIM5 $\alpha$ /TRIMCyp effector mechanisms (2), and co-localization between HIV-1 cores and the HIV-1-restrictive rhTRIM5 $\alpha$  is increased in presence of this drug (14). Likewise, we observed that MG132 treatment caused a nearly 3-fold increase in the % of capsid foci associating with WT omkTRIMCyp in MDTF cells following infection with the WT HIV-1 vector (Figure 3A). This result suggests that as expected from the rhTRIM5 $\alpha$  data, MG132 does not prevent HIV-1 cores interaction with omkTRIMCyp nor a possible sequestration in CBs, but that it protects these HIV-1 cores from either proteasomal degradation or accelerated decapsidation or both. This experiment confirmed that we could detect variations in omkTRIMCyp-CA co-localization in response to treatments that are known to affect restriction.

We then performed similar experiments in conditions in which omkTRIMCyp-mediated restriction of HIV-1 was functional or was disrupted pharmacologically or through mutations in omkTRIMCyp or in HIV-1 CA (Figure 3B). In addition, control infections were done in the presence of NH<sub>4</sub>Cl. The % of omkTRIMCyp-CA co-localization in the absence of NH<sub>4</sub>Cl was repeatedly higher than in the presence of this

drug. Specifically, in cells expressing WT omkTRIMCyp infected with WT HIV-1, 1.53% and 2.18% of CA foci co-localized with omkTRIMCyp bodies in the presence or the absence of NH<sub>4</sub>Cl, respectively. In cells expressing WT omkTRIMCyp infected with G89V HIV-1, the % of co-localization in the presence and absence of NH<sub>4</sub>Cl were 1.01% and 3.48%, respectively. Finally, in H436Q omkTRIMCyp-expressing cells infected with WT HIV-1, the % of co-localization in the presence and absence of NH<sub>4</sub>Cl were 1.05 % and 2.18%, respectively (Figure 3B). These results suggest the existence of a basal level of co-localization false positives, or noise, in our experiment. However, this noise seems to be constant at around 1.2%, strongly suggesting that co-localization rates of 2% or higher, which are seen in the absence of NH<sub>4</sub>Cl, are relevant. Surprisingly, none of the interventions that are known to abrogate the molecular interactions between omkTRIMCyp and HIV-1 CA, and that were confirmed to abrogate restriction by omkTRIMCyp (Figure 1), reduced the co-localization of omkTRIMCyp and CA foci. Specifically, the % of observed WT omkTRIMCyp-WT CA co-localization (2.18%) was increased 1.3-fold by CsA treatment (2.97%), 1.6-fold by the G89V mutation in CA (3.48%) and was not modified by the omkTRIMCyp H436Q mutation (Figure 3B). We conclude from the data in Figure 3B that the association of omkTRIMCyp and incoming HIV-1 cores, as reflected by the ratio of CA foci co-localizing with omkTRIMCyp, does not require direct molecular interactions between omkTRIMCyp and CA, nor does it correlate with restriction.

*Arsenic trioxide treatment increases the co-localization of HIV-1 CA cores and omkTRIMCyp*

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) inhibits TRIM5 $\alpha$ /TRIMCyp restriction in some cell lines by a yet unknown mechanism (6, 41). In restrictive conditions, As<sub>2</sub>O<sub>3</sub> treatment increases the amounts of viral DNA (6), suggesting that it acts to counteract the same restriction mechanism that MG132 also impedes (2). As<sub>2</sub>O<sub>3</sub> has no effect on the restriction of HIV-1 by omkTRIMCyp in MDTF cells (data not shown), but it is known to inhibit omkTRIMCyp when expressed in human HeLa cells (33). In HeLa cells stably expressing omkTRIMCyp, HIV-1 replication was severely inhibited and As<sub>2</sub>O<sub>3</sub> rescued HIV-1 infection by about 20-fold (Figure 4A). IF microscopy experiments showed that

As<sub>2</sub>O<sub>3</sub> had no obvious effects on the aspect or numbers of HIV-1 CA cores (Figure 4B). In contrast, omkTRIMCyp CBs were fewer and larger, on average, in As<sub>2</sub>O<sub>3</sub>-treated cells (Figure 4B). We noticed that As<sub>2</sub>O<sub>3</sub> caused a large increase in the localization of CA foci at omkTRIMCyp CBs, as exemplified by the white arrows in Figure 4B. We quantified the levels of omkTRIMCyp-CA co-localization and found it to be of 0.4% in the absence of drug (Figure 4C), thus relatively close to what was seen in the murine cells (Figure 3). As expected, MG132 increased the % of CA associated with omkTRIMCyp bodies (3.3-fold), whereas As<sub>2</sub>O<sub>3</sub> had an even greater effect (9.4-fold) (Figure 4C). Additionally, treatment with both MG132 and As<sub>2</sub>O<sub>3</sub> did not have an additional effect compared with As<sub>2</sub>O<sub>3</sub> alone (Figure 4C). This result supports the hypothesis that As<sub>2</sub>O<sub>3</sub> counteracts the same omkTRIMCyp-dependent restriction mechanism that is also inhibited by MG132.

## Discussion

Previous imaging analyses showed that rhTRIM5 $\alpha$  CBs and HIV-1 cores formed complexes in acutely infected cells (14). The experiments shown here, studying the restriction of HIV-1 by omkTRIMCyp, support the idea that omkTRIMCyp CBs associated with HIV-1 cores represent a restriction intermediate, as proposed by others (14, 27). Specifically, when downstream effector mechanisms are impeded by treatment with MG132 or with As<sub>2</sub>O<sub>3</sub>, then these complexes are more stable and co-localization markedly increases as a result (Figures 3, 4). Surprisingly however, we found that following interventions that abrogated omkTRIMCyp-CA interactions as well as restriction, a significant amount of HIV-1 cores still co-localized with omkTRIMCyp CBs (Figure 3B). Our experiment was done in the absence of MG132, and one might argue that differences between restrictive and non-restrictive conditions would have been revealed in the presence of a proteasome inhibition. However, Campbell *et al.* reported that the co-localization of rhTRIM5 $\alpha$  and incoming HIV-1 CA cores was several times greater than with a CA binding-deficient version of rhTRIM5 $\alpha$ , in the presence but also in the absence of MG132 (14). Thus, our results suggest that rhTRIM5 $\alpha$  and omkTRIMCyp behave differently in their interactions with their

common target, the incoming HIV-1 CA core. Of note, differences between these two restriction factors have been reported before. For instance, restriction of HIV-1 by rhTRIM5 $\alpha$  is more dependent on the presence of the RING domain at the N-terminal extremity of the protein (18). Also, TRIM5 $\alpha$  shuttles between cytoplasm and nucleus while omkTRIMCyp does not (17). Differences in methodologies, such as different markers used for the detection of viral cores, may also help explain the different conclusions, in addition to differences between TRIM5 $\alpha$  and omkTRIMCyp.

As with any microscopy-based study, a number of methodological caveats call for caution in interpreting the data. For instance, we used only one marker (CA) for the detection of HIV-1 cores, and it did not discriminate between fused and unfused particles. On the other hand, the robustness of our IF microscopy analyses is supported by the following points: (i) we used multiplicities of infection (MOI) of 0.5 or 2.0 (as calculated in permissive cells), at which restriction is not saturated (Figure 1); (ii) we observed that as expected from the Campbell paper (14), MG132 increases the localization of incoming HIV-1 CA cores at omkTRIMCyp bodies; (iii) we included a control defective for fusion (NH<sub>4</sub>Cl treatment) which allowed us to quantify the unspecific co-localization background. We used three different approaches to abrogate restriction by omkTRIMCyp (mutations H436Q, G89V and CsA treatment), and none of them resulted in the disruption of omkTRIMCyp-CA association. Therefore, our observations suggest that indeed, incoming HIV-1 cores can localize at omkTRIMCyp CBs even in the absence of restriction, and this calls for further investigation.

How could omkTRIMCyp co-localize with incoming HIV-1 cores in the absence of molecular interactions between omkTRIMCyp and CA? An explanation could be that both omkTRIMCyp and CA interact with other factors that determine their sub-cellular distribution. Indeed, both TRIM5 $\alpha$  CBs and incoming HIV-1 cores have been observed to associate with the microtubule network (13, 29). Recently, we have observed that nearly 40% of TRIM5 $\alpha$  or omkTRIMCyp CBs associate with microtubules (manuscript submitted). Therefore, we speculate that both omkTRIMCyp CBs and incoming HIV-1 cores interact with a yet unidentified cytoskeleton-related factor, such as microtubules or

a microtubule-associated transporter complex. This would explain the phenotypes observed here, and it would hint at a mechanism by which TRIM5 proteins can target incoming retroviruses in a non-random fashion.

## **Conclusions**

Our results show that omkTRIMCyp, like rhTRIM5 $\alpha$ , forms complexes with incoming HIV-1 cores that can be visualized by IF microscopy and that are stabilized by proteasome inhibition or by As<sub>2</sub>O<sub>3</sub> treatment. Our results also suggest that initial contacts between omkTRIMCyp and post-entry retroviral cores take place whether restriction occurs or not and whether omkTRIMCyp and CA proteins directly interact or not. This would imply the existence of unknown determinants for their co-localization, perhaps related to an intracellular transport system.

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## **Methods**

### *Plasmid DNAs*

pMIP-TRIMCyp expresses a C-terminal FLAG-tagged version of the corresponding protein from Owl monkey and has been extensively described before (7, 41). PCR-based “sewing” mutagenesis was performed to introduce H436Q in the CypA motif of omkTRIMCyp. The mutagenic primers used were: 5'-ATGGCAAGCAAGTGGTCTTTGGCAAGG-3' and 5'-CAAAGACCACTTGCTTGCCATCCAACC-3' and the inserted fragment containing the mutation was verified by sequencing. pMD-G, p $\Delta$ R8.9, p $\Delta$ R8.9 G89V, pCL-Eco and pTRIP-CMV-GFP have all been extensively described elsewhere (3-6, 30, 54).

### *Cells*

Human embryonic kidney 293T cells, human adenocarcinoma cervical cells (HeLa) and *Mus dunni* tail fibroblasts (MDTF) 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).

### *Virus production*

MLV and HIV-1-based vectors were produced through transient transfection of 293T cells with polyethylenimine (Polysciences) and collected as previously described (7, 39). To produce the MLV-based vectors MIP, cells were transfected with the relevant pMIP plasmid and co-transfected with pCL-Eco and pMD-G. All stably expressing cell lines were produced as previously described (7). To produce WT or mutant HIV-1<sub>TRIP-CMV-GFP</sub>, cells were co-transfected with WT or G89V p $\Delta$ R8.9, pMD-G and pTRIP-CMV-GFP. Normalization of virus amount was based on their titer in control permissive cells.

### *Viral challenges and drugs*

For the infectivity assays, cells were plated in 24-well plates at 30,000 cells /well (MDTF cells) or 50,000 cells/well (HeLa cells) and infected with either WT or G89V HIV-1<sub>TRIP-CMV-GFP</sub>, with or without CsA (5  $\mu$ M), NH<sub>4</sub>Cl (20 mM) or As<sub>2</sub>O<sub>3</sub> (15  $\mu$ M). Drugs (all from Sigma) were added just prior to the infection and treatments were done for 12 to 16 hours, after which supernatants were changed for fresh medium in order to remove drugs and viruses. For infections in the presence of NH<sub>4</sub>Cl, however, cells were maintained in the presence of the drug for the whole duration of the infection. Two days post-infection, cells were trypsinized and fixed in 2% formaldehyde in a PBS solution. The % of GFP-positive cells were then determined by analyzing 10,000 to 25,000 cells with a FC500 MPL cytometer (Beckman Coulter) using the CXP software (Beckman Coulter).

### *Immunofluorescence Microscopy*

Cells were plated at 100,000 cells (MDTF) or 150,000 cells (HeLa) on microscope cover glasses (Fisherbrand) in 6-well plates. The next day, cells were exposed to virus and drugs for 6 hours. Cells were washed with PBS, fixed for 10 min in 4% formaldehyde-PBS at 37°C, washed three times in PBS and permeabilized with 0.1% Triton X-100 for 2 min on ice. Cells were then washed again with PBS twice and treated with 10% normal goat serum (Vector laboratories) in PBS for 30 min at RT. This saturation step was followed by incubation with primary antibodies against FLAG (rabbit polyclonal DYKDDDDK Tag Antibody; Cell Signaling) and p24-CA (mouse monoclonal antibody purified in our laboratory from the 183 hybridoma clone, AIDS Research and reference Reagent Program, NIAID, NIH) at a 1:200 dilution in PBS with 10% normal goat serum. Fluorescent staining was done by using an Alexa594-conjugated goat anti-rabbit antibody and an Alexa488-conjugated goat anti-mouse antibody (Molecular Probes) both at a 1:200 dilution. Cells were washed 4 times in PBS before mounting in Vectashield (Vector Laboratories). Hoechst-33342 (0.8 µg/ml; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Pictures were generated using an AxioObserver microscope with apotome module and the Axiovision software. Imaging parameters were set to identical values across samples within each experiment, and analyses were done on randomly chosen fields.



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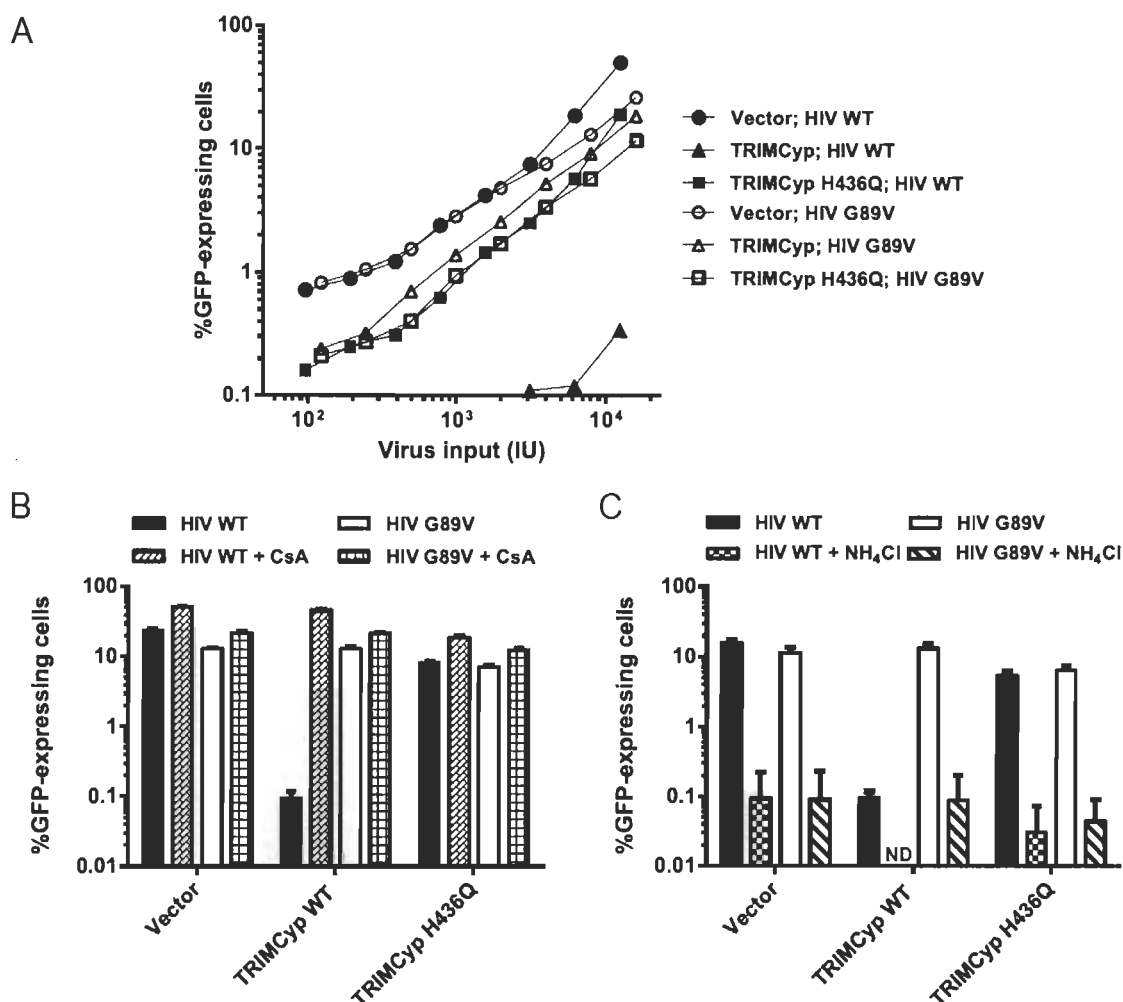
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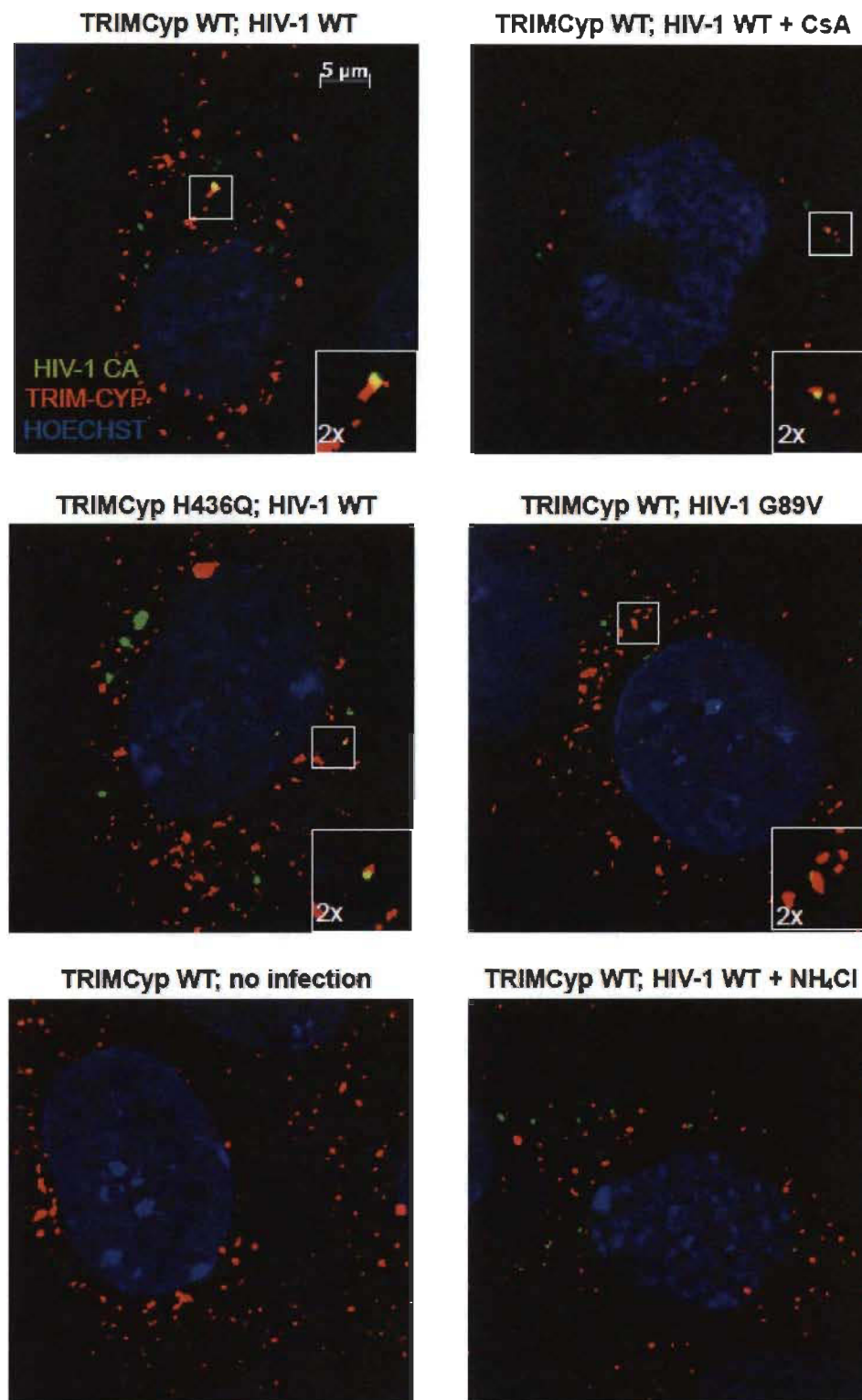
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**Figure 1** Pharmacological and mutagenic inhibition of Owl monkey omkTRIMCyp-mediated restriction of HIV-1.

(A) Murine MDTF cells stably expressing WT or H436Q omkTRIMCyp, as well as control cells transduced with the empty vector, were infected with multiple doses of VSV-G pseudotyped WT or CA-G89V HIV-1<sub>TRIP-CMV-GFP</sub>. Percentages of infected (GFP-expressing) cells were determined by FACS 48 hours post-infection. (B, C) MDTF cells stably expressing WT or H436Q omkTRIMCyp were infected in triplicates with a single dose of WT or G89V HIV-1<sub>TRIP-CMV-GFP</sub> in the absence or presence of 5  $\mu$ M of CsA (B) or 20 mM of NH<sub>4</sub>Cl (C). Infection yields were analyzed as above. ND indicates that we did not detect statistically significant levels of infected cells.

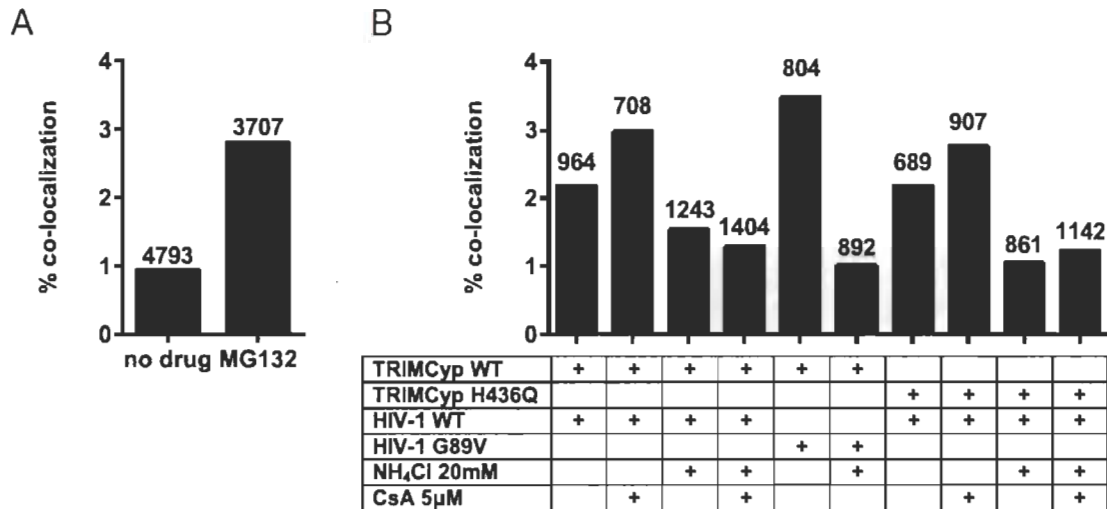


**Figure 2** Imaging of omkTRIMCyp and viral CA in MDTF cells shortly after exposure to an HIV-1 vector.

MDTF cells stably expressing WT or H436Q omkTRIMCyp-FLAG were infected for 6 hours with a single dose (MOI = 0.5) of WT or

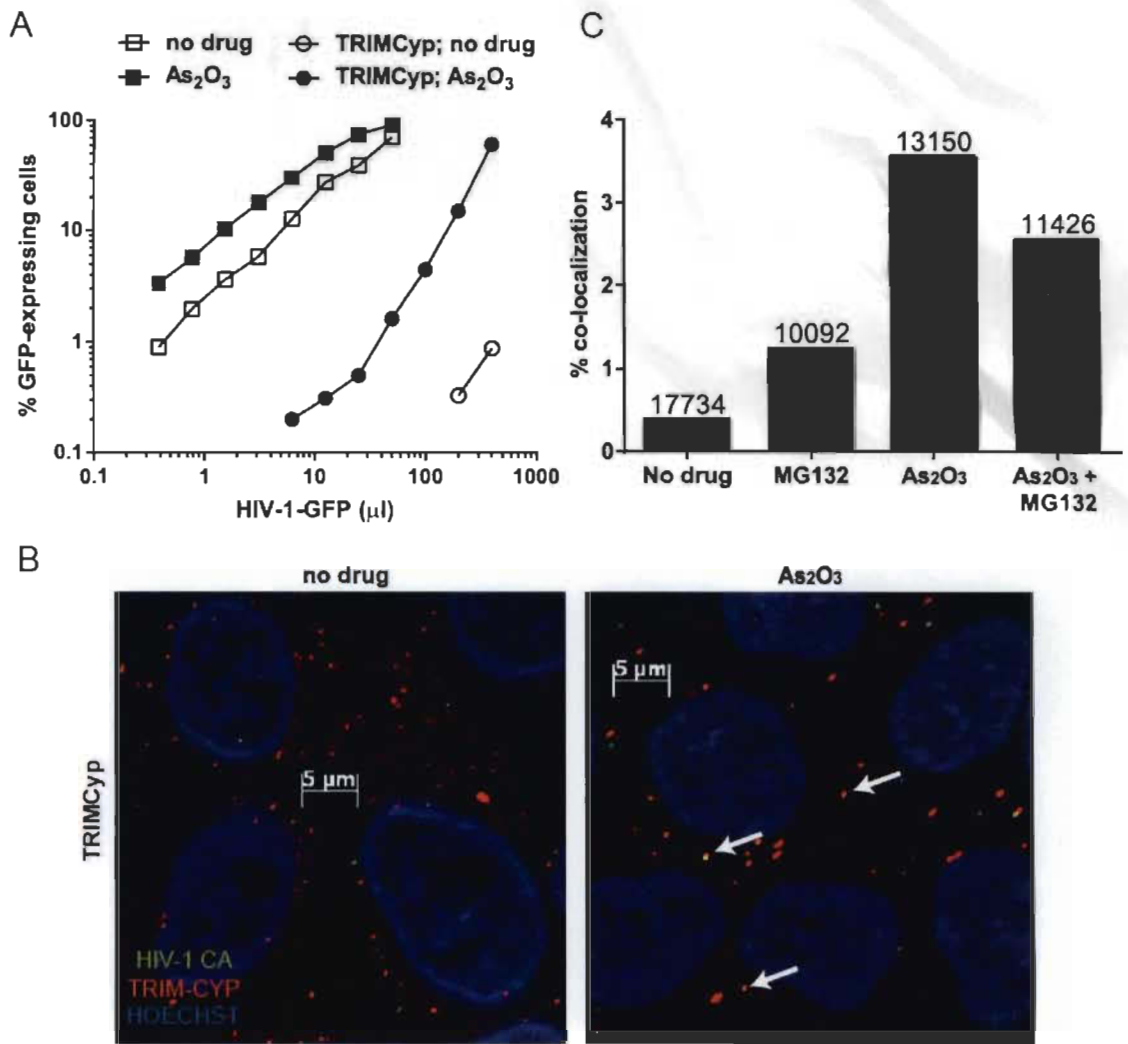


G89V HIV-1<sub>TRIP-CMV-GFP</sub>. Infections were done in the presence of CsA (5  $\mu$ M) or NH<sub>4</sub>Cl (20 mM) where indicated. Cells were fixed with formaldehyde 6 hours post infection and then stained with a monoclonal mouse antibody directed against HIV-1 CA (green) and a polyclonal rabbit antibody against FLAG (red). The cellular DNA was stained with Hoechst-33342 (blue). Representative IF microscopy images are shown. Enlarged areas show examples of omkTRIMCyp-CA co-localizations.



**Figure 3** Rates of omkTRIMCyp-CA co-localization in restricted and unrestricted acute infection of MDTF cells.

(A) MDTF cells expressing WT omkTRIMCyp were infected with HIV-1<sub>TRIP-CMV-GFP</sub> (MOI of 2), in the presence or the absence of MG132 (1 μg/ml). Cells were processed for IF microscopy as shown in Figure 2. The frequency of co-localization events was calculated as a % of the total number of discrete, visible CA foci in 20 randomly chosen fields. (B) MDTF cells expressing WT or H436Q omkTRIMCyp were infected with a single dose of WT or G89V HIV-1<sub>TRIP-CMV-GFP</sub> (MOI of 0.5) in the presence or absence of CsA (5 μM) or NH<sub>4</sub>Cl (20 mM). Cells were processed for IF microscopy as above. The frequency of co-localization events was calculated as a % of the total number of discrete, visible CA foci in 20 randomly chosen fields. Numbers on top of the bars represent the total amount of CA foci counted in the 20 fields analyzed.



**Figure 4** **As<sub>2</sub>O<sub>3</sub> treatment increases the frequency of omkTRIMCyp-CA co-localization in acutely infected HeLa cells.**

(A) HeLa cells stably expressing omkTRIMCyp, and control cells transduced with the empty vector, were infected with multiple doses of HIV-1<sub>TRIP-CMV-GFP</sub>. Infections were done in presence or absence of As<sub>2</sub>O<sub>3</sub> (15 μM) for 12 hours and the percentages of infected cells were analyzed by FACS at 48 hours post-infection. (B) Representative IF microscopy images of omkTRIMCyp-expressing HeLa cells infected for 6 hours with HIV-1<sub>TRIP-CMV-GFP</sub> (MOI of 2) and in the absence or the presence of As<sub>2</sub>O<sub>3</sub> (15 μM). Arrows show instances of omkTRIMCyp-CA co-localization. (C) The frequency of omkTRIMCyp-CA co-localization events in omkTRIMCyp-expressing HeLa cells infected for 6 hours with HIV-1<sub>TRIP-CMV-GFP</sub> (MOI of 2) with or without MG132 (1 μg/ml) and As<sub>2</sub>O<sub>3</sub> (15 μM). Co-localization events were calculated as in Figure 3.