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THÈSE présentée par :

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Importance des facteurs cellulaires LSD1 et HIC1 dans la restriction de l'expression du VIH-1 dans les cellules microgliales.

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lignes peuvent être assurés que j'aurais eu une pensée pour eux en les écrivant et c'est bien l'essentiel (si vous lisez ce passage fortuitement et que je ne vous connais pas ou que je ne vous aime pas, ça ne compte pas !). Néanmoins, je ferais une exception pour Damien, mon compagnon d'infortune (depuis nos aventures scientifiques en Suisses qui avaient déjà bien donné le ton de la suite…) qui m'aura, épaulé de A à Z. Merci à toi, bonne chance pour la suite mec ! Tu pourras toujours compter sur moi pour te renvoyer l'ascenseur !

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Liste des abbréviations :

AcK28/50 : acétylation du résidu lysine 28/50
ARM : Arginine Rich Motif
ARNm : ARN messager
ARNpolII : ARN polymérase II
ARV : Antirétroviraux
AZT : Azidothymidine
cART : Combination antiretroviral therapy
BTB/POZ : BR-C, ttk and bab/ Pox virus and Zinc finger
CDK9 : Cyclin-dependent kinase 9
CT1 : Cyclin T1
CtBP : C-terminal Binding Protein
COMPASS : Complex Proteins Associated with Set1
CTD : Carboxy terminal domain
CTIP2 : COUP-TF interacting protein 2
Gp160/120/41 : Glycoprotéine 160/120/41
H3K4me3 : Tri-méthylation de la lysine 4 de l'histone H3
H3K9me3 : Tri-méthylation de la lysine 9 de l'histone H3
HAT : Histones acetyl-transferases
HDAC : Histone déacétylase
HIC1 : Hypermethylated in cancer 1
HiRE : HIC1 Responsive Element
HMT : histone méthyltransférase
HP1 : Heterochromatin Protein 1
HPBP : Human Phosphate Binding Protein
INTI : Inhibiteurs nucléosidiques de la transcriptase inverse
INNTI : Inhibiteurs non-nucléosidiques de la transcriptase inverse
LSD1 : Lysine specific demethylase 1
LT CD4+ : Lymphocytes T CD4+
LTR : Long terminal repeat
miARN : micro ARN
NF-κB : Nuclear factor κB
NES : Nuclear export signal
NLS : Signal de localisation nucléaire
OMS : Organisation Mondiale de la Santé
PIC : Complexe de pré-intégration
P-TEFb : Positive transcription elongation factor-b
RRE : Reve responsive element
RTC : Reverse Transcription Complex
SIDA : Syndrome de l'immunodéficience acquise
SIRT1 : Sirtuin (silent mating type information regulation 2 homolog) 1
SIV : Simian immunodefiency virus

SNC : Système nerveux central
SUV39h1 : suppressor of variegation 3-9 homolog 1
TAR : Transactivation response element
TAT : Transactivateur de la transcription
VIH : Virus de l'immunodéficience humaine
VPR : Viral protein r

Introduction

A. Généralités

A1. La pandémie du SIDA.

Fin des années 1970, des médecins américains s'étonnent de la recrudescence de patients touchés par des infections pulmonaires à Pneumocistis Carinii et de la multiplication d'un rare cas de cancer de la peau, le sarcome de Kaposi.

Le Center for Disease Control (CDC) confirmera l'existence d'un problème sanitaire associé à une immunodéficience sévère en 1981, après avoir remarqué un nombre croissant de personnes immunodéprimées. Après quelques errances communautaristes, ce syndrome est renommé SIDA (syndrome de l'immunodéficience acquise) en 1982.

Peu de temps après, des cas de SIDA sont avérés un peu partout dans le monde, plus de 3000 seront recensés en 1983. Cette même année, un virus lymphotropique, le LAV (lymphadenopathy-associated virus), est identifié et étroitement associé avec le SIDA par les chercheurs de l'Institut Pasteur (Barre-Sinoussi et al., 1983) sans réussir à assurer un lien de causalité entre les deux. En 1984, par contre, le CDC affirme avoir enfin isolé l'agent étiologique du SIDA, le HTLV-III (humanT-cell lymphotropic virus) (Gallo et al., 1984). Il s'avérera que LAV et HTLV-III sont en fait un seul et même virus, renommé officiellement VIH (virus de l'immunodeficience humaine) en 1986 par le « international comittee on the taxonomy of viruses », mettant un terme aux luttes de paternité de l'identification du virus, entre laboratoires français et américains.

Cependant, même si la prise de conscience de l'épidémie trouve sa source aux Etats-Unis, l'origine du virus serait très probablement camerounaise. En effet, en 1999, des analyses phylogénétiques établissent un lien entre les groupes M, N et O du

VIH-1 avec le SIVcpz (simian immunodefiency virus), une souche infectant des chimpanzés sauvages, principalement retrouvés dans les forêts sud-camerounaise (Gao et al., 1999). Le VIH aurait donc dérivé d'une zoonose du SIVcpz à l'homme. Plus récemment, le gorille, et plus précisément le SIVgor, a été ajouté dans l'équation, comme étape intermédiaire évolutif entre chimpanzé et humain (Takehisa et al., 2009), et même comme source d'un nouveau VIH, le groupe P (Plantier et al., 2009). Le moment et l'endroit exact de cette zoonose restent des questions ouvertes et assujetties à controverses. Même si elles permettraient d'en savoir plus sur le virus et comment le combattre, répondre à ses deux questions peu aussi avoir l'effet pervers de stigmatiser une communauté de personnes en lui faisant porter la culpabilité de ce qui, en 2002, a été décrété comme pandémie par l'OMS.

A2. Le SIDA aujourd'hui

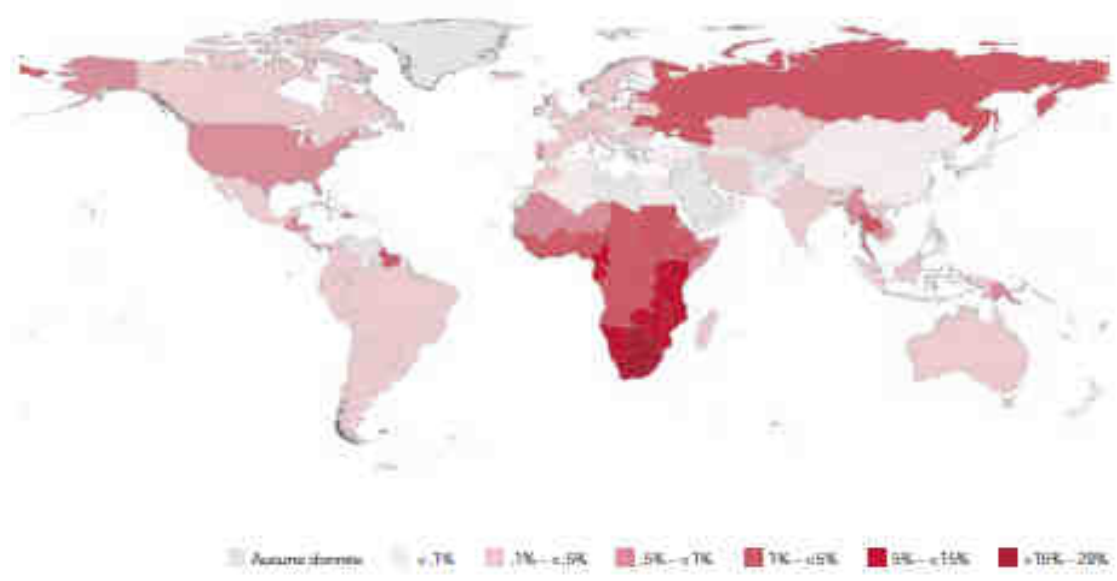


Figure 1: Prévalence du VIH dans le monde en 2009 **(Rapport ONUSIDA 2010)**

Nous voilà trente ans plus tard et les dernières données dressent le triste constat de 34 millions de personnes atteintes du VIH à travers le monde en 2010 et entre 2.4 et 2.9 millions de personnes nouvellement infectées cette même année (Figure 1) (données ONUSIDA 2011).

La pandémie continue, mais la situation va en s'améliorant depuis 1997, les nouvelles infections étant à la baisse, contrairement aux investissements. En 2011, 16.6 milliards de dollars ont été débloqués pour financer la lutte mondiale contre le SIDA contre 15 milliards en 2010. En juin 2011, les états membres se sont accordés sur la nécessité de mettre, chaque année, 24 milliards de dollars à disposition de la lutte mondiale et ce à compter de 2015 (Figure 2).

La France à promis une aide à hauteur de 1.4 milliards de dollars sur les trois prochaines années.

Des sommes d'autant plus considérables quand on considère le contexte de crise économique.

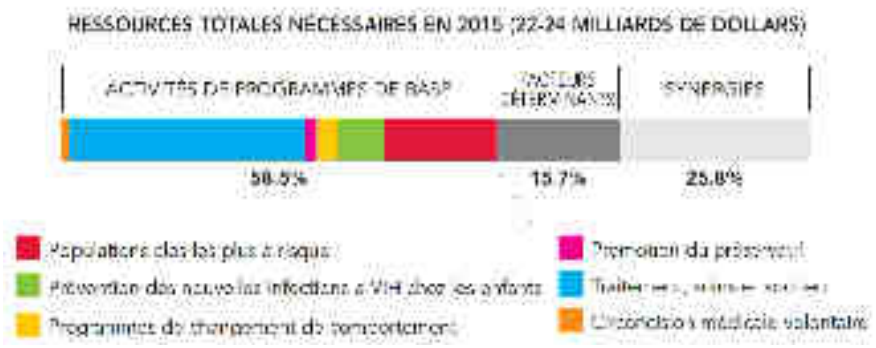


Figure 2: Plan financier de 2015 pour la lutte contre le SIDA **(OnuSIDA 2012)**

A3. Déroulement de la maladie

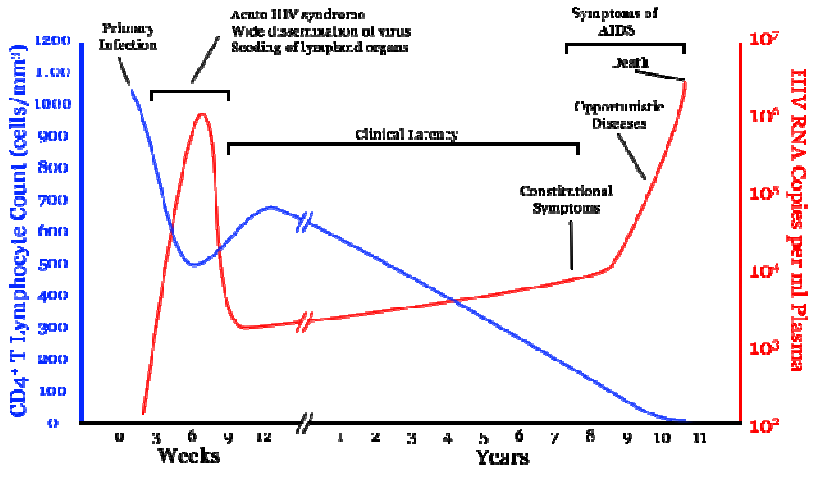


Figure 3: **Evolution de la maladie.** Au cours de la maladie, on observe une variation du taux de virus circulant (rouge) et du nombre de LT CD4+ (bleu), durant, dans l'ordre, la primo infection, l'infection aigue, la phase asymptomatique et enfin le stade SIDA. (Sanao/Licence Creative Commons)

La phase de primo-infection dure de 3 à 6 semaines et s'accompagne de symptômes proches de la mononucléose infectieuse. Durant cette phase, le virus se réplique de façon exponentielle, tout en entrainant la réduction du nombre de lymphocytes T CD4+ (LT CD4+), la cible privilégiée du virus, dans le plasma. (pour revue, Elspert et al., 2007)

Au terme de la primo-infection, le système immunitaire se redresse, la quantité de LT CD4+ remonte à un taux normal et la séroconversion s'amorce.
S'en suit une phase dite asymptomatique, d'une durée de 6 à 11 ans en l'absence de traitement, où le système immunitaire est renouvellé constamment et maintient la charge virale plasmatique à un faible niveau (Chun et al., 1997). L'existence d'une réplication résiduelle permet la production de virus laissant le système immunitaire dans un état d'hyperactivation constant. La persistance d'une inflammation chronique va être à l'origine du déclin progressif des LT CD4+. (Aziz et al., 1999 ; Aukrust et al., 1995 ; Breen et al., 1990 ; pour revue Lederman et Margolis, 2008)

Arrivé sous le seuil de 200 LT CD4+/ml, le système immunitaire n'est plus en mesure de répondre aux challenges des maladies opportunistes. Le patient, immunodéprimé, rentre en phase SIDA jusqu'à son décès.

A4. La vaccination au point mort

Les scientifiques ont pris l'habitude de rendre la politesse aux virus à grand coup de vaccins préventif et/ou curatif. Le VIH-1 ne déroge pas à la règle, et les stratégies conventionnelles de vaccination ont été déployées dès 1984 ; les premiers essais cliniques étaient alors attendus pour 1986/1987 (Brandt, 1984).

Malheureusement, le nombre élevé de groupes et sous-types de virus, ainsi que les méchanismes d'évasions déployés par le virus rendent la production d'un vaccin préventif très compliquée. Notamment, la protéine de surface, et plus particulièrement de la sous-unité gp120, cible privilégiée dans les stratégies vaccinales, présente des caractéristiques lui permettant de contourner les défenses immunitaires.
En effet, les épitopes potentiels sont masqués par des glycosylations, par des changements conformationnels rapides et du fait de la trimérisation de la protéine. A cela s'ajoute l'evolution rapide de la gp120 ainsi que l'apparition de nouveaux variants. Ainsi, il est difficile d'induire une réaction immunitaire capable de couvrir l'intégralité des variants, tandis que le trimère de gp120 a peu d'antigénicité. Ces deux phénomènes évolutifs sont inhérents à la haute variabilité du génome viral (pour revue, Burton et al., 2004).

La complexité lié à l'infection par le VIH, tant au niveau de son évolution rapide que de son action délétère sur le système immunitaire, pousse la recherche à ré-inventer la manière d'aborder la vaccination en mettant la communauté scientifique au pied du mur :
Le Pr Montagnier déclarait en mai 2012 « (le vaccin préventif) je n'y crois plus […] il y a besoin d'un mouvement pour révolutionner ou faire évoluer la médecine » (source : *France info)*.

Pendant ce temps, les campagnes de prévention et de dépistage ont permis de responsabiliser et de limiter le nombre de nouvelles infections, tandis que la recherche a déployé un éventail de molécules ciblant différentes étapes du cycle de réplication du virus. Les anti-rétroviraux (ARV) ont été introduits pour traiter les patients infectés en l'absence de vaccin, qui manque cruellement pour enrayer définitivement l'épidémie.

B. Le cycle réplicatif du VIH et les Anti-retroviraux.

B1. La particule virale

Le VIH est un virus de la famille des *Retroviridae* et du genre lentivirus. En tant que rétrovirus, le génome viral est porté par une une molécule d'ARN monocaténaire de polarité positive. Deux copies d'ARN sont recouvertes de la protéine de nucléocapside, p7.

L'association ARN/p7, ainsi que des protéines virales essentielles au cycle réplicatif, mais aussi des protéines d'origine cellulaire, sont enfermés dans une capside conique composée d'une oligomérisation d'environ 1500 protéines p24.

La capside elle même se trouve dans une cavité délimité par la protéine de matrice p17, protéine qui se fixe par l'intermédiaire de son extrémité N-terminale myristillée aux lipides de l'enveloppe virale.

L'enveloppe virale est une bicouche lipidique, provenant du bourgeonnement des virus depuis les cellules humaines infectées. Cette bicouche lipidique est traversée par 14 trimères de protéines d'enveloppes, formées d'une sous unité transmembranaire de gp41 et d'une sous unité de surface de gp120 (Clapham et al., 2001).

Le tout donnant une particule virale d'une taille s'échelonnant de 80 à 120nm (pour revue Ganser-Pornillos et al., 2008 ; Pornillos et al., 2012) (Figure 4)

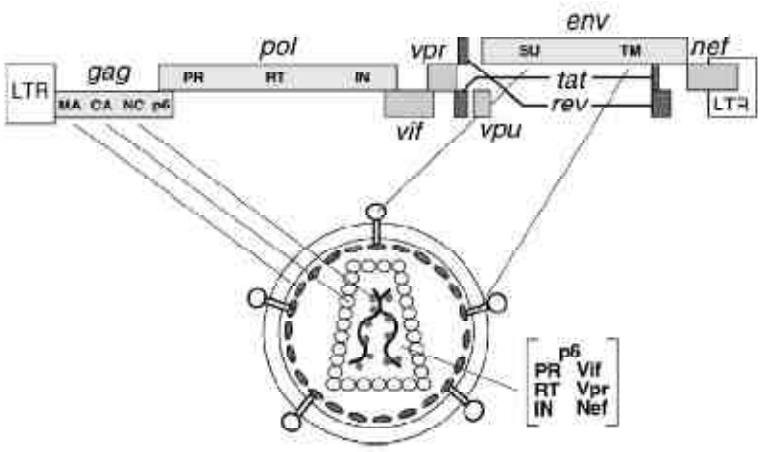


Figure 4 : Organisation du génome et de la particule virale. (Frankel et Young, 1998)

B2. Le génome viral

L'information génétique du virus est portée par deux copies d'une molécule monocaténaire d'ARN à polarité positive d'environ 9,7 kB chacun. Les retrovirus ont la particularité de retro-transcrire la molécule d'ARN encapsidée en une molecule d'ADN bicaténaire grâce à la transcriptase inverse virale, incluse dans le virion.

Une fois retro-transcrit la molécule d'ADN est flanquée de deux régions non codantes identiques en 5' et 3', appelées séquence LTR (Long Terminal Repeat). Ces régions participent à l'intégration du génome viral dans l'ADN de la cellule hôte et encadrent 9 gènes viraux, régulés par l'activité promotrice dans le LTR en 5'.

Les gènes *Gag*, *Pol* et *Env* codent pour les protéines fondamentales. Chacun d'eux donnera naissance à des polyproteines qui seront ensuite clivées soit par la protéase virale p11, dans le cas des précurseurs GAG et POL, soit par la protéase cellulaire furine, dans le cas de la polyprotéine ENV. (Figure 4)

Autour du gène *Env* se trouve des gènes codant pour des protéines régulatrices, *Tat* et *Rev*, et pour des protéines accessoires (ou auxiliaires), *Vif*, *Vpr*, *Vpu* et *Nef*. Ces gènes sont transcrits par décalage du cadre de lecture (pour revue, Frankel et Young, 1998 ; Tang et al., 1999).

Le génome viral comporte aussi des séquences non-codantes. Les LTRs, déjà abordées précédemment, la séquence CTS (Central Terminaison Signal), riche en AT, qui permet d'achever la transcription inverse et la séquence RRE (REV Responsive Element) impliquée dans la transcription et l'export des ARN messager (ARNm) viraux.

B3. Le cycle de réplication

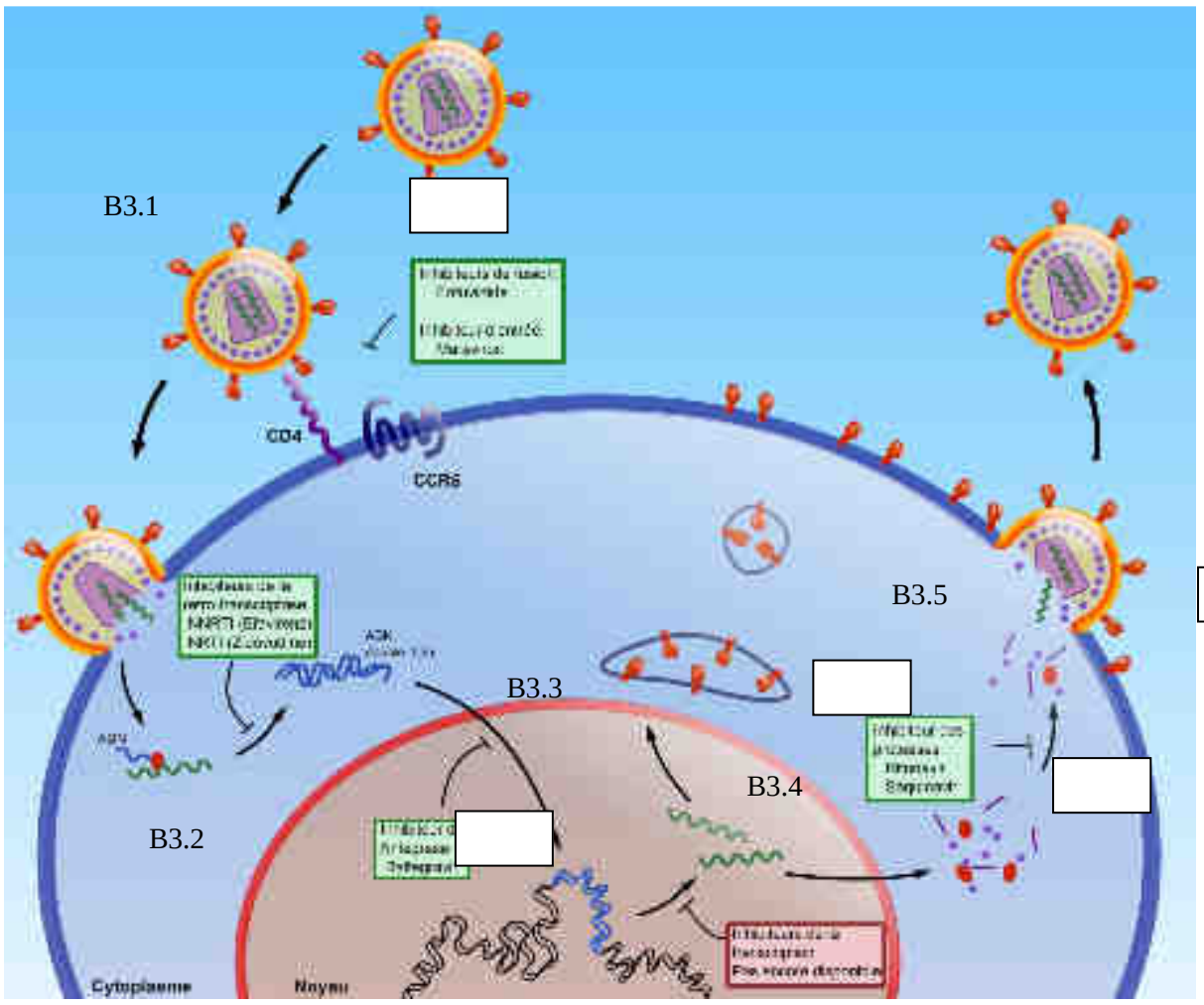


Figure 5: Les étapes du cycle de réplication du virus. Le cycle viral se décompose en différentes étapes : l'entré du virus (chapitre B3.1), la transcription inverse (chapitre B3.2), l'intégration (chapitre B3.3), l'expression du provirus et la production des protéines virale (chapitre B3.4), l'assemblage de la particule virale, l'encapsidation des deux copies d'ARNv, le bourgeonnement de nouveaux virions et leur maturation (chapitre B3.5). Des molécules sont capables de cibler certaines étapes de ce cycle et sont actuellement utilisées lors des multi-thérapies. (Source personnelle)

B3.1. L'entrée du virus

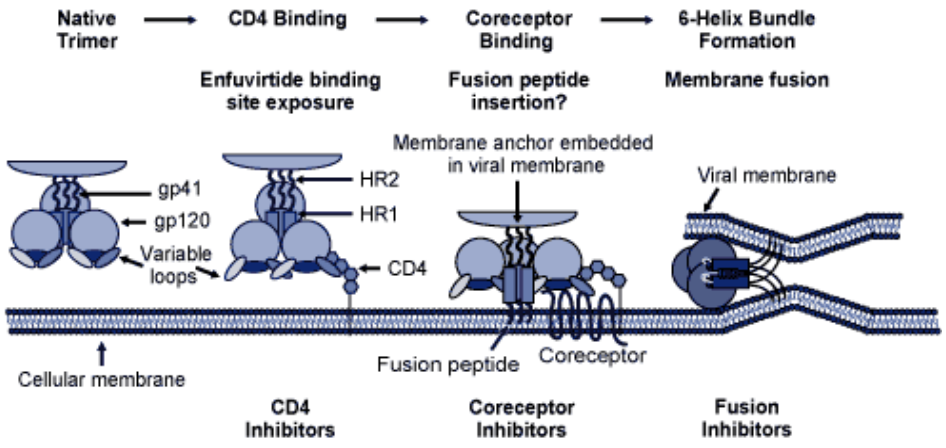


Figure 6: Les interactions séquentielles des récepteurs cellulaires/ récepteurs viraux (Doms, 2012)

La sous-unité gp120 initie le processus d'entrée du VIH-1 en se fixant aux récepteurs CD4, présents à la surface des lymphocytes et des macrophages. Cette fixation révèle la boucle hypervariable V3 de la gp120. Suivant le tropisme viral, cette boucle porte un site de fixation au co-récepteur à chémokine CCR5 ou CXCR4. La fixation simultanée de la gp120 au récepteur et au co-récepteur engage la sous unité gp41 dans le processus d'entrée. La partie N-terminale de la gp41, appelé le peptide de fusion, s'insère alors dans la membrane de la cellule hôte. Un repliement subséquent de la gp41 entraine le rapprochement des membranes cellulaire et virales et aboutit à la fusion des deux bicouches lipidiques (Willen et al., 2012). (Figure 6)

La compréhension de ce mécanisme à permis la mise sur le marché de l'Enfuvirtide®, ou T20. Cet analogue du peptide de fusion, avec qui il entre en compétition, empêche l'étape de repliement de la gp41 et par voie de conséquence l'entrée du virus dans la cellule hôte. Bien qu'élégante, cette approche est facilement mise à mal par l'évolution rapide du virus. En effet, quelques mutations de la gp41 suffisent à rendre le T20 inefficace (Carmona et al., 2005). De plus, le T20 doit être administré par voie intra-veineuse pour atteindre une efficacité optimale, ce qui en fait un protocole lourd pour les patients. Du fait de ces limitations, la pérennité de cette stratégie a été remise en cause par Roche en 2010 avec l'abandon du

développement du T1249, inhibiteur de fusion de seconde génération (Eggink et al., 2009 ; pour revue, Berkhout et al., 2012)

Une autre molécule, le Maraviroc® se fixe sur le co-récepteur CCR5 pour empêcher l'interaction gp120/CCR5. Malheureusement, des résistances à cette molécule peuvent apparaitre du fait de l'hypervariabilité de la boucle V3. (Yuan et al., 2011 ; pour revue Gilliam et al., 2011).

B3.2. La transcription inverse.

Une fois entrée, la capside du virus est acheminée vers le noyau grâce au réseau de microtubules de la cellule hôte (Arhel et al., 2006). Pendant le trajet, le RTC (Reverse Transcription Complex) commence la transcription inverse de l'ARN viral. L'ARNt lys 3, préalablement intégré dans la capside, sert d'amorce à la transcriptase inverse. A l'issu de l'étape de transcription inverse, le génome viral est converti en ADN bicaténaire linéaire, encadré par les LTRs et contient un chevauchement d'ADN dans le centre du brin codant. Du fait de ce chevaument, l'ADN viral nouvellement forme est appelé ADN flap. La synthèse de l'ADN flap et l'arrivé à proximité du noyau, servent de signal à la décapsidation, privant le RTC de la p24 qui va dès lors évoluer en complexe de pré-intégration (PIC) (Telesnitsky et al., 1997 ; Zennou et al., 2000 ; Arhel et al., 2007 ; pour revue Nisole et Saïb, 2004). (Figure 7)

Etape clé du cycle réplicatif, la transcription inverse a rapidement été la cible d'ARV. On distingue deux catégories de molécules ciblant la transcriptase inverse : les inhibiteurs nucléosidiques et non-nucléosidiques de la transcriptase inverse (respectivement INTI et INNTI).

Les INTIs font office d'initiateurs de la lutte anti-VIH basé sur les ARV. En effet, l'azidothymidine (AZT) a été la première molécule anti-retrovirale mise sur le marché. Le principe des INTIs repose sur la terminaison précoce de l'étape de transcription inverse. Les INTIs sont des analogues des nucléotides cellulaires privés

d'extrémité 3'OH sur le motif désoxyribose. Une fois incorporés à la chaine de nucléotides en formation, ils font office de terminateurs et la transcriptase inverse est dans l'incapacité de poursuivre la synthèse du brin d'ADN (pour revue Vivet-Boudou et al., 2006)

Les INNTIs sont quant à eux des inhibiteurs non-compétitifs de la transcriptase inverse. Les modifications conformationnelles de la transcriptase induites par la fixation d'un INNTI entrainent une perte d'affinité de l'enzyme pour les nucléotides. Pour palier l'emergence de variants résistants aux INNTIs de première génération, comme l'Efavirenz®, les molécules de seconde génération, comme l'Etravirine®, ont une flexibilité structurelle accrue, qui leur permet de passer outre une partie des mutations apparaissant dans la transcriptase inverse (Minuto et al., 2008 : pour revue Sarafianos et al., 2009).

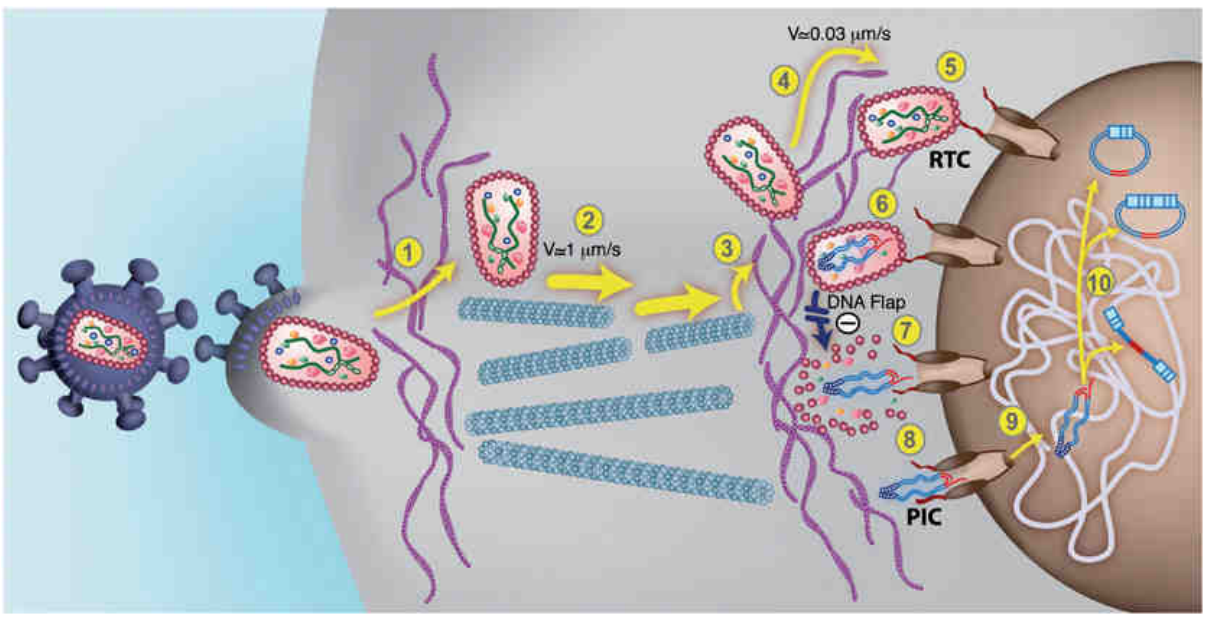


Figure 7: Migration du RTC vers le noyau et décapsidation. La capside virale est prise en charge par le système de microtubules de la cellule infectée. La capside migre vers le noyau et le RTC (Reverse Transcription Complex) réalise la transcription inverse, qui permet la synthèse de l'ADN viral, l'ADNflap. A l'issue de la migration au noyau, la capside est destabilisée, le RTC évolue en PIC (Pre-Integration complex) (Arhel et al., 2007)

B3.3. L'intégration

Des protéines virales, membres du PIC, permettent l'import de l'ADN flap dans le noyau, et ce par des voies multiples et redondantes. La protéine VPR (viral protein r) peut importer l'ADN viral soit en interagissant avec des nucléoporines soit en destabilisant la membrane nucléaire (de Noronha et al., 2001 ; pour revue Morellet et al., 2009), tandis que l'intégrase et p17, par l'intermédiaire de leur signal de localisation nucléaire (NLS) permettent l'entrée du complexe ADN/PIC en liant l'importine-α (Figure 8) (Haffar et al., 2000 ; Ao et al., 2010). Une fois dans le noyau, l'intégrase, liée aux extrémités de l'ADN flap, va, par attaque nucléophile, rabotter les LTRs de deux nucléotides et cliver l'ADN de la cellule hôte. Cette étape est facilitée par des protéines cellulaires membres du PIC, telle que LEDGF et HMG-I (pour revue Delelis et al., 2008). Le chevauchement dans l'ADN flap est ensuite corrigé par la protéine cellulaire, FEN1 (Rumbaugh et al., 1998).

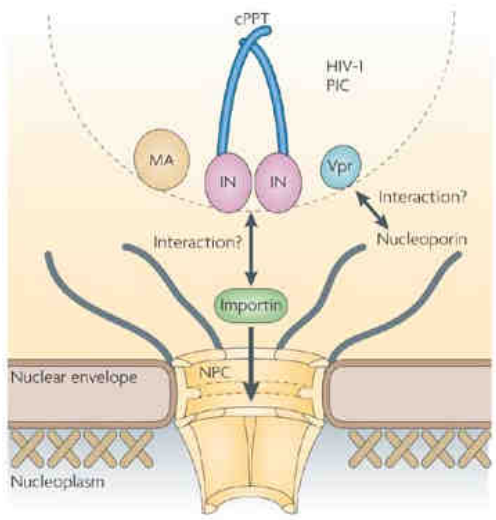


Figure 8: Mécanisme d'import du PIC dans le noyau de la cellule infectée. Vpr intéragit avec les nucléoporines, tandis que l'intégrase peut intéragir avec l'importine, ce qui permet la migration du PIC dans le noyau de la cellule hôte. (Suzuki et Craigie, 2007)

L'unique inhibiteur de l'intégrase que l'on peut considérer sur le marché, est le Raltégravir®. Mis sur le marché depuis 2007, les effets sur le long terme n'ont pas

encore pu être totalement évalués, même si des études commencent à émerger et à pointer du doigt des effets secondaires, notamment des problèmes rénaux ou un rare syndrome de Stevens-Johnson (Vassallo et al., 2012). La seule alternative, l'Elvitégravir®, n'est proposé qu'aux patients en échec thérapeutique, bien qu'ayant un profil de résistance identique au Raltégravir®. La barrière génétique des ces ARV est faible, une seule mutation est délétère, ce qui les rend sensibles à l'échappement virologique (Markowitz et al., 2007 ; Quercia et al., 2009).

L'avenir se tourne maintenant vers les inhibiteurs de deuxième génération, comme le Dolutégravir®, actuellement en phase clinique III, qui s'avère efficace sur les virus résistants au Raltégravir® et l'Elvitegravir®. Des recherches sur des inhibiteurs de l'interaction intégrase/LEDGF sont actuellement en cours. (pour revue Quashie et al., 2012)

B3.4. la transcription virale

A ce stade débute la phase post-intégrative. Le génome viral intégré, ou provirus, détourne la machinerie transcriptionnelle cellulaire afin de produire les transcrits viraux, en deux étapes distinctes, la phase précoce et la phase tardive.

La phase précoce

Dans un premier temps la transcription des gènes est dépendante des facteurs de transcription cellulaires. Lors de l'activaton des LT CD4+, les facteurs activateurs NF-κB et NFAT migrent dans le noyau et se fixent dans la région LTR du provirus sur leurs motifs de reconnaissances respectifs. Par la suite, les facteurs NF-IL6, CREB, USF et Ets sont recrutés pour l'activation complète de la transcription.

Dans les cellules microgliales, la fixation seul de NF-κB semble suffisante pour activer la transcription. (Kinoshita et al., 1998 ; Barboric et al., 2001 ; pour revue Rohr et al., 2003). Pendant cette phase la vaste majorité des transcrits produits sont courts du fait de la déstabilisation précoce de l'ARN polymérase II (ARNpolII) (Kao et al.,

1987 ; Kessler et Mathews, 1992). Les quelques transcrits complets produits sont ensuite multi-épissés, migrent dans le cytoplasme et mènent à la synthèse des protéines virales régulatrices TAT et REV. Quand les quantités de ces deux protéines sont suffisamment élevées, la phase tardive de transcription s'amorce.

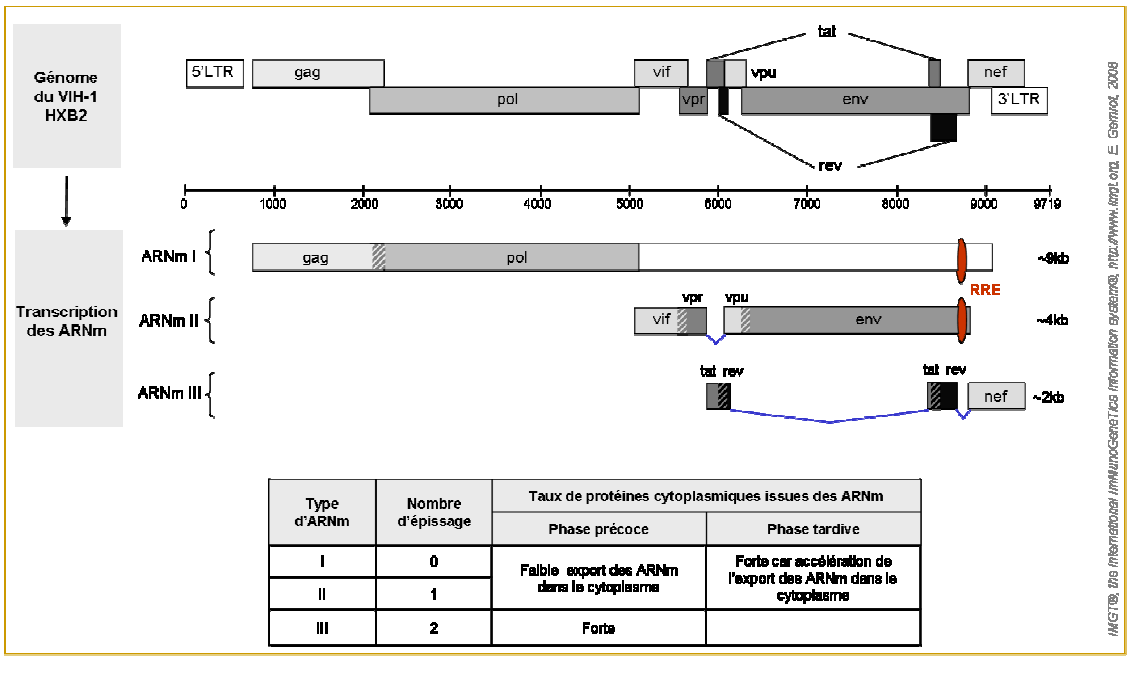
La phase tardive REV- et TAT-dépendante

Au début de l'étape de transcription, le transactivateur TAT se fixe à l'ARN viral naissant au niveau d'une séquence en structure tige-boucle, la TAR (Trans-Activation Responsive element) (Berkhout et al., 1989). TAT recrute et agit comme intermédiaire entre P-TEFb, acteur majeur dans la transcription des gènes de classe II, et l'ARNpolII (Zhu et al., 1997).

L'activité kinase de la sous-unité CdK9 de P-TEFb agit sur l'ARNpolII, d'une part en augmentant sa processivité, en phosphorylant les résidus du domaine C-terminal, et d'autre part en augmentant sa stabilité, en phosphorylant les facteurs négatifs de l'élongation NELF et NSIF. (Price et al, 2000 ; Ping et Rana, 2001 ; Zhang et al.,2007). La phase d'élongation favorisée, des transcrits complets du génome viral sont produits.

Tout comme TAT, REV peut se fixer avec une haute affinité à une structure tige boucle dans l'ARN viral, le site RRE, situé dans le gène *Env* (Malim et al, 1989 ; Mann et al., 1994 ; Watts et al., 2009). REV comporte un site site NES (Nuclear Export Signal) et un site NLS, qui vont interagir respectivement avec le récepteur d'export CRM1 (Chromosome maintenance region 1) et l'importine-β (Henderson et al. 1997 ; pour revue Köhler et Hurt., 2007). Une fois fixé sur le RRE, REV va augmenter la stabilité des transcrits et permettre leur export hors du noyau, en contournant les mécanismes cellulaires de rétention des transcrits n'ayant pas achevés leur épissage (Figure 9).

A partir des transcrits mono- ou non épissés, les dernières protéines virales sont produites.



Type d'ARNm	Nombre d'épissage	Taux de protéines cytoplasmiques issues des ARNm	
		Phase précoce	Phase tardive
I	0	Faible export des ARNm dans le cytoplasme	Forte car accélération de l'export des ARNm dans le cytoplasme
II	1		
III	2	Forte	

Figure 9: Les différents types ARNm produits selon leur niveau d'épissage. Le génome viral permet la production d'ARNm multi-épissés (ARNm III) pendant la phase précoce. Pendant la phase tardive, la protéine REV se fixe sur la structure d'ARN RRE (en rouge) et permet l'export des ARNm non-épissés et mono-épissés (respectivement ARNm I et ARNm II). (Korber et al., 1998)

Aucune molécule n'est pour l'instant disponible sur le marché pour interférer avec cette partie du cycle réplicatif.

B3.5. L'assemblage, bourgeonnement et maturation.

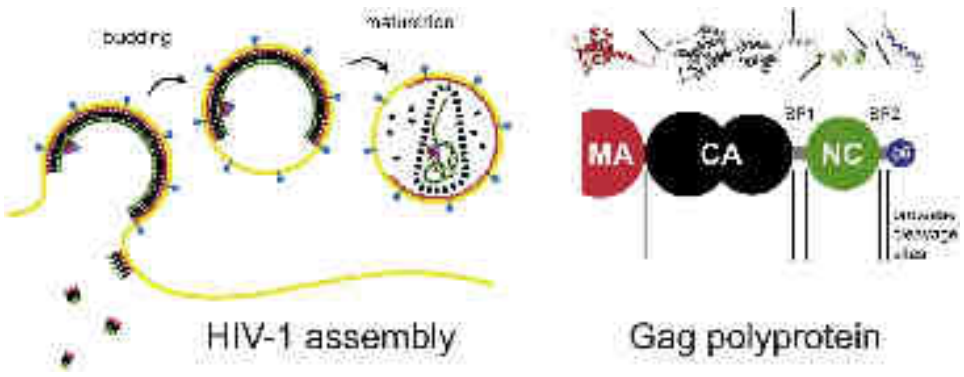


Figure 10 : Du bourgeonnement à la maturation de la particule virale. Gauche : bourgeonnement suivie de la maturation Droite : Les sites de clivages dans le précurseurs GAG sont ciblés par la protéase virale lors de la maturation. (Briggs et Kraeusslich, 2011)

Les transcrits mono- et non-épissés servent de matrice à la production des polyprotéines ENV, GAG et GAG-POL.

La polyprotéine ENV est clivée en gp120 et gp41 par la furine dans l'appareil de Golgi pendant son transport vers des rafts lipidiques de la membrane cytoplasmique (Hallenberger et al., 1997). Parallèlement, les précurseurs GAG et GAG-POL migrent et oligomérisent au niveau de la face interne de la membrane plasmique, sous ces mêmes rafts (Ono et Freed, 2001 ; pour revue Ono 2010 ; Chazal et Gerlier 2003). La concentration croissante de GAG-POL sous-membranaire, environ 1500 copies, induit une courbure de la membrane qui amorce bourgeonnement viral (figure 10) (Provitera et al., 2001). GAG-POL, par son domaine p6, achève le processus en recrutant des protéines cellulaires impliqués dans le transport vésiculaire (Garrus et al., 2001).

La particule virale produite est qualifiée d'immature jusqu'à ce que la protéase virale, p11, clive GAG/GAG-POL en protéines de structure (Figure 9)(Ross et al., 1991 ; Wiegers et al., 1998). Une fois maturée, la particule virale devient infectieuse et peut recommencer un cycle réplicatif (pour revue Bieniasz, 2009)

Introduit en 1995, les inhibiteurs de p11, comme le Saquinavir®, première molécule de la gamme disponible, ont pour but d'empêcher la formation et la maturation de la particule virale (Kitchen et al., 1995). Ces inhibiteurs sont couramment utilisés dans les multi-thérapies, bien qu'ayant une biodisponibilité sous-optimale, notamment dans le cerveau et le testis, et de nombreux effets indésirables (Kim et al., 1998 ; Huisman et al., 2001 ; Gohsn et al., 2004)

Une molécule alternative, le Bevirimat®, inhibe la maturation du précurseur GAG en entravant le site de coupure de la p24. Le développement de cet inhibiteur, facilement mis à mal par des mutations dans le site de clivage, a depuis été abandonné (Lu et al., 2011).

B4. Les anti-rétroviraux ne permettent pas d'éradiquer le virus.

Les ARV ont pour objectif de réduire la charge virale au dessous des seuils de détectabilité (<50 copies d'ARN viral/ml) et de maintenir le taux plasmatique de LT CD4+ au dessus des 500 cellules/ml. Grâce aux traitements, la période asymptomatique et l'espérance de vie des patients sont conjointement prolongées, passant d'environ 10 ans en absence de traitement à environ 45 ans sous ARV (May et al., 2011).

Les traitements sont pris sous forme de combinaisons de plusieurs ARV de différentes classes, on parle de multi-thérapie ou cART (combination antiretroviral therapy). En général, le traitement de première ligne consiste en une association de deux INTIs avec soit un inhibiteur de la protéase soit un INNTI. Ces multi-thérapies ont gagné en confort, en se limitant désormais à un comprimé unique quotidien, contre 25 par jour au début des protocoles (Sterrantino et al., 2012).

Mais là où le bât blesse, c'est que la cART ne guérit pas de la maladie, elle permet uniquement de la contrôler et en fait une maladie chronique. Cette chronicité impose au patient une observance à vie de son traitement, dont le coût est estimé à hauteur de 10.000€/an dans les pays occidentaux, où l'accès aux ARV est facilité (communiqué de presse GBI Research du 04/05/2012 – 12829$/an en 2010). On regrettera ainsi que plus de la moitié des personnes infectées dans le monde n'aient pas les moyens d'être traité, même avec la production de médicaments génériques.

De surcroit, l'observance des traitements doit être stricte. En effet, en cas d'interruption, on observe rapidement un rebond de la virémie, une chute du taux de LT CD4+ et la possibilité d'émergence de virus résistants qui rendent le traitement en cours obsolète. Dans ce dernier cas de figure, on parle d'échappement thérapeutique ou virologique. (Harrigan et al., 1999 ; Zhang et al., 2000)

B4.1. Echappement thérapeutique

L'échappement provient de l'apparition de nouveaux variants, résistants aux traitements, d'où l'intérêt du couplage de plusieurs molécules dans les thérapies pour limiter ce phénomène. La poussée de ces variants est le résultat d'une retro-transcriptase peu fidèle, avec un taux d'erreur estimé d'une toutes les 10.000 paires de bases, soit 1 mutation dans le génome de chaque virions nouvellement produit (Mansky and Temin., 1995).

C'est paradoxalement la fidélité médiocre de la reverse transcriptase qui va permettre au virus d'évoluer rapidement. La chute de fitness viral éventuelle étant largement compensée par la capacité de perdurer dans l'organisme.

Les données de l'OMS indiquent que 6% des patients ont une pharmaco-résistance avant les traitements de premières lignes, 10% sont en échec virologique après un an de traitement, de ces 10%, 4% restent en échec en passant sur un deuxième régime anti-rétroviral et, enfin, 69% des patients présentent des résistances détectables, mais ne conduisant pas à un échec thérapeutique (Jordan et al., 2011).

Nonobstant, chez les patients répondeurs, des techniques de détection ultra-sensible révèlent une virémie résiduelle (Palmer et al., 2003). Le déclin de la quantité d'ARN viral plasmatique amorcé par les traitements ne concrétise pas l'éradication totale du virus.

Cette réplication de fond peut s'expliquer par la présence de virus dans des zones de l'organisme où la biodisponibilité des ARV est moindre (les réservoirs anatomiques) ou par résurgence, ou « blips de réplication », provenant de cellules infectées de façon latente (les réservoirs cellulaires) (Rong et Perselson, 2009 ; Conway et Coombs, 2011).

Les deux phénomènes ne sont pas exclusifs, mais amènent à des considérations différentes vis-à-vis des ARV. Dans le premier cas, l'amélioration de la pénétration des molécules dans les réservoirs solutionnera ce problème, tandis que dans le second cas, les ARV seuls ne permettront jamais d'atteindre la guérison des patients, le virus latent y étant virtuellement insensible.

C. Réservoir et latence

Les virus issus de la réplication résiduelle ne présentent pas de signes notables d'évolution dans leur génome (Hermankova et al., 2001). Ces observations corroborent la ré-apparition de souches sauvages lors de la levée des traitements (Wong et al., 1997). La réapparition de ces virus sauvages, pourtant inadaptés à l'environnement thérapeutique, s'explique par la présence de réservoirs viraux.

C1. Les réservoirs anatomiques

Les réservoirs anatomiques, ou sanctuaires viraux sont définis comme des zones à immunologies privilégiées, où le virus aura une cinétique de réplication plus stable que celle des virus réplicativement actif dans le reste de l'organisme (Blankson et al., 2002).

Des barrières anatomiques séparent les sanctuaires viraux du sang et des organes lymphoïdes, réduisant la diffusion des ARV dans ces sites (Solas et al., 2003). Cette particularité permet au virus de poursuivre une réplication résiduelle et d'entretenir un état d'inflammation permanent des tissus.

On distingue trois principaux réservoirs :

Le tractus génital et le système nerveux central (SNC), isolés respectivement par la barrière hémato-testiculaire et la barrière hémato-encéphalique et les organes lymphoïdes (principalement le tractus digestif), lieu de résidence des lymphocytes T à mémoire.

C2. Les réservoirs cellulaires

L'hypothèse de l'existence de réservoirs viraux cellulaires latents a rapidement été validée. Dans les LT CD4+ quiescents, le nombre de cellules infectée de façon latente est estimé à une cellule par million (Finzi et al., 1997 ; Chun et al., 1997). Ces

réservoirs proviennent soit de l'infection directe de lymphocytes T mémoire, soit de l'infection de LT CD4+ activés, en court d'évolution vers la quiescence. (Figure 11)

La persistance et le métabolisme ralenti des LT CD4+ mémoire, en font un réservoir de virus non productif de longue durée.

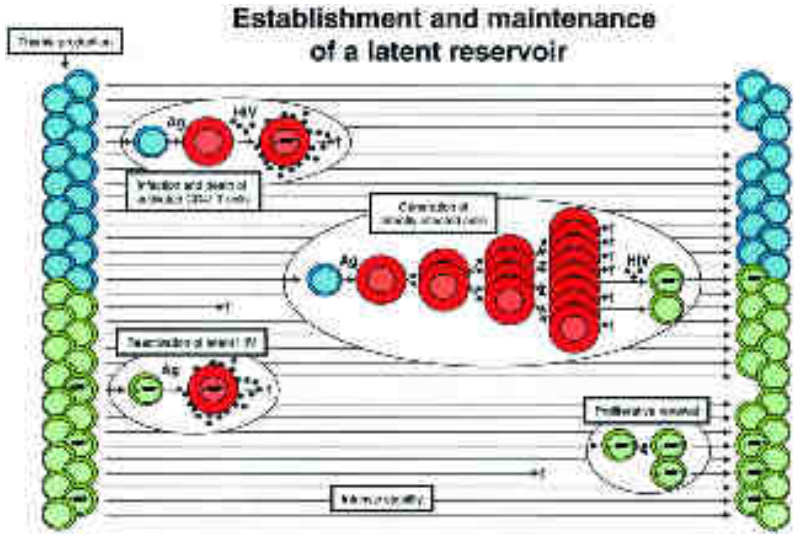


Figure 11: Etablissement et maintien du réservoir cellulaire LT CD4+. Les cellules T naïves (bleues) peuvent se différencier en lymphocytes T activés (rouges). La part de LT CD4+ ne succombant pas des effets cytopathiques de l'infection peuvent évoluer en cellule mémoire (vertes), quiescentes, qui renouvellent le stock de cellules réservoirs. (Persaud et al., 2003)

Par ailleurs, des analyses génétiques, consécutivement à un rebond virémique après interruption des traitements ARV, indiquent que les LT CD4+ ne sont pas l'unique zone d'archivage de virus latent (Bailey et al., 2006 ; Chomont et al., 2009). Fondé sur ces observations, les cellules de la lignée monocyte/macrophage ont été proposées comme source de latence virale à leur tour (Figure 12). En effet, la réplication y est possible et ces cellules peuvent persister dans l'organisme pour de longues périodes.

Pour plus de détails cf Annexe- publication 5 (Le Douce et al., 2010).

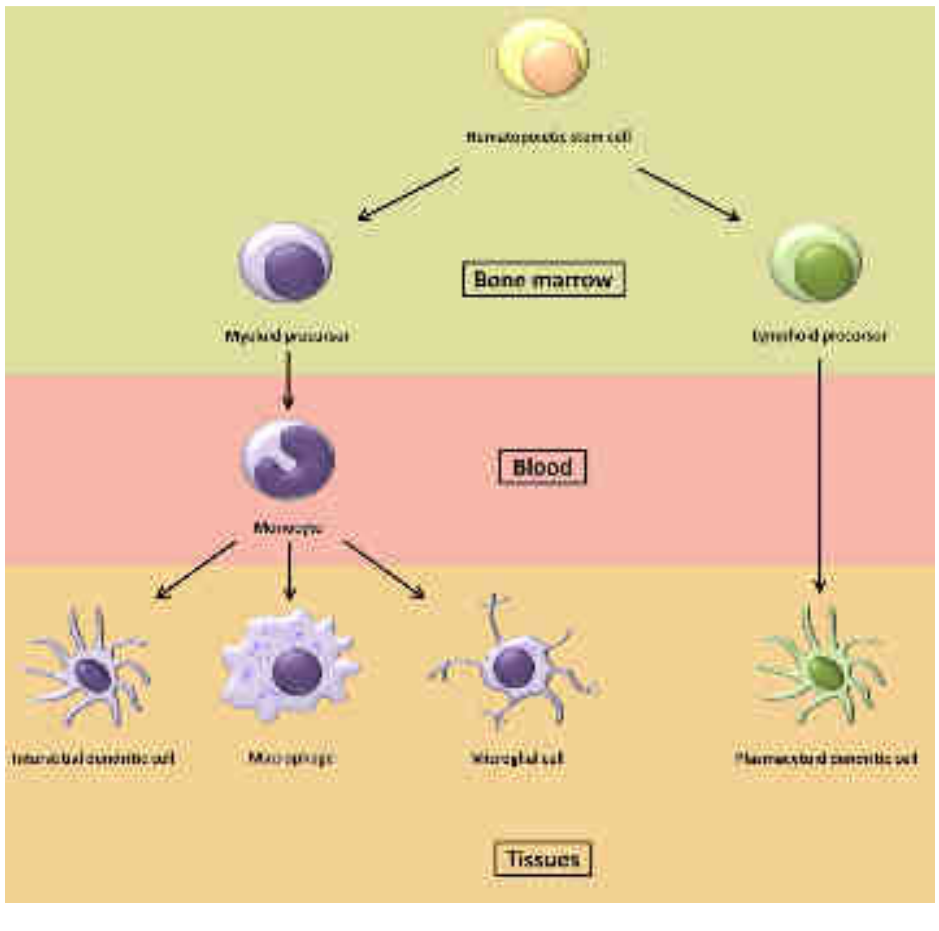


Figure 12: **Cellules de la lignée monocyte/macrophage.** Issue d'une cellule progénitrice hématopoïetique, les cellules de cette lignée compte comme représentantes les cellules dendritiques interstitielles et plasmacytoïdes, les cellules microgliales et les macrophages. (Le Douce et al., 2012)

C3. Les cellules microgliales

Les cellules microgliales sont des macrophages résident du SNC, capables de proliférer *in-situ* et perdurant pendant la durée de vie de l'individu (Suh et al., 2005). Ces cellules, cibles principales du VIH dans le SNC, sont infectées très précocement pendant la phase aiguë de la maladie (He et al., 1997 ; Jordan et al., 1991). La réplication virale y est ensuite rapidement stoppée, provoquant l'entrée en latence du virus et faisant des microglies le principal réservoir cellulaire cérébral (Davis et al., 1992 ; Barber et al., 2006).

Dans les dernières étapes de la maladie, l'inflammation provoquée par le rebond virémique réactive ce réservoir et le nombre de microglies productivement infectées augmente drastiquement (Cosenza et al., 2002). Le nombre de macrophages

cérébraux activés est par ailleurs étroitement lié au syndrome de démence induite par le VIH en phase SIDA (Glass et al., 1995).

C4. Les nouvelles stratégies et traitements de demain

Les ARV, aussi efficace soient-ils, accusent clairement de leurs limites. Echappement viral, sanctuaire anatomique et latence virale, pour n'en citer que trois, font que leur utilisation sur le long le terme ne sera pas la réponse pour aboutir à la guérison.

La nécessité de développer de nouvelles stratégies, complémentaires des ARV ou non, n'est plus à démontrer à ce stade. (pour revue, *Annexe-publication 7, Le Douce et al., 2012*)

C4.1. La thérapie génique

Pour l'heure il n'existe qu'un cas observé de guérison, le « patient de Berlin ». Séropositif et atteint de leucémie, il subit une greffe de moelle osseuse provenant d'un donneur homozygote pour la mutation delta 32 du récepteur CCR5, rendant le récepteur inexploitable par le virus lors de l'étape d'entrée (Hutter et al., 2009). A la suite de cette thérapie audacieuse, le niveau d'ARN viral plasmatique devient indétectable, son pool de LT CD4+ est restauré et une population de macrophages, résistante à l'infection, a remplacé l'ancienne.

En juin 2012, lors de la conférence « Challenges inherent in detecting HIV persistence during potentially curative interventions : a study of the Berlin patient», le Pr Yukl présente les résultats croisés de plusieurs laboratoires indiquant que l'ARN et l'ADN du virus sont à nouveau détectables, respectivement dans le plasma et dans le rectum du « patient allemand ». Parmi les hypothèses émises, une nouvelle infection, court-circuitant la protection allouée par le CCR5 Δ32 ou une résurgence depuis des réservoirs viraux.

Bien que remise en cause, mais pas encore réfutée, et surtout non-applicable à grande échelle, la méthode employé sur le « patient allemand » valide l'éventuel recours aux thérapies géniques (Lewin et Rouzioux, 2011). Les premiers essais de thérapie cellulaires à base de LT CD4+ génétiquement modifiés sont en cours de réalisation. (Perez et al., 2008 ; Holt et al., 2010).

C4.2. Améliorer et compléter la cART

C4.2.1. Inhibition de la transcription

L'échappement virologique est imputable aux mutations apparaissant dans le génome viral lors de la réplication résiduelle. Ajouter des molécules, ayant pour vocation l'inhibition de l'étape de transcription virale, aux cocktails d'ARV actuels permettrait un contrôle accru de la virémie, en réduisant/abolissant les blips, et par voie de conséquences de limiter l'apparition de mutants résistants. *In fine*, en limitant les effets d'inflammations provoqués par la virémie de fond, et en augmentant la barrière génétique des ARV, la qualité de vie des patients serait améliorée.

Des inhibiteurs de facteurs transcriptionnels recrutés au promoteur viral paraissent constituer de bons candidats pour compléter les thérapies. (pour revue Baba, 1997 ; Baba 2006, Stevens, 2006). Notre laboratoire s'interesse par ailleurs depuis peu aux protéines de la famille Ding qui présentent une activité anti-VIH en regulant négativement la transcription des gènes viraux.

C4.2.2. HPBP et la famille Ding.

Les protéines Ding présentent un motif conservé DINGG dans leur partie aminoterminale, d'où leur nom, et pour l'instant n'ont aucun gène identifié dans le génome humain (Berna et al., 2009). Dans notre cas, ce qui est notable est que la quantité de ces protéines est significativement plus élevée chez les patients infectées (Djeghader et al., 2012). Récemment, des protéines de la famille Ding ont tour à tour été présentées comme répresseurs transcriptionnels du VIH-1. Notamment,

p27StJohn est capable d'interagir avec deux activateurs de la transcription virale, TAT et NF-IL6, et de limiter l'activité transcriptionnelle du virus en déphosphorylant l'extrémité CTD de la ARNpolII. (Darbinian-Sarkissian et al., 2006 ; Darbianian-Sarkissan et al. 2011). X-DING-CD4 est quant elle produite par des cellules resistantes à l'infection et agit comme facteur de résistance humain (HFR) (Lesner et al., 2009). La Human Phosphate Binding Protein (HPBP) a été récemment découverte dans le plasma, co-purifiée avec une paroxonase humaine (hPON1), et a une structure très proche des transporteurs de phosphate de la famille ABC (Figure 13) (Fokine et al., 2003 ; Morales et al., 2006 ; Contreras-Martel et al., 2006 ; Elias et al., 2011). HPBP est retrouvée dans de nombreux tissus de différents types, avec une localisation aussi bien cytoplasmique/nucléaire qu'exclusivement nucléaire, suivant les types cellulaires (Collombet at al., 2010).

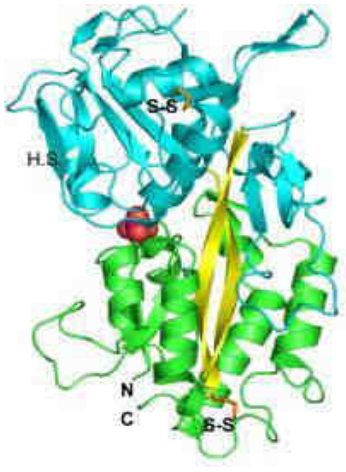


Figure 13: **Représentation en ruban de la protéine HPBP.** Les deux domaines de la protéine (vert et bleu) sont connectés par une charnière (jaune). Au centre de la protéine se trouve les molécules de phosphate (rouge). (modifié depuis Morales et al., 2006)

Ces protéines présentent une activité anti-VIH très prononcée, et apparait plus particulièrement être un puissant répresseur de la transcription (*Annexe-Publication 4 Cherrier et al., 2011*). L'étude approfondie des activités anti-virales de la HPBP et des zones de la protéine impliquée dans l'inhibition transcriptionnelle du promoteur viral, pourrait mener à la synthèse de petits peptides (comme dans le cas du T20) ou de molécules pour complémenter les multi-thérapies.

C4.2.3. Purger les réservoirs.

L'obstacle majeur barrant la route qui mène vers la guérison sous multi-thérapie est la présence de réservoirs viraux latents. Adjoindre des molécules, permettant une purge des réservoirs viraux, aux multi-thérapies actuelles pourrait être une solution. Les thérapies actuelles ne sont en mesure de cibler que les virus avec un cycle réplicatif productif et non le provirus. Restaurer l'activité transcriptionnelle des virus latents, permettrait de les rendre sensibles aux ARV. Pour cela on peut chercher à stimuler les voies d'activations ou bien interférer les mécanismes d'établissement et de maintien de la latence.

Mais avant de trouver les molécules susceptibles de stopper la mise en place des réservoirs, nous devons connaître les acteurs cellulaires et viraux mis en jeu dans le déroulement de ces étapes.

D. La latence moléculaire

On distingue deux formes de latence, la latence pré-intégrative et post-intégrative.

Dans la latence pré-intégrative, le provirus ne s'intègre pas dans le génome de la cellule infectée (Zack et al., 1990). Ce phénomène peut venir d'un défaut dans l'import du provirus dans le noyau ou d'une perturbation de l'étape de transcription inverse. L'activité de la transcriptase inverse peut être dérangée par un pool de dNTPs disponibles insuffisant ou l'hypermutation du génome viral en cours de production par la protéine cellulaire APOBEC3 (Zack et al., 1992 ; Bukrinsky et al., 1992).

Cette forme de latence est régulièrement observée dans les lymphocytes CD4+, mais n'explique pas l'existence des réservoirs de longue durée. En effet, la demi-vie des ADN viraux non intégrés n'y est que d'une journée. Ce n'est cependant pas le cas dans les macrophages, où l'ADN viral non intégré peut perdurer jusqu'à deux mois et même y être transcrit (Gillim-Ross et al., 2005 ; Kelly et al., 2008).

D1. La latence post-intégrative

Un blocage post-transcriptionnel peut être à l'origine de la latence post-intégrative. En effet, les ARNm viraux peuvent être retenus dans le noyau ou ciblés par des miARNs, empêchant ainsi la production des protéines virales et le déroulement complet du cycle de réplication (Lassen et al., 2006 ; Huang et al., 2007).

Même si les mécanismes de blocages post-transcriptionnels ont un rôle non négligeable dans le maintient de la latence, nous nous attarderons plus particulièrement sur les évènements intervenant au niveau de la transcription du provirus.

D2. L'hétérochromatine

L'hypothèse la plus simple pour expliquer la latence transcriptionnelle est l'intégration du provirus dans des zones organisées en hétérochromatine. L'hétérochromatine est une structure condensée de l'ADN, contrairement à l'euchromatine dans un état relâché. La compaction des gènes dans un environnement hétérochromatinien inactive leur transcription. (Figure 14)

L'unité fondamentale de la chromatine, le nucléosome, est un octamère de protéines, les histones. Ces histones peuvent être modifiées post-traductionnellement par des acétylations, phosphorylations, méthylations, SUMOylations et ubiquitinations. Les résidus ciblés, les marques post-traductionnelles et la combinaison de résidus portant une marque, permettent de définir l'état de la chromatine. On parle de code histone. Ces modifications d'histones ne sont pas irréversibles, ce qui rend l'état chromatinien labile et augmente la complexité de l'activité transcriptionnelle des gènes. Ces modifications ayant un impact sur le profil d'expression des gènes sans modifier le génome, on les appelle modifications épigénétiques (Kouzarides 2007).

L'acétylation des histones, par des histones acetyl-transferases (HAT) est associée à la formation d'euchromatine, tandis que la dé-acétylation, par des histones déacétylases (HDAC), mènent à la formation d'hétérochromatine. L'état d'acétylation est donc directement corrélé à l'état d'activation transcriptionnelle (pour revue Csordas, 1990 ; Hebbes et al., 1994 ; Van Lint et al, 1996). Inversement, la SUMoylation entraine la formation d'hétérochromatine (Shiio and Eisenman, 2003 ; Nathan et al., 2006). (Figure 14)

Dans le cas des méthylations, phosphorylations et ubiquitinations le code est plus complexe. Le ou les résidus impliqués vont orienter vers la formation d'une des deux formes de la chromatine (pour revue Fischle et al., 2003).

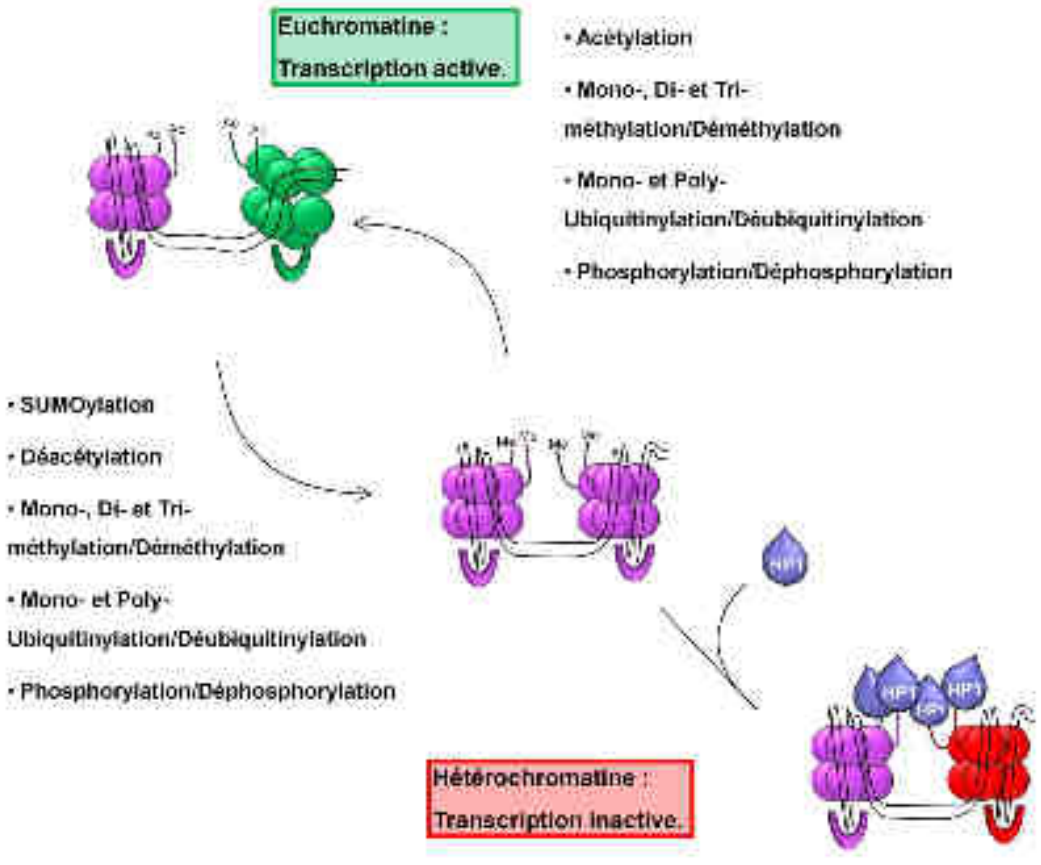


Figure 14: **Modifications épigénétiques et contrôle de la transcription.** Certaines marques post-traductionnelles sont associées à une transcription active (euchromatine, verte), tandis que d'autres sont liées à une extinction de l'expression génique (hétérochromatine, rouge) (modifié depuis *Annexe-Publication 6 Redel et al., 2010*)

D3. La méthylation des lysines

Les résidus lysines de la queue N-terminale des histones peuvent être mono-, di- ou tri-méthylées par des histones méthyl transférases (HMTs) ou déméthylés par des déméthylases.

La méthylation de certain résidus est associée à un relâchement de la structure chromatinienne, tandis que d'autres permettront la condensation de la chromatine. Cependant, le code histone n'est ni totalement figé, ni compris, à l'heure actuelle. En effet, certaines de ces marques ont un rôle ambivalent, *ie* suivant le type cellulaire ou le contexte épigénétique déjà en place, leur rôle sera activateur ou répresseur.

Par exemple, la tri-méthylation de la lysine 9 de l'histone H3 (H3K9me3) par la HMT SUV39h1 est communément responsable de la mise en place

d'hétérochromatine en permettant la fixation d'HP1 (Heterochromatin Protein 1) stabilisant l'environnement hétérochromatinien (Nakayama et al., 2001 ; Lachner et al., 2001). Pourtant elle a aussi été associée, dans les lymphocytes, à une transcription active du provirus (Mateescu et al., 2008).

Dans l'autre sens, la tri-méthylation de la lysine 4 de l'histone H3 (H3K4me3) a toujours été associé à la transcription des gènes (Sims et al., 2006). Mais la marque H3K4me3 à récemment été associée à l'inactivation de la transcription de gènes cryptiques. (Pinskaya et al., 2009 ; Le Douce et al., 2012)

D4. Le site d'intégration

Cependant, l'intégration du provirus dans des zones hétérochromatiniennes n'est pas la seule explication à la latence transcriptionnelle. En effet, il a été établi que le provirus s'intègre majoritairement (93%) dans des introns appartenant à des zones transcriptionnellement actives du génome hôte (Han et al., 2004).

Des mécanismes d'interférences transcriptionnelles peuvent alors expliquer l'absence de transcription du provirus : par compétition de promoteurs - le promoteur du gène dans lequel le provirus est intégré s'accapare les éléments amplificateurs du promoteur viral ; par occlusion promotrice - lorsque le promoteur cellulaire est plus fort que le promoteur viral, et s'approprie l'ARNpolII au détriment du provirus ; par collision des ARNpolII - lorsque le promoteur cellulaire et le promoteur viral se font face (Eszterhas et al, 2002 ; pour revue : Lassen et al, 2004). (Figure 15)

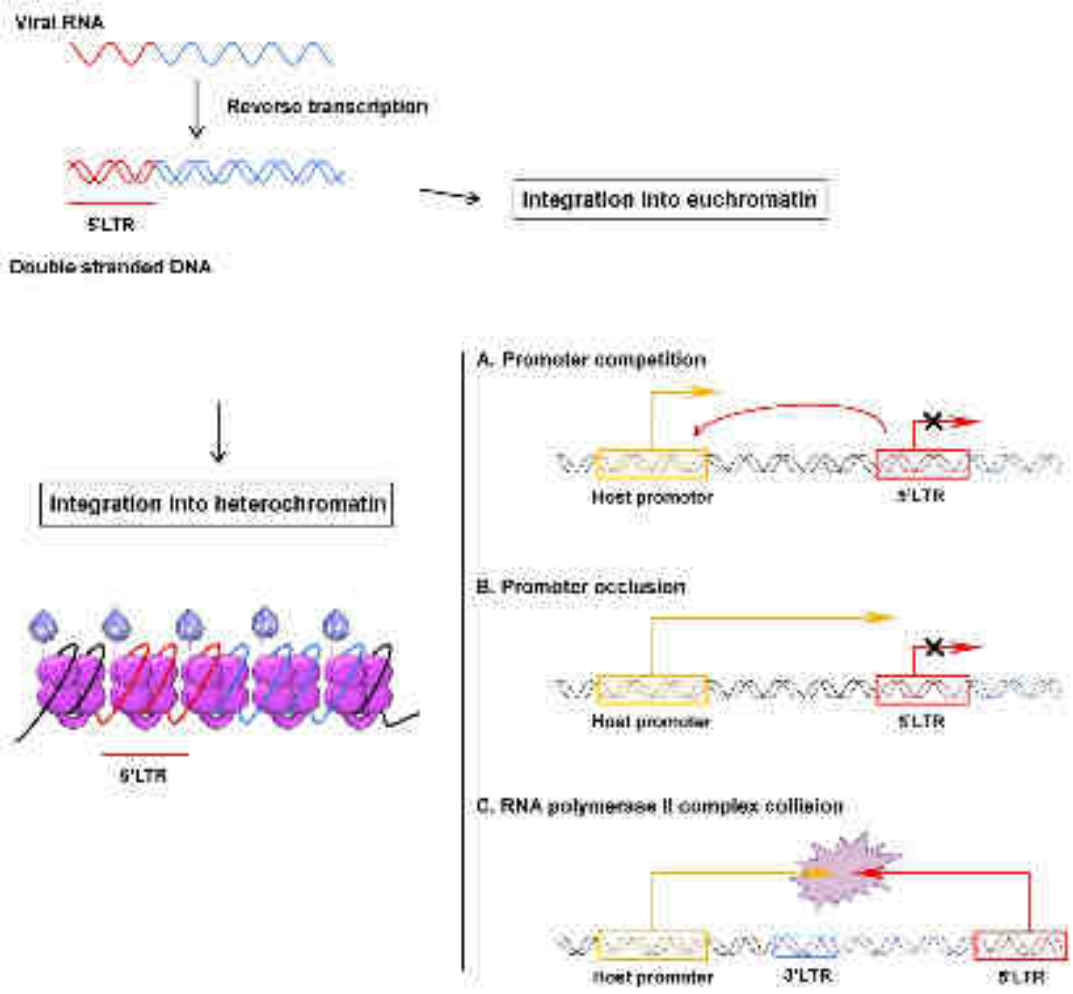


Figure 15: L'influence du site d'intégration sur l'expression du génome viral. L'ADN flap viral peut soit intégrer une zone hétérochromatinienne (partie gauche) ou une zone euchromatinienne (droite). Dans le second cas de figure, des interférences transcriptionnelles peuvent provoquer l'extinction de l'expression du provirus. (*Annexe-Publication 6 Redel et al, 2010*)

D5. Le promoteur viral

Le promoteur viral, ou LTR, contient de nombreux sites pour des activateurs et répresseurs transcriptionnels cellulaires.

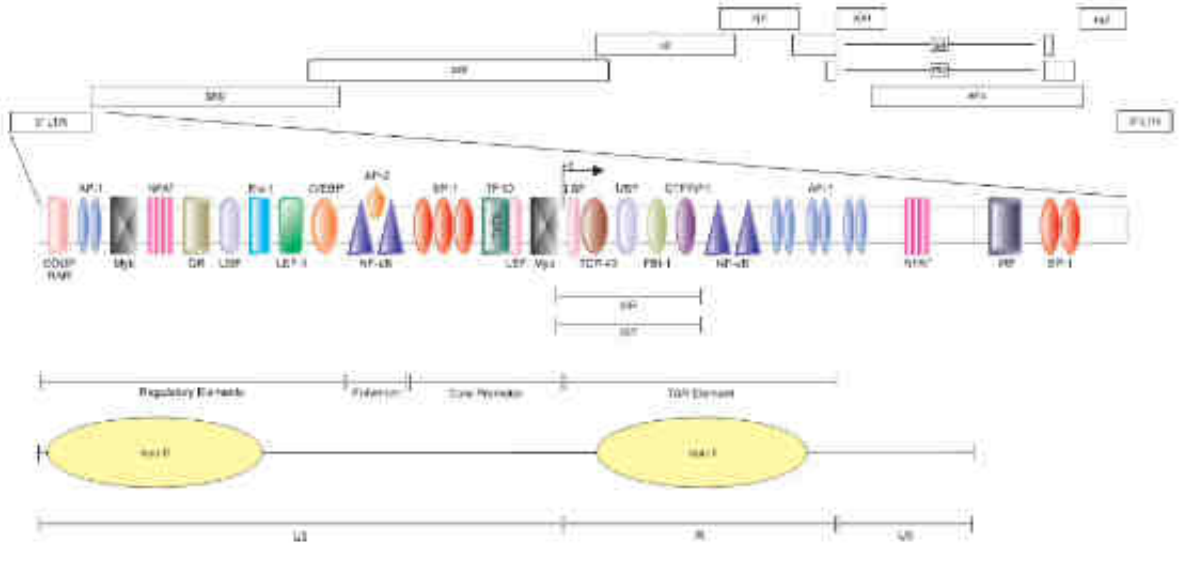


Figure 16: **Le promoteur viral.** Le promoteur LTR du VIH contient 3 zones contenant chacunes une pléthore de sites de fixation pour des régulateurs cellulaires de la transcription. Invariablement, l'organisation chromatinienne du promoteur virale s'organise autour de deux nucléosomes Nuc-0 e Nuc-1 (jaune). (Stevens, 2006)

Le promoteur viral est structuré en 3 régions, comprenant 4 zones, respectivement, de 5' en 3' (Figure 16):

La zone régulatrice, de -454 à -104, contient des sites de fixation à des cis-répresseurs et des cis-activateurs servant à nuancer l'activité transcriptionnelle. Cette zone est, quelque soit le site d'intégration, le siège du nucléosome 0 (Nuc-0), qui va limiter l'accès des protéines régulatrices.

La zone amplificatrice, de -105 à -79, contient des sites de fixations en tandem pour l'hétérodimère NF-κB, facteur de transcription primordial. Entre les deux sites NF-κB est enchâssé un site pour la protéine AP-2, un autre activateur de la transcription virale.

Le promoteur basal, de -78 à -1, est l'unité minimale pour l'initiation de la transcription. On y trouve à la fois une boite TATA et un initiator-like

region, tous deux sites de fixation de l'ARNpolII. Cette zone contient également trois sites pour la protéine Sp1 qui va servir de plateforme d'ancrage à d'autres protéines régulatrices. (Berkhout and Jeang, 1992 ; pour revue Rohr et al., 2003 ; Stevens et al., 2006)

Ces trois zones sont contenues dans la région U3, tandis que la séquence transactivatrice TAR, dernière des quatres zones, est contenue dans la région R du LTR. Cette zone va donner naissance à la structure tige boucle du même nom lors de l'initiation de la transcription. Cette zone servira ensuite à recruter le transactivateur viral Tat, intéragissant avec le facteur d'élongation P-TEFb, nécessaire à l'amélioration de la processivité de l'ARNpolII. De plus, tout comme la région U3, cette région est invariablement le siège d'un nucléosome, Nuc-1, qui va obstruer le provirus et empêcher l'ARNpolII de démarrer la transcription (Verdun et al., 1993 ; Van Lint et al., 1996). (Figure 16)

Au-delà de Nuc-1, se trouve la région U5, qui contient d'autres sites de fixations pour des facteurs de transcriptions, AP-1, SP1, NF-AT et IRF-1 (Rohr et al., 2003). (Figure 16)

La zone entre les nucléosomes Nuc-0 et Nuc-1 contient la zone amplificatrice et le promoteur basal. Cette séquence, tout comme la zone au-delà du Nuc-1, est accessible aux modulateurs transcriptionnels et va être le siège de compétitions entre facteurs activateurs et répresseurs. Ces compétitions entre facteurs transcriptionnels sont à l'origine des modifications épigénétiques de Nuc-1 et menent à la fermeture et l'ouverture de la chromatine au niveau du LTR.

Le promoteur viral est donc cible de nombreux facteurs cellulaires qui vont établir ou lever la latence du provirus. Nous sommes donc face à un choix binaire, d'entrée ou non en latence, sous le contrôle d'une régulation fine faisant intervenir une pléthore de facteurs de régulation. La question qui se pose alors ensuite, est de savoir ce qui favorise, ce qui oriente vers un système productif ou au contraire vers l'extinction transcriptionnelle.

D6. Le circuit stochastique de TAT

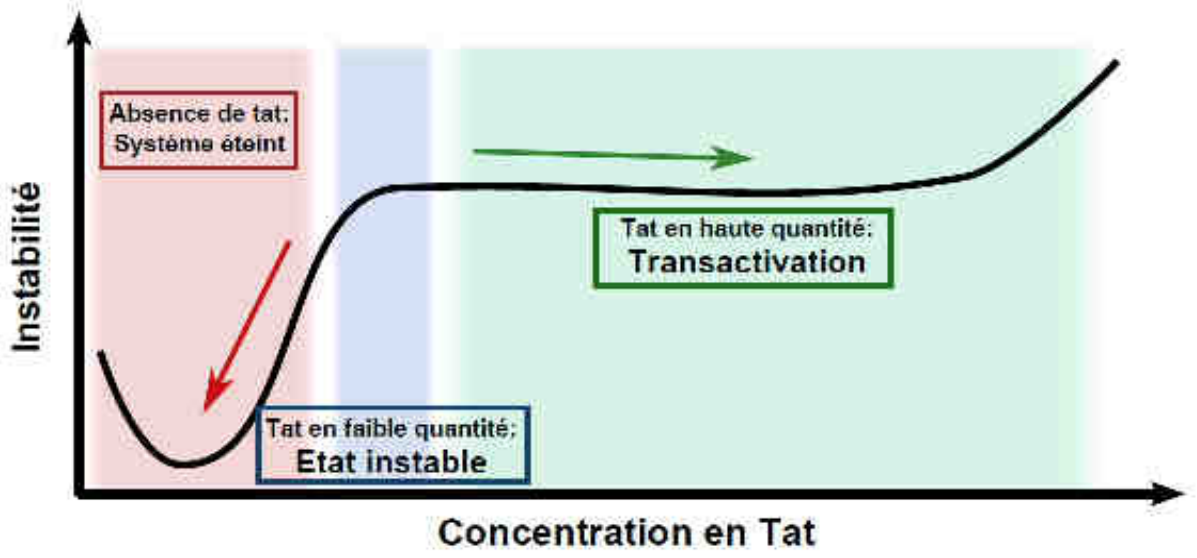


Figure 17: Modèle stochastique de l'état bi-directionnel de la transcription virale. L'état reste instable lorsque la concentration de TAT est faible (bleu) et peu évoluer soit vers une chute de la quantité de tat menant à l'extinction de la transcription (rouge) soit vers la transactivation, permettant l'entretien d'une haute quantité de Tat dans le système. (source personnelle, d'après Weinberger et al., 2005)

Précédemment nous avons abordé comment TAT était capable de transactiver la transcription du provirus en permettant l'augmentation de la stabilité et de la processivité de l'ARNpolII. Une fois produit, TAT va augmenter la quantité de transcrits viraux synthétisés et donc rétro-contrôler positivement sa propre production.

Parler d'activation transcriptionnelle peut paraître saugrenue dans un chapitre de latence post-intégrative. Et pourtant TAT fait office d'interrupteur entre transcription active et inactive.

Pour expliquer ce système à deux sorties potentielles, des modèles mathématiques, basés sur le rétro-contrôle opéré par TAT, ont été proposés (Weinberger et al., 2005 ; Weinberger et al., 2008 ; pour revue Razooky et Weinberger, 2011). Des expériences *in-vitro* ont ensuite infirmé le modèle bistable, qui usuellement explique ce genre de circuit décisionnel, mais ont corroboré le modèle d'expression stochastique, dans lequel la destinée du provirus dépend de la force et

la durée du rétrocontrôle positif. A terme, il apparaît que ce système décisionnel tend inexorablement vers l'entrée du provirus en latence. Pour faire simple, lorsque la quantité de TAT est suffisamment faible le système a l'opportunité d'évoluer vers deux issues opposées l'une à l'autre. Si les fluctuations stochastiques de la transcription virale tendent à affaiblir la transactivation et qu'il n'y a pas assez de TAT dans le système pour contrebalancer cet effet, alors l'extinction transcriptionnelle s'accélère et aboutit à la latence virale (figure 17, partie rouge). Par contre, si les fluctuations stochastiques de la transcription virale permettent de maintenir une quantité suffisante de TAT, la transactivation se poursuit. Réciproquement, tant que la transactivation est entretenue, TAT continue d'être produit. Dès lors il y a un effet boule de neige qui va allonger dans le temps la période transitoire de transcription virale (Figure 17, partie verte).

Lorsque la période productive est suffisamment longue et entretenue, le cycle réplicatif se poursuit et s'achève concomitamment avec la destruction de la cellule hôte. La lyse de la cellule hôte ne laisse alors pas le temps au système de décliner suffisamment vite pour évoluer vers l'arrêt de la transcription et la latence (pour revue Rasooky et Weinberger, 2011).

Ce serait donc les variations dans la production de TAT qui permettraient dans un premier temps au système de ralentir jusqu'à son immobilisation totale. A la suite de quoi, d'autres mécanismes viennent établir la mise en place durable de la latence, par l'intermédiaire de marques épigénétiques favorisant la formation d'hétérochromatine.

E. Définir les prochaines cibles des ARVs.

Pour définir les nouvelles cibles, il est critique de connaître les acteurs et les mécanismes régissant l'étape de transcription du provirus.

Le modèle stochastique d'établissement de la latence établi par le Dr. Weinberger et son équipe met TAT au cœur du circuit décisionnel.

E1. Le cycle de transactivation de TAT.

TAT fait partie des premières protéines virales produites par le virus. Une fois produit, le transactivateur va subir une succession de modifications post-traductionnelles dans son motif ARM (Arginine Rich Motif) lui permettant d'interagir ou de libérer des protéines cellulaires importantes pour les étapes d'initiation et d'élongation de la transcription du provirus.

Le modèle séquentiel proposé dans les lymphocytes T se déroule de la manière suivante :

TAT recrute l'acétyltransférase cellulaire PCAF. PCAF acetyle tat au niveau du résidu lysine 28 (K28). L'acétylation en K28 (AcK28) provoque la libération de PCAF, laissant TAT libre et acétylé (Kiernan et al., 1999 ; Brès et al., 2002).

Le facteur d'élongation P-TEFb est majoritairement séquestré dans le snRNP 7SK, où il est inactivé par HEXIM1 ou 2. L'AcK28 augmente l'affinité de TAT pour la sous-unité Cyclin T1 (CT1) du facteur d'élongation P-TEFb et TAT va être en mesure de recruter P-TEFb dans le pool inactif (Krueger et al., 2010).

L'interaction TATAcK28/P-TEFb induit une modification de la structure de TAT lui permettant de se fixer sur la TAR néo-formée (d'Orso et Frankel, 2009).

Le seconde sous-unité de P-TEFb, la Cyclin-dependent kinase 9, phosphoryle les résidus sérine 2 et 5 de l'heptapeptide du CTD de l'ARNpolII, améliorant sa processivité (Zhou et al., 2000 ; Kim et al., 2002).

La lysine 51 (K51) préalablement méthylée par SET7/9/KMT7 est déméthylée par LSD1 (Lysine Specific Demethylase 1) et le complexe coREST (RE1-silencing transcription factor), ce qui permet à l'acétyl-transférase CBP/P300, fixée sur le LTR, d'acétyler TAT au niveau du résidu lysine 50 (K50) (Figure 18&19) (Sakane et al., 2011). Le but et le moment de la méthylation de la K51 restent des questions sans réponse à l'heure actuelle, il apparait juste que sa déméthylation est requise à l'acétylation de la K50. TAT, qui est alors double acétylé en K28 et K50, se sépare de la TAR et, une fois libérée se fixe au niveau de l'ARNpolII, puis y recrute de nouveau PCAF (Figure 19) (Dorr et al., 2002).

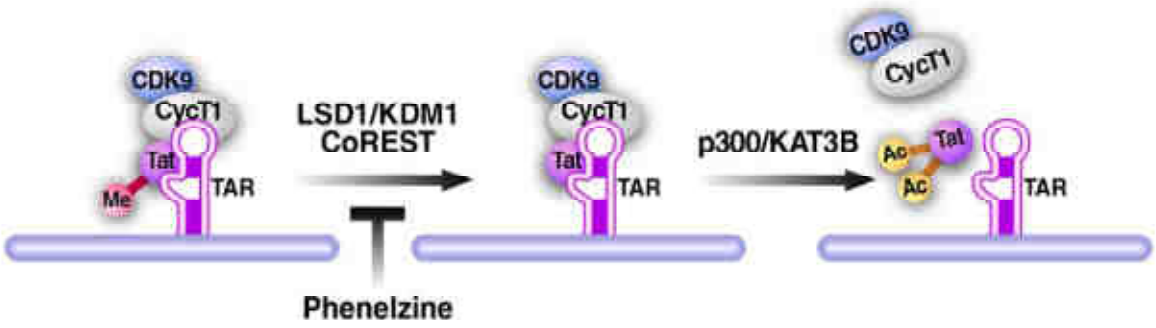


Figure 18: La déméthylation de la lysine 51 de TAT permet son acétylation à la lysine 50. En déméthylant la K50, LSD1 permet à p300 d'acétyler TAT au niveau de la lysine 50. TAT doublement acétylée, en K28 et K50, se sépare de P-TEFb et poursuit le cycle de transctivation. (Sakane et al., 2011)

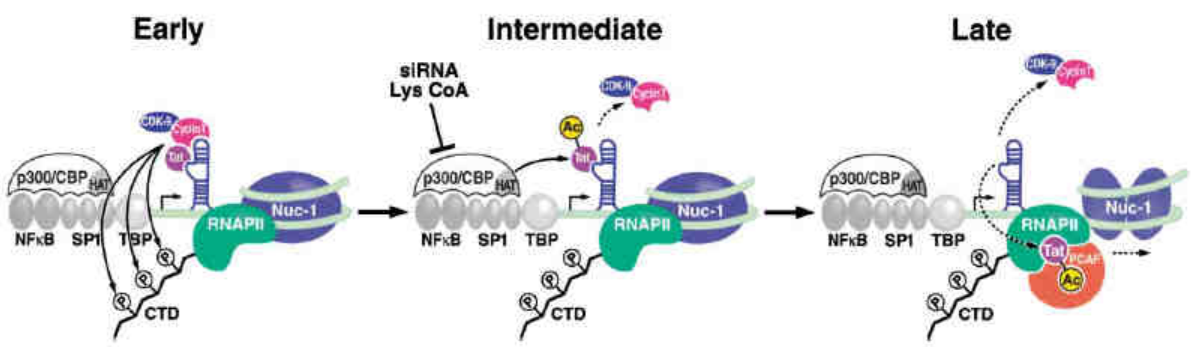


Figure 19 : Acétylation de la lysine 50 par P300/CBP. L'acétylation de la K50 permet à TAT d'être déplacé depuis la TAR vers l'ARNpolII, où il recrute PCAF. (Kaehlcke et al., 2003)

L'interaction entre TAT et PCAF pourrait moduler l'activité enzymatique de cette dernière pour, par exemple, l'amener à déstabiliser les nucléosomes, favorisant ainsi l'élongation (Hamamori et al., 1999). Dans la même optique, l'acétylation de K50 permet la mobilisation du complexe SWI/SNF, qui déstabilise la structure des nucléosomes (Agbottah et al., 2006)

A la fin de l'étape d'élongation, la déacétylase de classe III SIRT1 ôte les marques acétyles de K28 et K50, ce qui permet le recyclage de TAT. TAT est alors disponible pour un nouveau cycle de transactivation (Blazek et Peterlin, 2008).

Les résidus sérines 16 et 46 de TAT sont par ailleurs soumis à la phosphorylation. Ces modifications post-traductionnelles auraient vraisemblablement une importance lors de la transcription virale (Ammosova et al., 2006)

Comprendre les mécanismes régissant le cycle de transactivation et identifier les partenaires de TAT à cette occasion permettraient de définir de nouvelles cibles thérapeutiques.

E2. Le répresseur transcriptionnel CTIP2

Le facteur CTIP2 (COUP-TF-Interacting Protein 2) est un facteur de transcription, impliqué dans la différentiation et le développement du système immunitaire et du système nerveux central, en induisant la formation d'hétérochromatine sur ses promoteurs cibles.

Notre laboratoire a mis en évidence le rôle de CTIP2 dans la répression de la transcription virale. CTIP2 est en effet capable d'inhiber la transcription TAT-dépendante en induisant et relocalisant le transactivateur viral dans des sous-structures nucléaires denses contenant la protéine HP1α, indicatrice de zones hétérochromatiniennes (Rohr et al., 2003). En plus de la perturbation de la voie de transactivation, nous avons démontré que CTIP2 était capable de favoriser

l'établissement de marques épigénétiques induisant la formation d'hétérochromatine au niveau de Nuc-1. Pour cela CTIP2 recrute toute une machinerie de modifications des histones comprenant des HDACs (1 et 2) et la méthyltransferase SUV39h1 (Marban et al., 2007). Le remodellage chromatinen est initié par la suppression des marques d'acétylation sur Nuc-1, suivie de l'établissement de la marque répressive H3K9me3. La marque H3K9me3 permet le recrutement de la protéine HP1α qui va stabiliser la structure hétérochromatinienne du nucléosome Nuc-1 et permettre la compaction en cascade des nucléosome suivants tout le long du provirus (Figure 20).

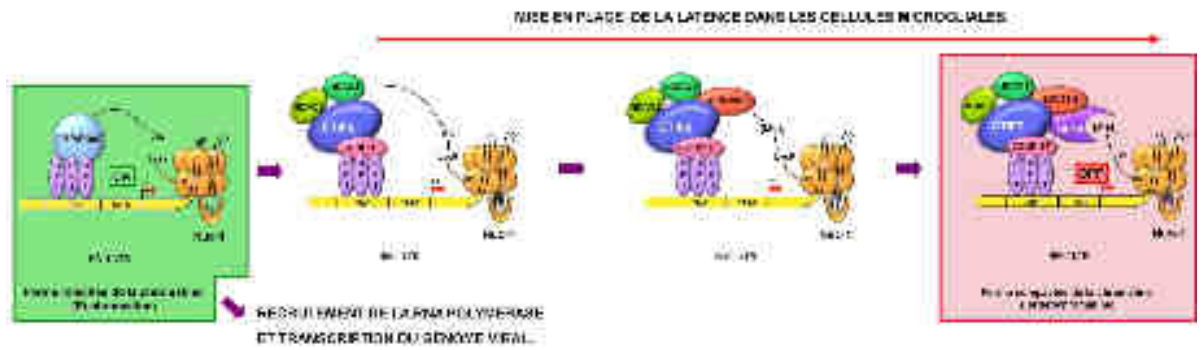


Figure 20: Etablissement de la latence virale dans les cellules microgliales. CTIP2 va séquentiellement recruter des activités HDACs (HDAC1 et 2), qui vont ôter la marque activatrice H3K9Ac, suivi du recrutement de la méthyltransférase SUV39h1, qui va installer la marque répressive H3K9me3. La protéine HP1α va ensuite maintenir l'état hétérochromatinien. (Schwatz et al., 2010)

En tant que facteur de restriction de la transcription du VIH-1, notre laboratoire s'est également intéressé aux effets indirects de CTIP2 sur la régulation de l'expression du génome viral. Notre équipe a ainsi pu mettre en évidence le rôle de CTIP2 dans l'extinction du gène codant pour le contrôleur du cycle cellulaire p21, décrit comme un facilitateur du cycle de réplication virale.

La protéine p21 est un inhibiteur de kinase cycline-dépendante et cible des complexes cycline-CdK. La protéine p21 permet ainsi l'arrêt du cycle cellulaire en phase G1, G2 ou M (Niculescu et al., 1998 ; Radahkrishnan et al., 2004). L'activité de p21 est majoritairement régulée au niveau transcriptionnel (Gartel et Radahkrishnan, 2005). Ainsi, la modification des marques épigénétiques au voisinage du promoteur

de *p21* est de première importance (Suzuki et al., 2000 ; Lagger et al., 2003 ; Gartel et Radahkrishnan 2005 ; pour revue Gartel et Tyner, 2002).

Par ailleurs, p21 permet une facilitation du cycle réplicatif du VIH-1 dans les macrophages en bloquant le cycle cellulaire dans des conditions favorables à sa transcription (Thierry et al., 2004 ; Vazquez et al., 2005). Dans les macrophages, c'est la protéine virale VPR, recrutée au promoteur du gène *p21* par l'intermédiaire de SP1, qui induit la production de p21 (Figure 22) (Amini et al., 2004).

Notre équipe à récemment montré que CTIP2 est capable, à l'instar de VPR, de se fixer au promoteur de *p21* via la protéine SP1. Une fois au promoteur, CTIP2 va recruter le complexe multi protéique de remodelage de la chromatine précédemment évoqué (Figure 22). Les activités HDACs (HDAC1 et 2) et méthyltransférase vont établir des marques associées à l'hétérochromatine au niveau des histones du promoteur *p21*. Ainsi, CTIP2 est capable de réprimer la production de p21, lui permettant de réguler la transcription virale de façon indirecte (Cherrier et al., 2009)

De plus, nous avons récemment pu mettre en évidence que CTIP2 est aussi capable d'entraver la transcription virale TAT-dépendante en séquestrant P-TEFb dans le pool inactif liée au snRNP7SK ou en inhibant l'activité kinase de la CdK9 dans le pool actif (Figure 21). Dans le premier cas, CTIP2 limite le stock de P-TEFb actif disponible, ce qui empêche la transactivation de la transcription virale médiée par TAT. (Cherrier et al., unpublished *cf Annexe publication 3*). CTIP2 interagit avec HEXIM1 et le snRNA 7SK (au niveau de la boucle 2), permettant ainsi la stabilisation du complexe inactif dans lequel P-TEFb est détenu (Figure 21).

Autrement, CTIP2 est aussi en mesure de réprimer l'activité kinase de CdK9 lorsque CTIP2 fait parti du complexe CTIP2/P-TEFb/TAT. En inhibant la CdK9, CTIP2 limite la phosphorylation du CTD de l'ARNpolII, contrecarrant ainsi le pouvoir transactivateur de TAT, même si la protéine virale a pu extraire P-TEFb du complexe inactif snRNP 7sk. (Cherrier et al., unpublished *cf Annexe publication 3*).

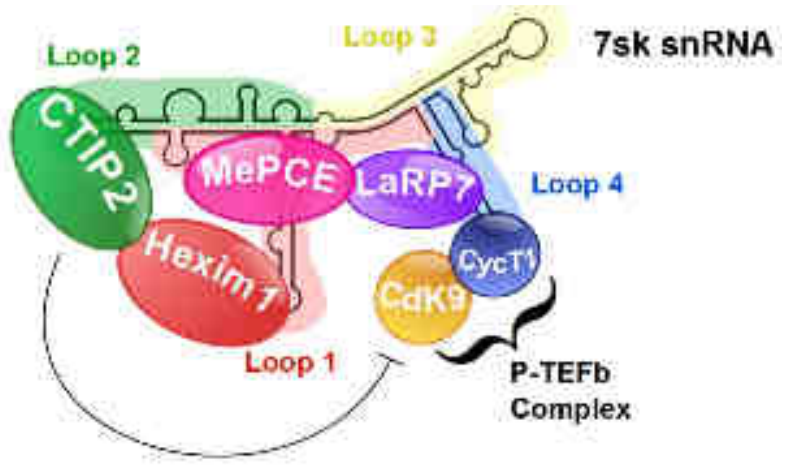


Figure 21 : CTIP2 est membre du complexe snRNP7sk. CTIP2 intéragit avec la boucle 2 de l'ARN 7sk et la protéine Hexim1, elle-même interagissant avec l'ARN 7sk au niveau de la boucle 1. P-TEFb est séquestré dans ce complexe structuré par l'association des différentes protéines sur l'ARN 7sk (Source personnelle *cf Annexe publication 3 - Cherrier et al, unpublished.*)

A lui seul, CTIP2 est capable de ralentir directement l'activité transcriptionnelle du VIH-1 en établissant un environnement compact de la chromatine au niveau du promoteur viral et en limitant le pouvoir transactivateur de TAT. D'autre part, CTIP2 à un aussi un rôle indirect, en provoquant l'extinction de l'expression de *p21,* ou en séquestrant P-TEFb dans le stock inactif associée au complexe snRNP7SK.

Les effets pleiotropiques de CTIP2 semblent ne pas s'arrêter là : des résultats préliminaires semblent indiquer que CTIP2 est en mesure de réguler la production de cytokines dans les cellules microgliales, ce qui pourrait limiter leur sensibilité à l'infection.

CTIP2 est un répresseur transcriptionnel cellulaire d'importance majeure dans l'extinction de la production virale. Il semble donc nécessaire de comprendre l'étendue de ses actions sur la transcription virale et la mise en place de la latence post-intégrative.

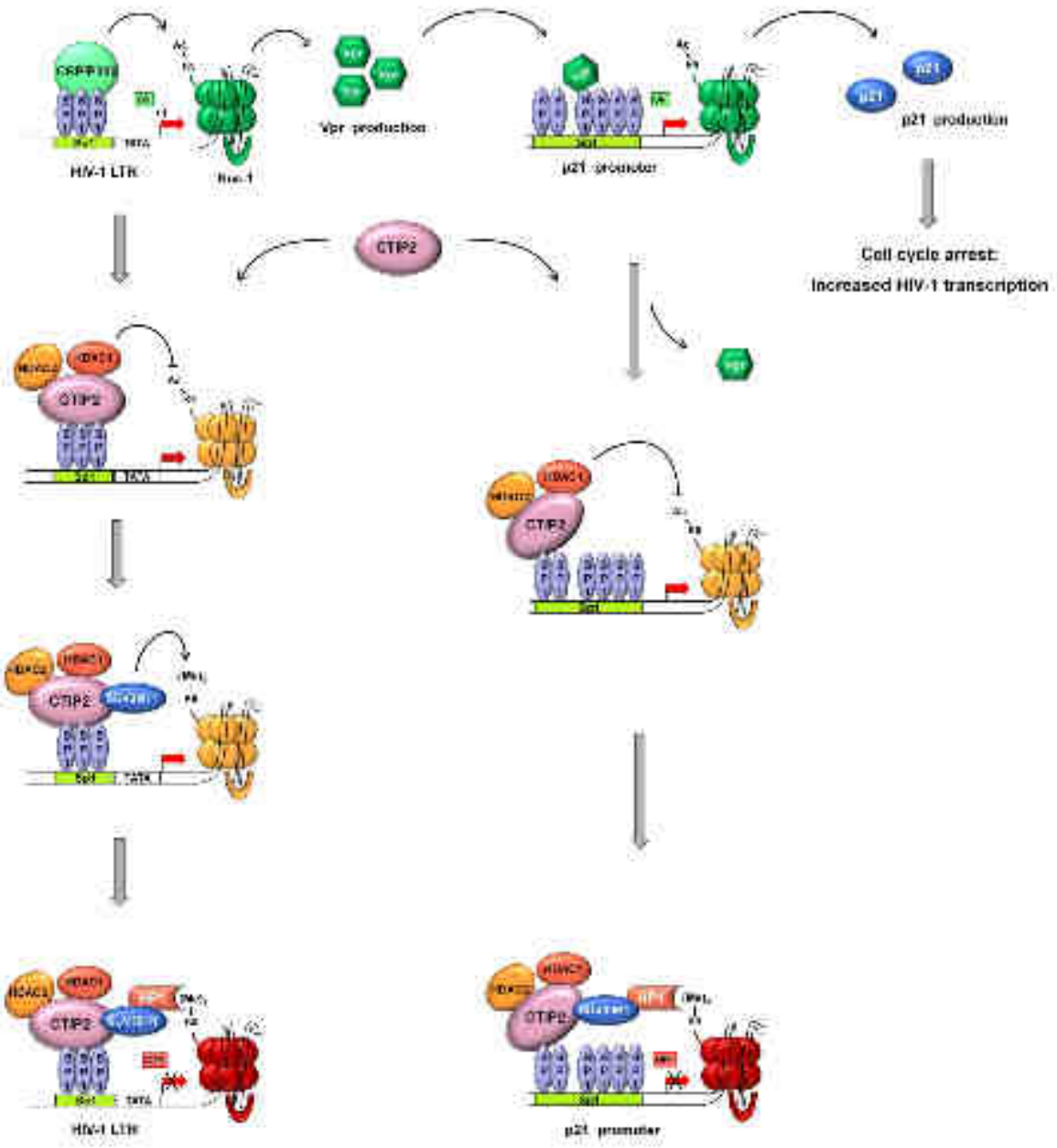


Figure 22 : Fonctions de CTIP2 dans la régulation de la transcription du VIH-1. CTIP2 recrute séquentiellement des activités HDACs, la methyltransferase SUV39h1 et la protéine HP1 au niveau du promoteur viral pour promouvoir l'extinction de la transcription virale (gauche) et du gène codant pour la p21 (droite). CTIP2 contrecarre l'effet de VPR, consistant à stimuler la transcription de *p21*. (Le Douce et al., 2012).

E3. Projet LSD1

Dans l'exemple de CTIP2, la formation d'hétérochromatine au niveau du provirus provient en substance de l'activité méthyltransférase de SUV39h1 parallèlement à la déacetylation des histones.

La question que nous nous sommes alors posée était de savoir si des activités demethylases pouvaient aussi être recrutées au promoteur LTR. Soit dans le but de faciliter, ou soit pour reverser, l'activité répressive de CTIP2.

Dans les deux cas, LSD1 apparait comme un bon candidat. LSD1 est une déméthylase capable de cibler les lysines 4 et 9 de l'histone H3 mono- et di-méthylées (Shy et al., 2004 ; Metzger et al., 2005).

L'activité répressive ou activatrice de LSD1 dépend du substrat ciblé (H3K4 ou H3K9). La spécificité du substrat elle-même est modulée par les interactants de LSD1. Ainsi, lors de son interaction avec le complexe coREST/HDAC1/HDAC2, LSD1 va permettre la transition d'un état activé vers un état inactivé des gènes cibles (Shy et la., 2003 ; Shy et al., 2005). Dans ce cas de figure, LSD1 promeut l'établissement de marques répressive (Figure 23).

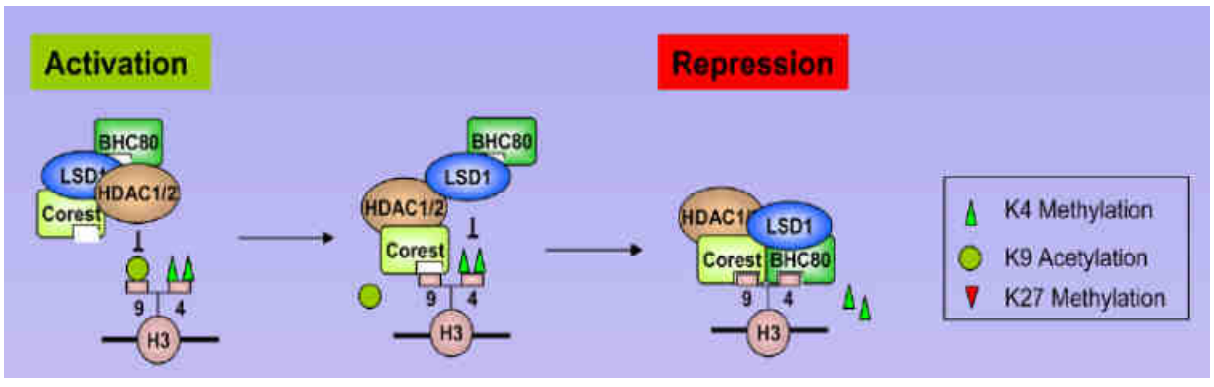


Figure 23: Mécanisme d'établissement de marques épigénétiques répressives par l'intermédiaire de LSD1. LSD1 recrute séquentiellement des HDACs, qui vont enlever la marque activatrice H3K9Ac, le complexe coREST, déméthylant la H3K4, et enfin la protéine BHC80, qui maintien la H3K4 dans un état hypométhylé. (Revue Lan et al., 2008)

Au contraire, lorsque LSD1 est recruté par le récepteur aux androgènes (AR), il coopère avec la démethylase JMJ2DC. L'association LSD1/JMJ2DC permet de passer séquentiellement de la marque répressive H3K9me3 à H3K9m2 (JMJ2DC), puis à H3K9me1/0 (LSD1). L'activité demethylase a pour résultat l'activation transcritpionnelle des gènes cibles de AR (Metzger et al., 2005 ; Wissmann et al., 2007). L'expression aberrante de LSD1, et donc l'activation constitutive des voies activatrice de l'AR, a même été proposée comme biomarqueur d'agressivité des cancers de la prostate (Kahl et al., 2006).

Par ailleurs, LSD1 est aussi capable de réguler l'expression génique en deméthylant des protéines non-histones. Par exemple, le transactivateur viral TAT, que nous avons déjà abordé auparavant (Sakane et al., 2011). Mais aussi des régulateurs cellulaire de la transcription, comme le répresseur de tumeur p53 ou l'ADN methyltransferase Dnmt1. En démethylant p53, LSD1 réprime sa fonction pro-apoptotic en abolissant son interaction avec son co-activateur 53BP1, alors que la démethylation de Dnmt1 stabilise la protéine et lui permet d'échapper à la dégradation (Figure 24) (Huang et al., 2007 ; Wang et al., 2009).

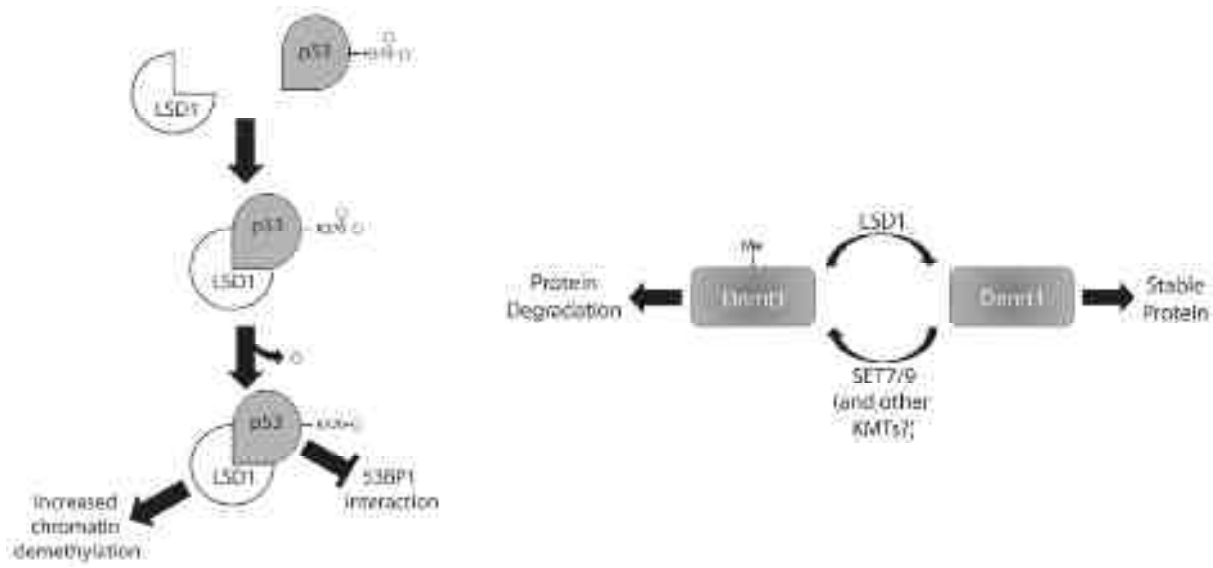


Figure 24: Demethylation de protéines non-histone par LSD1. LSD1 peut réprimer l'activité pro-apoptotique de p53 (gauche) ou, au contraire, stabiliser Dnmt1 et augmenter temporellement l'activité de la protéine (droite). (Nicholson et Chen, 2009)

Dans la première partie de ce travail de thèse nous avons cherché à déterminer d'une part, l'éventuel impact de LSD1 sur la transcription virale dans les cellules microgliales, d'autre part, le rôle que pourrait avoir LSD1 dans l'établissement et le maintien de la latence dans le cadre du mécanisme induit par CTIP2 et enfin, les partenaires de LSD1 dans ce contexte.

E4. Projet HIC1

Toujours dans l'optique de trouver les partenaires de CTIP2 dans la régulation du VIH-1, nous nous sommes interessés au répresseur de tumeur HIC1. En effet, HIC1 et CTIP2 sont capables d'intéragir, cependant cette intéraction n'a pour l'instant pas encore été associée à un mécanisme physiologique, ni même à d'autres protéines qui pourraient faire partie de ce complexe.

Le locus 17p13.3 est fréquemment méthylé ou perdu dans un grand nombre de cancers humains (médulloblastome, carcinome ovarien, cancer gastrique, du sein, de la prostate…) (Rood et al., 2002 ; Pieretti et al., 1995 ; Kanai et al., 1998 ; Fujii et al., 1998 ; Yamamakam et al., 2003). L'étude de ce point chaud, altéré dans de nombreux cancers, a permis l'identification d'un gène codant pour un suppresseur de tumeur, appelé Hypermethylated In Cancer 1 (HIC1) (Wales et al., 1995). Ainsi, HIC1, exprimé de façon ubiquitaire dans les tissus sains, voit son expression drastiquement diminuée dans les cas de cancers précédemment cités.

HIC1 est sous-divisé en 3 régions (Figure 25.A):

Une partie Aminoterminale contenant un domaine BTB/POZ (BR-C, ttk and bab/ Pox virus and Zinc finger), intervenant dans l'oligomérisation de HIC1. L'oligomérisation de HIC1 est une étape indispensable qui initie le recrutement du co-répresseur CtBP (C-terminal Binding Protein), la fixation coopérative des oligomères sur l'ADN et l'ajout de la marque post-traductionnelle SUMO (Deltour et al., 2002 ; Pinte et al., 2004 ; Stankovic-Valentin et al., 2007).

Ce domaine a également une activité répressive autonome en recrutant et coopérant avec l'HDAC de classe III, SIRT1 (Chen et al., 2005).

Un domaine central, avec une séquence peu conservée inter-espèce, si ce n'est pour quelques motifs protéiques, dont deux ont été caractérisés.

Le motif GLDLSSK/R, séquence qui permet l'interaction de HIC1 avec le co-répresseur CtBP. Cette interaction confère à la zone centrale de HIC1 une activité répressive autonome, faisant intervenir des HDACs de classe I et II. Une mutation ponctuelle dans ce motif, $L^{225}A$, suffit à abolir cette interaction et, dès lors, à diminuer le pouvoir répresseur de HIC1 (Deltour et al., 2002 ; Stankovic-Valentin et al., 2006).

Le motif ψKxEP, dont la lysine 314 (K^{314}) est la cible de modifications post-traductionnelles. Ainsi, la K^{314} fait office de commutateur entre absence de marque, acétylation et SUMOylation de HIC1. L'acétyl-transferase P300 acétyle la K^{314} tandis que SIRT1 la déacetyle. Suite à la déacétylation, HDAC4 facilite la SUMOylation de la K^{314} par un mécanisme encore non identifié (Figure 25.B) (Stankovic et al., 2007). L'acétylation diminue l'intéraction entre HIC1 et CtBP, tandis que la SUMOylation lui permet d'interagir avec des complexes de modifications des histones, tel que NuRD (Stankovic-Valentin et al., 2007 ; Van Rechem et al., 2010).

La partie carboxyterminale contient quant à elle 5 doigts de zinc krüppel-like. Alors que le premier d'entre eux, à l'écart, ne semble pas intervenir dans la fixation de HIC1 à l'ADN, le cluster des 4 doigts de zinc restants permet par contre la reconnaissance d'un motif HiRE (HIC1 Responsive Element). Le HiRe a une séquence consensus 5'-C/GNGC/GG**GGCA**C/ACC-3' s'articulant autour d'un motif central GGCA (Pinte et al., 2004).

En marge de l'interaction de SIRT1 avec le domaine BTB/POZ de HIC1, pour recruter son acitvité HDAC, HIC1 est aussi capable de se lier à SIRT1 par l'intermédiaire de ses doigts de zinc krüppel-like. Dans ce cas de figure, SIRT1 va permettre de déacétyler la K^{314} et modifier post-traductionnellement HIC1. (Dehennaut et al., 2012).

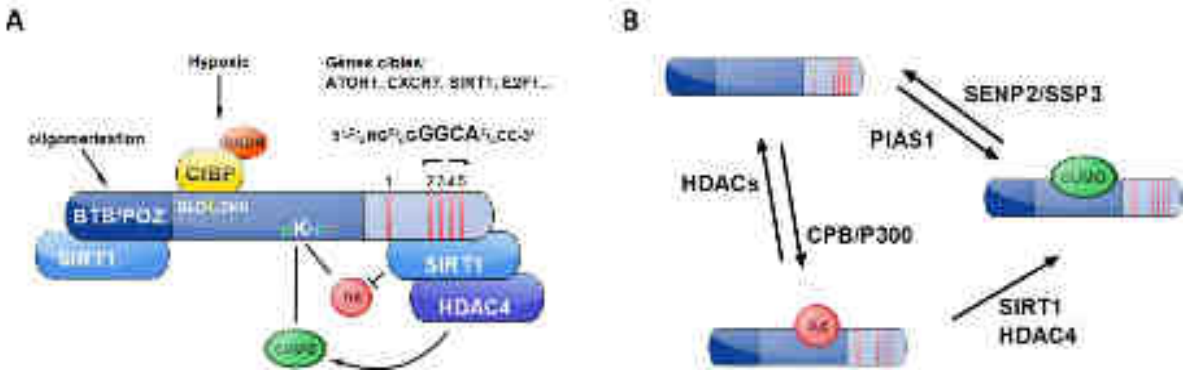


Figure 25 : Partenaires, modifications post-traductionnelles et organisation de la protéine HIC1. HIC1 possède trois domaines. Un domaine N-terminal BTB/POZ, un domaine central avec deux motifs conservés et un domaine C-terminal contenant 5 doigts de zincs (Fig25.A). HIC peut être acetylé ou SUMOylé au niveau de la K^{314} par des protéines cellulaires (Fig25.B). (Source personelle)

Par l'intermédiaire de ses doigts de zinc, HIC1 est capable de se fixer sur les sites Hires présents sur les promoteurs de ses gènes cibles (*Cxcr7*, *E2f1*, *Atoh1*, *Sirt1*…) et d'y recruter des complexes multi protéiques répresseurs (SWI/SNF, NuRD, Polycomb Repressive Complex 2) (Van Rechem et al., 2009 ; Jenal et al., 2009 ; Briggs et al., 2007 ; Zhang et al., 2007 ; Van Rechem et al., 2009b ; Van Rechem et al., 2010 ; Boulay et al., 2012)

La répression de la transcription de certaines protéines, ayant des effets pleiotropiques, est accompagnée de boucles de rétrocontrôle qui permettent d'en moduler plus finement l'expression.

HIC1 intervient ainsi dans les voies de réparation de l'ADN, l'appauvrissement calorique, la migration et le développement cellulaire. (pour revue Fleuriel et al., 2009)

L'une de ces boucles de rétrocontrôle concerne le suppresseur de tumeur p53 et SIRT1. Dans ce paradigme, l'augmentation de HIC1, suite à des dommages à l'ADN, permet l'extinction transcriptionnelle de *Sirt1*, un de ses gènes cibles. La diminution de SIRT1 permet une augmentation du taux de p53 acétylé, version active de la protéine, qui va pouvoir éteindre l'expression d'oncogènes et activer celle de suppresseurs de tumeur, dont HIC1. Parallèlement, SIRT1 interagit avec HIC1 pour

diminuer sa propre transcription, permettant des ajustements fins de la boucle de régulation (Figure 26). (pour revue Dehenaut et Leprince, 2009).

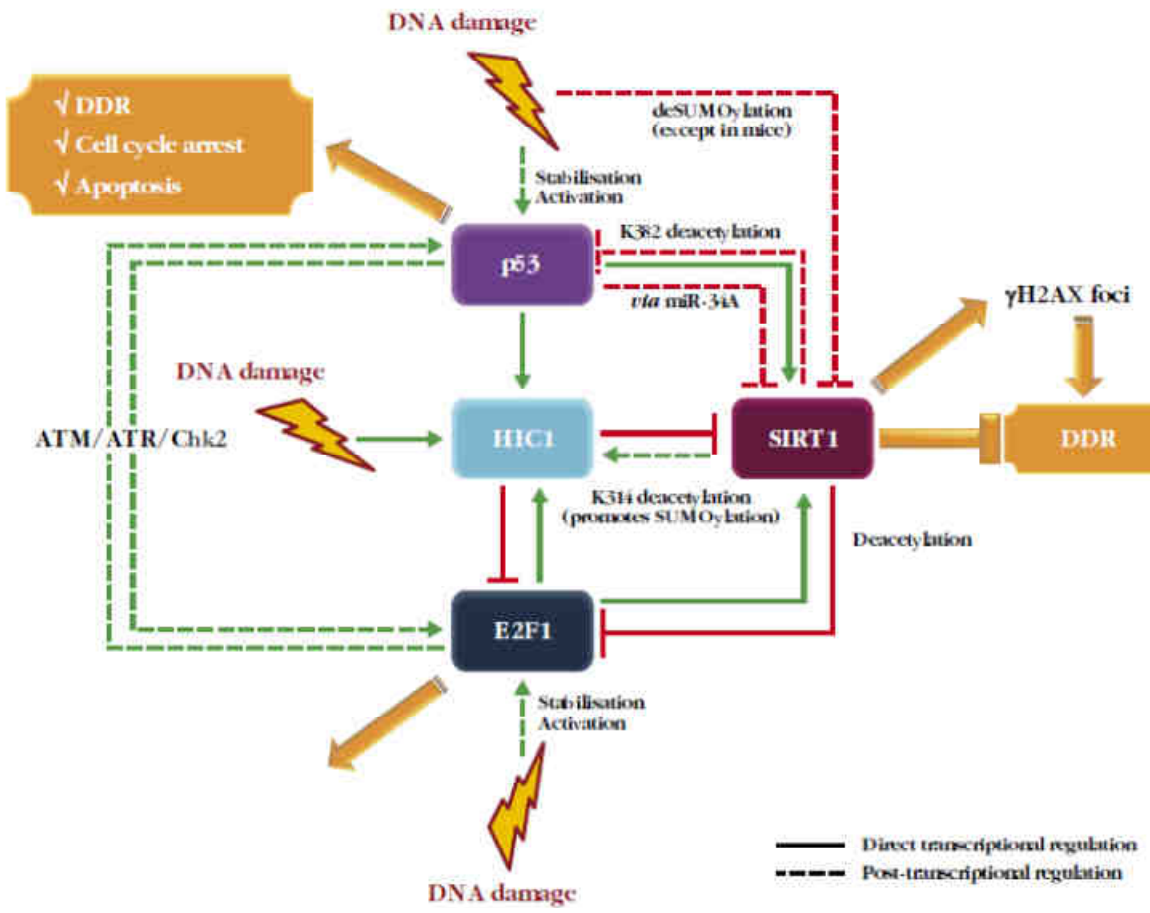


Figure 26: Les boucles de régulation et de rétrocontrôle en réponse aux dommages à l'ADN. Les dommages à l'ADN provoquent l'activation des protéines p53 et E2F1, qui vont activer la transcription du gène HIC1. La protéine HIC1 va stopper la transcription de *Sirt1* et *E2f1*, ce qui va permettre la régulation positive du suppresseur de tumeur p53. (Dehennaut et Leprince, 2009)

On sait depuis peu que p53 permet l'inhibition de la transcription TAT-dépendante du VIH-1 dans les cellules microgliales en limitant la phosphorylation de la sérine 2 du CTD de l'ARNpolII (Figure 27) (Mukerjee et al., 2010)

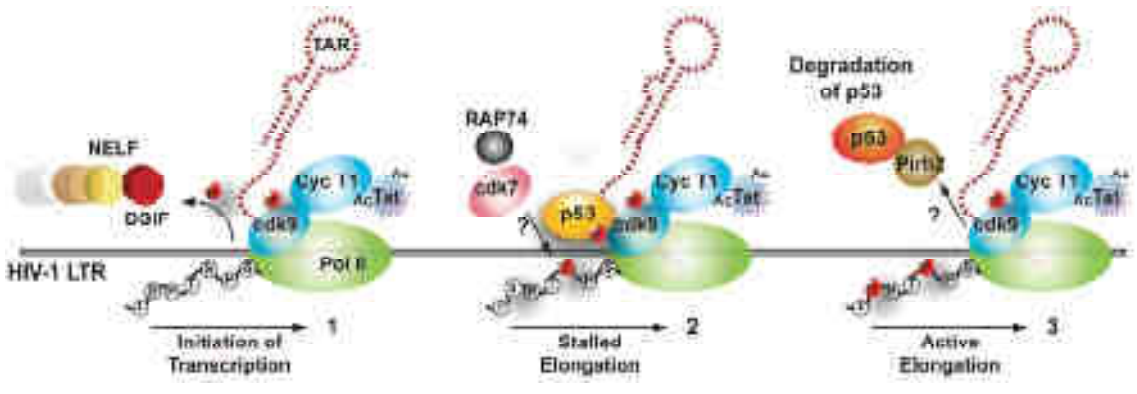


Figure 27 Mécanisme potentiel de l'activité de p53 sur la transcription du provirus. P53 semble en mesure de réduire l'activité kinase de la sous-unité CdK9 de P-TEFb en limitant la phosphorylation de la sérine 2 du CTD de la RNApol II. (Mukerjee et al., 2010)

HIC1 permet de contrôler l'expression de protéines ayant un rôle dans la régulation transcriptionnelle du VIH-1. De plus, nous savons que CTIP2 et HIC1 peuvent co-immunoprécipiter *in-vitro* (Leprince et al., unpublished). De ce constat, nous en sommes venus à nous demander si HIC1 pouvait lui aussi intervenir dans les mécanismes d'extinctions transcriptionnels du provirus et notamment ceux impliquant CTIP2.

J'ai donc cherché à savoir si HIC1 avait un impact sur la réplication et transcription virale et auquel cas les modalités et ses partenaires lors de son action sur le provirus.

Résultats

Publication 1

LSD1 cooperates with CTIP2 to promote HIV-1 trancriptional silencing.

Le Douce V, Colin L, Redel L, Cherrier T, Herbein G, Aunis D, Rohr O, Van Lint C, Schwartz C.
Nucleix Acid Research 2012

Les cellules microgliales sont la cible principale du VIH-1 lors de l'infection du système nerveux central (SNC) et constitue un important réservoir cellulaire de virus latents. L'établissement et le maintien de ces réservoirs dépend de la structure chromatinienne au niveau du provirus. Nous avons précédemment démontré que le co-facteur cellulaire CTIP2 induit la formation d'hétérochromatine et l'extinction transcriptionnelle du génome du VIH-1 en recrutant des activités histones deacetylase et histone méthyltransferase au promoteur viral. Les travaux suivants indiquent que la deméthylase LSD1 est capable de réprimer la transcription et l'expression du VIH-1 en coopérant avec CTIP2. Nos travaux font état d'un recrutement de LSD1 au promoteur viral proximal s'accompagnant des marques épigénétiques H3K4me3 et H3K9me3. Enfin, nos données suggèrent que la triméthylation de la lysine 4 de l'histone H3 induite par LSD1 est lié au recrutement de hSET1 au niveau du provirus.

J'ai effectué une partie des expériences initiales et la majeure partie du reviewing de cette publication.

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LSD1 cooperates with CTIP2 to promote HIV-1 transcriptional silencing

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ABSTRACT

Microglial cells are the main HIV-1 targets in the central nervous system (CNS) and constitute an important reservoir of latently infected cells. Establishment and persistence of these reservoirs rely on the chromatin structure of the integrated proviruses. We have previously demonstrated that the cellular cofactor CTIP2 forces heterochromatin formation and HIV-1 gene silencing by recruiting HDAC and HMT activities at the integrated viral promoter. In the present work, we report that the histone demethylase LSD1 represses HIV-1 transcription and viral expression in a synergistic manner with CTIP2. We show that recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic marks. Finally, our data suggest that LSD1-induced H3K4 trimethylation is linked to hSET1 recruitment at the integrated provirus.

INTRODUCTION

Eukaryotic DNA is wrapped around core histone proteins to form the chromatin (1). It is now well-established that the local state of chromatin influences transcription. A heterochromatin environment is more compact and structured than euchromatin, and is therefore repressive for transcription. On the contrary, euchromatin, a relaxed state of chromatin, is associated with active transcription. The compaction of chromatin and its permissivity for transcription depend on post-translational modifications of histones such as acetylation, methylation, sumoylation, phosphorylation and ubiquitinylation (2). It has been proposed that combination of such different covalent modifications of histone proteins may constitute a histone code and could be used to determine transcriptional status (3,4). The acetylation of a lysine in histones is mainly linked to gene activation, while lysine methylation can be associated to both gene activation and repression (5). For instance, methylation of H3K4 (Histone 3 Lysine 4), H3K36 and H3K79 have been associated to gene activation, whereas methylation of H3K9 and H3K27 have been linked to gene repression (6). The transcriptional activity of a gene is also regulated by the degree of histone methylation (mono, di or trimethylation). Trimethylation of H3K4 (H3K4me3) can exist in conjunction with H3K9 acetylation and is correlated to the activation of transcription (7,8), whereas dimethylation of H3K9 is linked to the recruitment of the deacetylase complex Set3, which induces gene repression (9). However, this epigenetic code is not always correlated with a corresponding transcriptional activity (10,11).

To date a great number of methyltransferases and demethylases has been shown to shape the pattern of lysine methylation. SUV39H1 has been involved in heterochromatin formation at the HIV-1 promoter and, as a consequence, in HIV silencing (12,13). The lysine specific demethylase (LSD1), discovered in 2004 (14), was initially associated to gene repression (15,16). This enzyme, which removes methyl groups from mono and dimethylated

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

H3K4, was characterized as a REST co-repressor. Additional binding partners of the LSD1-CoREST complex are histone deacetylases HDAC1 and HDAC2, which have been linked to transcriptional repression of several genes including the HIV-1 provirus (17). However, LSD1 has also been involved in the activation of transcription (18). Indeed, Metzger *et al.* (18) showed that LSD1 and the androgen receptor co-localize on promoters following hormone treatment. The recruitment of these two proteins did not alter H3K4 methylation but stimulated H3K9 demethylation, which led to transcriptional activation. Since LSD1 cannot remove methyl groups from trimethylated lysines, it has been proposed that LSD1 could serve as an anchored protein to recruit directly or indirectly H3K9 specific histone demethylases. Furthermore, both LSD1 and the H3K9 demethylase of the Jumonji-containing class belong to the same chromatin-remodelling complex, further supporting this hypothesis (19). However, the discovery that inhibition of LSD1 prevents lytic replication of the herpes simplex virus (HSV) as well as its reactivation from latency has added another level of complexity in our understanding of LSD1 function in gene regulation. Indeed, it was shown that HCF-1, which is a component of the SET1 and MLL1 H3K4 methyltransferase complexes, recruits LSD1 and induces H3K4 trimethylation and transcriptional activation of the HSV promoter (20–23). From an elegant approach that uses a variation of genome-wide chromatin immunoprecipitation called chromatin-immunoprecipitation (ChIP)-DSL, it appeared that LSD1 plays an even broader role in transcriptional activation as 80% of the 4200 LSD1-positive promoters were associated with RNA polymerase II and gene activation (24). These results underlined the dual role of LSD1 in gene activation and repression, and highlighted the complex role of lysine methylation in epigenetic regulation.

Here, we focused on the molecular mechanisms underlying HIV-1 transcription. We studied in more details the molecular mechanisms involved in the establishment and maintenance of HIV-1 latency in microglial cells, the main HIV-1 target cells in the central nervous system (CNS) (25). These long-lived latent reservoirs constitute a major obstacle to the eradication of HIV-1. Understanding the cell-type specific molecular mechanisms of establishment, maintenance and reactivation of HIV-1 latency is therefore crucial to achieve an efficient therapeutic intervention, in which the ultimate goal is to completely eradicate both latently and productively infected cells (26,27). We have previously shown that COUP-TF interacting protein 2 (CTIP2), a recently cloned transcriptional repressor that can associate with members of the COUP-TF family (28), inhibits HIV-1 replication in human microglial cells (29,30) by recruiting a chromatin-modifying complex (13). Indeed, our work showed a concomitant recruitment of histone deacetylases HDAC1 and HDAC2, and methyltransferase SUV39H1 to the viral promoter by CTIP2. Heterochromatin formation at the HIV-1 promoter has been linked to post-integration latency (26,31), suggesting that transcriptional repressors such as CTIP2 are involved in the establishment and maintenance of viral persistence and post-integration latency in the brain. The co-repressor CTIP2 has an even more pleiotropic action by regulating the expression of host genes in the infected cell. In this context, we have shown that CTIP2 silences p21 gene transcription by inducing epigenetic modifications, such as deacetylation and methylation of histones (32). This effect may indirectly favour HIV-1 latency since activation of the p21 gene stimulates viral gene transcription in macrophages (33). Moreover, CTIP2 counteracts HIV-1 Vpr which is required for p21 expression. In a recent article, we suggested that all these factors together contribute to HIV-1 transcriptional latency in microglial cells (32).

In the present work, we show that LSD1 represses HIV-1 transcription and viral expression in a synergistic manner with CTIP2. We report for the first time that recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic marks.

MATERIAL AND METHODS

Plasmids

Most of the constructs used in our assays have been described previously: pcDNA3, pFLAG-CTIP2 (28), pNL-4.3, pVSV.G, pRFP-CTIP2 (29), pTat-GFP (30), pshRNALSD1, pshRNA-Control and pFLAG-LSD1 were provided by E. Metzger and R. Schule (18). The episomal LTR-LUC, pshRNA-CTIP2, pSirenZsGreen-shRNA-CTIP2 plasmids have also been described (13).

Cell culture

The human microglial (provided by M. Tardieu, Paris, France) (34) and HEK 293 T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 100 U/ml penicillin–streptomycin. CTIP2 knocked-down microglial cells expressing shRNA-CTIP2 were stably established by infection of microglial cells with a pSirenZsGreen-ShRNA-CTIP2-based retrovirus as described by the manufacturer (Clontech Lab. Inc.). CTIP2 shRNA-expressing cells were sorted by flow cytometry for the concomitant expression of the ZsGreen protein and cultured in DMEM. The CTIP2 knock-down efficiency was controlled by western blot and ChIP experiments. The monocytic HIV-1 infected U1 cell line was maintained in RPMI 1640-Glutamax I medium supplemented with 10% fetal bovine serum and 50 U/ml penicillin–streptomycin.

Co-immunoprecipitation assays

HEK 293 T cells cultured in 100-mm diameter dishes were transfected using the calcium phosphate co-precipitation method with the indicated pFLAG-CTIP2 (30 µg), pFLAG-LSD1 (30 µg), pSuper control or pcDNA3-FLAG control (30 µg) vectors. Two days post-transfection, immunoprecipitations were performed using the standard technique with M2 anti-FLAG (Sigma) overnight at 4°C. Finally, the immunoprecipitated complexes were processed for SDS–PAGE and western blot analysis.

SDS–PAGE and western blot analysis

SDS–PAGE was performed using standard techniques. Proteins were detected using antibodies directed against the FLAG epitope (M2 mouse monoclonal from Sigma), LSD1 (Abcam), CTIP2 (bethyl) and β-actin (Sigma). Proteins were visualized by chemiluminescence using the Super Signal Chemiluminescence Detection System (Pierce).

Luciferase assays

Microglial cells cultured in 48-well plates were transfected with the indicated vectors using the calcium phosphate co-precipitation method. Total amounts of DNA were normalized with the corresponding control vector. Two days later, cells were collected and luciferase activity was determined using the Dual-Glo™ Luciferase Assay System (Promega). Values correspond to an average of at least three independent experiments performed in triplicate.

Viral replication

Microglial cells cultured in 12-well plates were transfected using the calcium phosphate co-precipitation method with HIV-1 pNL-4.3 and the expression plasmids as indicated. Total amounts of DNA were normalized with the corresponding empty vector. HIV-1 replication was monitored as described previously (30). Values correspond to an average of at least three independent experiments carried out in triplicate.

Pseudotyped virion production and single-round infection

The plasmid pNL-4.3-Env was co-transfected with the envelope plasmid encoding the pVSV.G envelope protein into HEK 293 T cells. Virions were collected 48 h post-transfection. For single-round infection, microglial cells were incubated with the VSV-pseudotyped HIV-1 NL4.3-Env virus for 24 h at 37°C.

Chromatin immunoprecipitation assays

Microglial and CTIP2 knocked-down microglial cells cultured in 150-mm diameter dishes were subjected to single-round infection by the VSV-pseudotyped viruses 24 h before being processed for ChIP experiments. HEK 293 T cells cultured in 100-mm diameter dishes were transfected using the calcium phosphate co-precipitation method with the indicated vectors or the corresponding control vectors. ChIP assays were performed using the ChIP assay kit (Upstate) 48 h post-transfection. Microglial and U1 cells were mock-treated or treated with PMA (100 nM) for 24 h before ChIP assays. The primary antibodies used for ChIP were as follows: anti-LSD1 (Abcam), anti-CTIP2 (bethyl), anti-RNA Pol II (Santa Cruz), anti-Sp1 (Upstate), anti-H3pan (Upstate), anti-Ac-H3 (Upstate), anti-H3K9me3 (Upstate), antiH3K4me3 (Upstate), anti-WDR5 (Abcam) and anti-hSET1 (Abcam). Immunoprecipitated DNA was subjected to real-time PCR quantification. The amplified regions of the provirus are indicated in the legend section. The specificity of the enrichment has been controlled by amplifications of the GAPDH gene (13).

Indirect immunofluorescence and confocal microscopy

Microglial cells cultured in 48-well plates were transfected or not using the calcium phosphate co-precipitation method with the pFLAG-LSD1, pRFP-CTIP2 or pTat-GFP expression vectors. Cells were fixed and permeabilized as described previously (30). The cover slips were then incubated for 1 h at room temperature with primary antibodies directed against LSD1 (Abcam), hSET1 (Abcam) or against the FLAG epitope (M2 mouse monoclonal; Sigma). The primary immunocomplexes were revealed by CY3- or CY5-labeled secondary anti-species antibodies. The stained cells were analysed by confocal microscopy using a Zeiss laser scanning microscope (model 510 invert) equipped with a Planapo oil (63×) immersion lens (numerical aperture = 1.4).

RESULTS

LSD1 represses HIV-1 replication and transcription in microglial cells

The function of LSD1 in HIV-1 infected cells was investigated by using an LSD1 knock-down strategy. We co-transfected microglial cells with a complete HIV-1 infectious provirus (pNL-4.3) and with or without a shLDS1 expressing vector. The efficiency of the knock-down of LSD1 was checked by western blot (Figure 1A). As shown in Figure 1A, the knock-down of LSD1 was associated with a 6-fold increase in p24 production, which argues in favour of a repressive role of LSD1 in HIV-1 replication. We next investigated whether LSD1 has a direct impact on transcription of the HIV-1 genes since this protein is involved in the transcriptional regulation of many cellular genes. Microglial cells were transfected with the episomal LTR-Luc vector with or without the shLSD1 expressing vector in the absence (Figure 1B) or presence (Figure 1C) of Tat. In the absence of Tat, LSD1 repressed LTR transcriptional activity in a dose-dependent manner (Figure 1B columns 2 and 3). When Tat was expressed together with the shLDS1 vector, we observed a synergistic activation of LTR-driven transcription (Figure 1C column 4 compared to columns 2 and 3). Thus LSD1 inhibits HIV-1 replication as a result of transcriptional repression occurring at both the early Tat-independent and the late Tat-dependent steps.

LSD1-mediated repression of HIV-1 is associated with the epigenetic marks H3K4me3 and H3K9me3

To investigate whether LSD1 is recruited at the HIV-1 promoter *in vivo*, we performed ChIP assays using microglial cells transfected with the pNL-4.3 provirus. Over-expression of LSD1 was associated to an increase of H3K9 trimethylation (H3K9me3) and more surprisingly to an increase of H3K4 trimethylation (H3K4me3) (Figure 2A columns 3 and 4) in the proximal region of the HIV-1 promoter. In agreement, knocking-down endogenous LSD1 expression disfavoured H3K9 and

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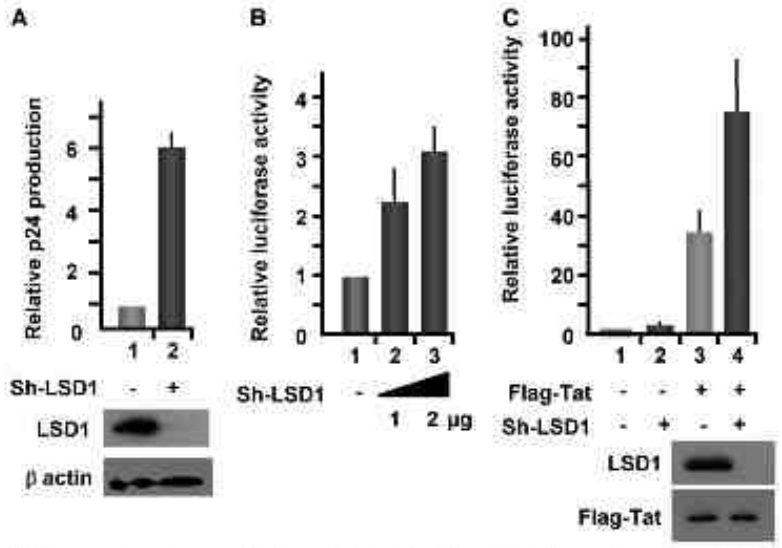

Figure 1. LSD1 represses HIV-1 gene transcription and viral replication. (**A**) Microglial cells were transfected with the pNL-4.3 and the indicated vectors. Culture supernatants were analysed for p24 Gag contents 48 h post-transfection. (**B** and **C**) Microglial cells were transfected with the episomal LTR-LUC and the indicated vectors. DNA amounts were normalized in all transfection assays with pshRNA-Control or peDNA3-FLAG control vectors. Luciferase activities were measured 2 days post-transfection and expressed relative to the value obtained with episomal LTR-LUC alone. (A and C) The knock-down efficiency of sh-RNA constructs (versus sh-control) has been controlled by western blot analysis.

H3K4 trimethylation (Figure 2A columns 3 and 4). Interestingly, knocking-down LSD1 in HIV-1 transfected microglial cells was associated with a strong increase of H3 global acetylation level (Figure 2A column 6) and with a stable amount of H3 histones associated to the viral promoter (Figure 2A column 5). Furthermore, knocking-down LSD1 strongly increased the recruitment of the RNA pol II to the HIV-1 promoter, thereby confirming the activated status of the viral promoter (Figure 2A column 7). From these results we speculated that HIV-1 reactivation in latently infected U1 cells could be associated with a release of LSD1 and a concomitant decrease of H3K9 and H3K4 trimethylation levels at the HIV-1 promoter. To test this hypothesis, histone methylation marks and LSD1 recruitment at the HIV-1 promoter were monitored in the latently infected U1 cell line after activation of viral gene transcription. As shown in Figure 2B, PMA (phorbol-12-myristate 13-acetate) treatment induced a release of the endogenous LSD1 from the viral promoter (column 2). Moreover, this phenomenon was associated with decreased trimethylation levels of H3K4 and H3K9 (columns 3 and 4 from Figure 2B, respectively). As expected, the release of the endogenous LSD1 following PMA treatment of the latently infected U1 cell line was associated with an increase of the global histone H3 acetylation level and with an increased RNA pol II recruitment to the HIV-1 promoter (Figure 2B columns 6 and 7). To verify whether LSD1 is specifically located at the promoter region of the HIV-1 genome, we performed several ChIP experiments with additional sets of primers hybridizing in adjacent regions of the viral genome. As shown in Figure 2C, LSD1 was only associated to the proximal promoter region (columns 1 and 2) and not with adjacent regions such as intragenic Gag or Vpr regions (columns 3 and 4). Moreover, the epigenetic marks associated with the loss of LSD1 we described above were also observed with other LSD1 regulated genes since we observed the same events with the LSD1-regulated gene CEBP alpha (Figure 2D, columns 5, 6 and 7), as previously described (35). In accordance with the literature (36), we showed that knock-down of LSD1 is correlated with an increase of H3K4me3 in the promoter region of LSD1 regulated genes such as SCN1A, SCN3A (data not shown) and SCN2A2 (Figure 2D columns 2, 3 and 4), suggesting that these LSD1-sensitive genes may be regulated by molecular mechanisms linked to the previously described enzymatic activity of LSD1. As a control, we showed that LSD1 is not associated to the promoter of the LSD1-insensitive gene GAPDH (Figure 2D). In addition, H3K4 trimethylation level at the GAPDH promoter was not sensitive to the modulation of LSD1 expression.

Taken together, these data suggest that LSD1 is recruited to the HIV-1 promoter and thereby represses its transcriptional activity. However, we were unable to show that this repression was linked to its previously characterized H3K4 or H3K9 demethylase activities.

LSD1 represses HIV-1 LTR-driven gene expression through the Sp1-binding sites of HIV-1 promoter

In order to identify the LTR region allowing the LSD1-mediated repression of HIV-1 transcription in microglial cells, we performed transient transfection experiments with 5′ truncations or mutations of the LTR-Luciferase

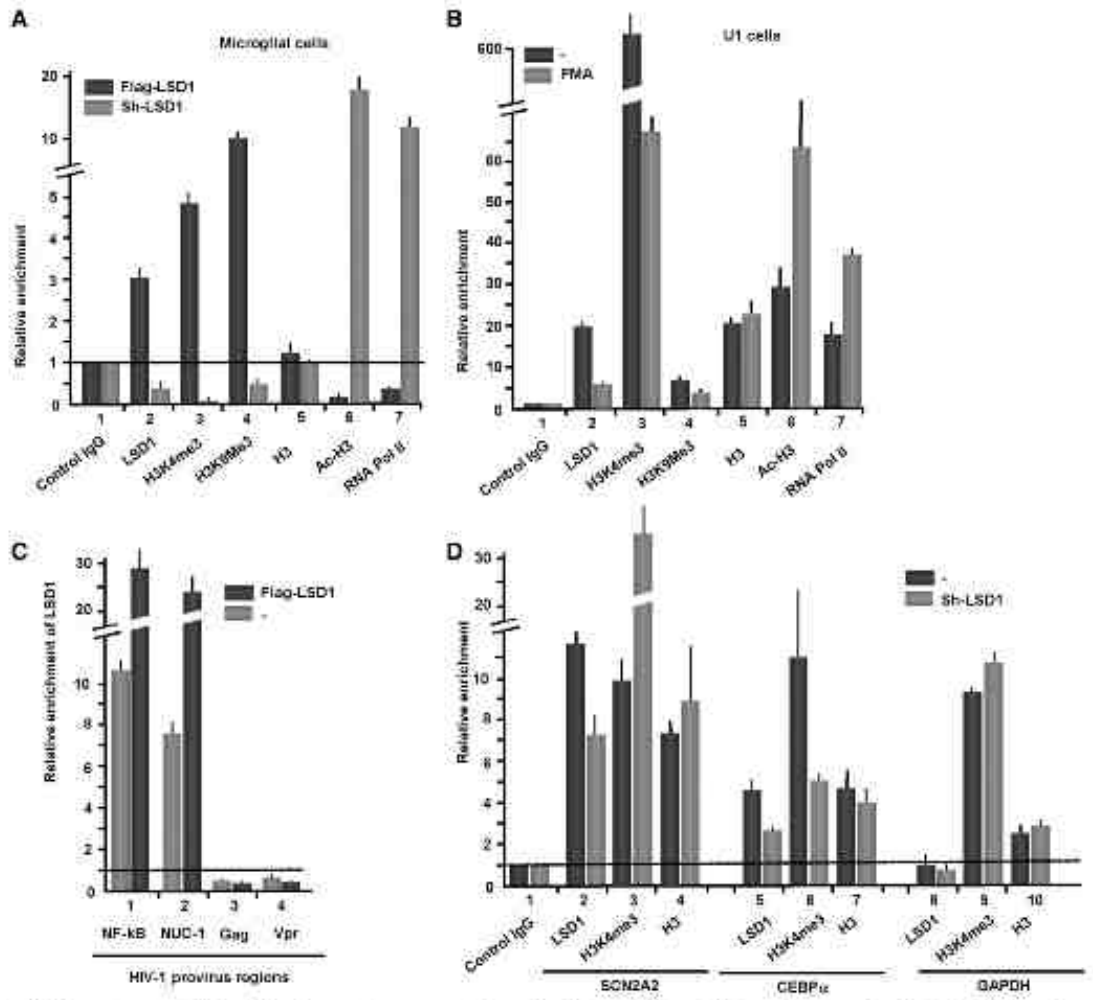

Figure 2. LSD1 association with the HIV-1 proximal promoter induces local trimethylation of histone 3 lysines 4 and 9. (**A**) ChIP experiments were performed on microglial cells transfected with the pNL-4.3 provirus in the presence of the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Nuc-1 region. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or depletion were expressed relative to the value obtained with the pcDNA3-FLAG or the pshRNA-control vectors, respectively. (**B**) Mock-treated and PMA-treated U1 cells were subjected to ChIP experiments with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Nuc-1 region. The amounts of immunoprecipitated material were normalized to the input DNA and presented relative to the non specific IgG precipitation. (**C**) pcDNA3 and pFLAG-LSD1 transfected microglial cells were subjected to ChIP experiments with the anti-LSD1 antibody. Specific enrichment of the NF-kB and the Nuc-1 regions of the promoter and the Gag and Vpr intragenic regions are presented relative to the non specific enrichment obtained with the control IgG setted at 1. (**D**) pshRNA-control and pshRNA-LSD1 transfected microglial cells were subjected to ChIP experiments with the indicated antibodies. Specific enrichment of the SCN2A2, CEBPα and GAPDH promoters are indicated relative to the control IgG.

vector in the context of ectopic LSD1 over-expression or endogenous LSD1 knock-down. While LSD1 over-expression repressed the luciferase expression of the full-length and the proximal LTR constructs, knocking-down LSD1 stimulated these transcriptional activities (Figure 3A lanes 1 and 2). Interestingly, mutation of the Sp1-binding sites abrogated LSD1-mediated repression, suggesting the involvement of this LTR region in LSD1 recruitment (Figure 3A lane 3). To establish whether the endogenous LSD1 associates with the viral promoter via the Sp1-binding sites, we performed ChIP experiments with the wild-type LTR-Luc (wild-type 1 789) and the Sp1-binding sites-mutated vectors. As shown in Figure 3B (column 3), mutation of the Sp1-binding sites abolished LSD1 recruitment at the vial promoter. Interestingly, we observed that the epigenetic mark H3K4me3 is preferentially detected in the wild-type LTR (pLTR-Luc wt) (Figure 3B column 4). Since it has been shown that

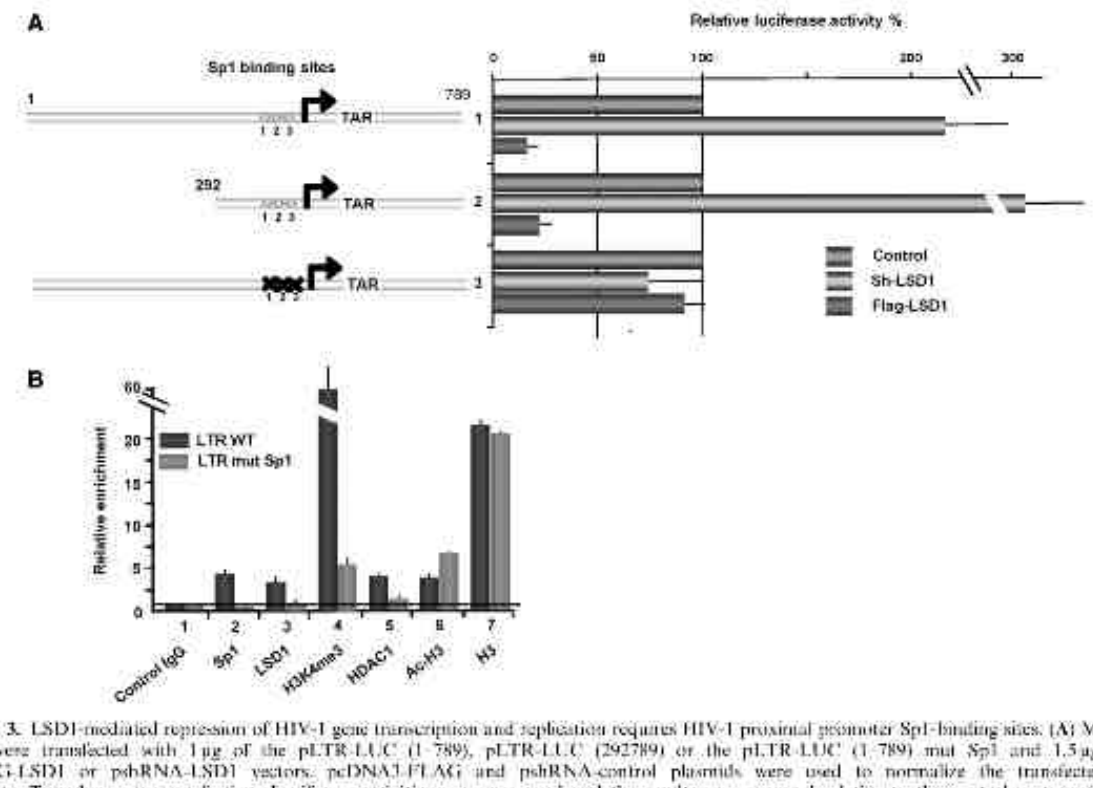

Figure 3. LSD1-mediated repression of HIV-1 gene transcription and replication requires HIV-1 proximal promoter Sp1-binding sites. (**A**) Microglial cells were transfected with 1 μg of the pLTR-LUC (1–789), pLTR-LUC (292–789) or the pLTR-LUC (1–789) mut Sp1 and 1.5 μg of the pFLAG-LSD1 or pshRNA-LSD1 vectors. pcDNA3-FLAG and pshRNA-control plasmids were used to normalize the transfected DNA amounts. Two days post-transfection, Luciferase activities were measured and the results are expressed relative to the control vectors. (**B**) HEK 293 T cells were transfected with the pLTR-LUC (1–789) or with the pLTR-LUC (1–789) mut Sp1 vector 48 h before being subjected to ChIP experiments with the indicated antibodies. Input and immunoprecipitated DNAs were quantified by real-time PCR using primers targeting the Sp1-binding sites region of the HIV-1 promoter. The amounts of immunoprecipitated material were normalized to the input DNA and results are presented relative to the non-specific control IgG.

Sp1 recruits HDAC1 (37), we compared the association of HDAC1 on the WT and the Sp1-mutated viral promoter. As shown in Figure 3B, the global recruitment of HDAC1 to the HIV-1 promoter was mostly abrogated by the mutation of the Sp1-binding sites (column 5). Interestingly, this was correlated with a small increase of the H3 acetylation level (Figure 3B column 6) and a strong decrease of the H3K4 trimethylation level (Figure 3B column 4). In a control experiment, we verified that the same amounts of histone H3 was found on both constructs (Figure 3B column 7).

LSD1 cooperates with CTIP2 to repress HIV-1 replication and transcription

We have previously shown that CTIP2 represses HIV-1 gene transcription in microglial cells (13). We therefore hypothesized that CTIP2 and LSD1 could cooperate to repress HIV-1 replication and transcription. As shown in Figure 4A, the knock-down of both factors synergistically activated HIV-1 replication (30-fold activation with the double knock-down compared to the 5-fold and 10-fold activations observed with the LSD1 and CTIP2 single knock-downs, respectively).

The transcriptional impacts of LSD1 and CTIP2 knock-downs were then assessed in the presence or absence of Tat. Single knock-down of CTIP2 stimulated transcription in the absence and presence of Tat (Figure 4B columns 2 and 3 and C columns 3 and 4). However, combination of LSD1 and CTIP2 knock-downs further increased HIV-1 gene transcription in the absence or presence of Tat (Figure 4B column 4 and 4 C column 5). These results strongly suggest a functional cooperation between LSD1 and CTIP2 in the repression of HIV-1 transcription in a chromatinized promoter. As controls, knock-down and over-expression efficiencies were verified by western blot (Figure 4D).

LSD1 interacts with CTIP2 and co-localizes with Tat and CTIP2 in the nucleus

Our data strongly suggest a functional cooperation between LSD1 and CTIP2. We therefore investigated whether these proteins could interact physically. To this end, we performed FLAG-targeted immunoprecipitation experiments with nuclear extracts from cells expressing FLAG-LSD1 or FLAG-CTIP2 proteins. As shown in Figure 5A, FLAG-CTIP2 and FLAG-LSD1 co-immunoprecipitated with endogenous LSD1 and CTIP2 proteins,

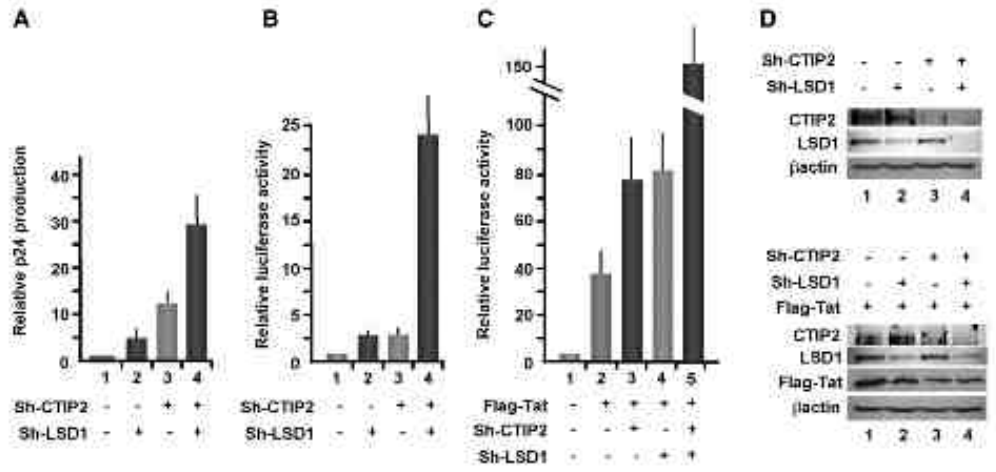

Figure 4. LSD1 cooperates with CTIP2 to repress HIV-1 gene transcription and viral replication. (**A**) Microglial cells were transfected with pNL-4.3 and the indicated plasmids (columns 2 to 4) or the pshRNA-control vector (column 1). Culture supernatants were analysed for p24 contents 48 h post-transfection. (**B** and **C**) Microglial cells were transfected with the episomal LTR-LUC and the indicated plasmids or the pshRNA-control vector. Luciferase activities were measured 2 days post-transfection and expressed relative to the value obtained with the episomal LTR-LUC and the control vectors (columns 1). DNA quantities were normalized with the pshRNA-control vector. (**D**) The knock down efficiency of sh-RNA constructs was controlled by western blot analysis. The control columns 1 of the panels correspond to extracts from cells transfected with the pshRNA-control vector.

respectively, arguing for a physical interaction between these two proteins. We next investigated whether LSD1 co-localizes with Tat as previously shown for CTIP2 (30). Cells transfected with a RFP-CTIP2 expressing vector in the presence or not of GFP-Tat were examined for endogenous LSD1 localization using confocal microscopy. Endogenous LSD1 expression was observed in both the cytoplasm and the nucleus (Figure 5B, pictures 4 and 6). As previously described (13), nuclear expression of CTIP2 harboured ball-like structures (Figure 5B, pictures 7 and 9). As shown in Figure 5C (pictures 5–8), LSD1 and CTIP2 co-localized in the CTIP2-induced nuclear structures (30), suggesting that CTIP2 relocated LSD1 into these structures. Interestingly, GFP-Tat expression relocated LSD1 from the cytoplasm to the nucleus (Figure 5C, pictures 1–4). Finally, observations of the concomitant expressions of RFP-CTIP2 and GFP-Tat revealed co-localization of both proteins with LSD1 in the nucleus (Figure 5D). Staining of genomic DNA are presented in Figure 5B. Altogether, these results support that CTIP2 and LSD1 interact physically and that LSD1 is re-localized by CTIP2 and Tat in dense sub-nuclear structures.

LSD1 is required for CTIP2 recruitment at the HIV-1 proximal promoter

We next asked whether LSD1 is required for CTIP2 recruitment to the HIV-1 promoter. To address this question, we performed additional ChIP experiments in the LSD1 over-expression or LSD1 knock-down contexts. As shown in Figure 6A, over-expression of LSD1 was associated with an increase of endogenous CTIP2 recruitment to the viral promoter. As expected, LSD1 knock-down decreased CTIP2 association with the viral promoter (Figure 6A). To further study LSD1 and CTIP2 recruitment at the HIV-1 promoter, we performed ChIP experiments with HIV-1 infected microglial cells expressing (control) or not CTIP2 (shCTIP2) (Figure 6B). As a control, we checked that CTIP2 is less recruited onto the HIV-1 proximal promoter in the infected shCTIP2 microglial cell line (Figure 6B column 2 compared to column 1) compared to the control cell line. Unexpectedly, knocking-down CTIP2 slightly increased LSD1 recruitment to the LTR (Figure 6B column 3). Moreover, this recruitment was correlated with an increased H3K4 trimethylation (Figure 6B column 4). These results suggest that LSD1 is required for CTIP2 recruitment to the HIV-1 proximal promoter.

LSD1-mediated repression and H3K4 trimethylation are associated with the recruitment of hSET1 and WDR5 to the HIV-1 proximal promoter

The epigenetic mark H3K4me3 has been shown to be associated with LSD1 recruitment (20,21). This association results from the interaction of LSD1 with a methyltransferase complex containing WDR5 and hSET1 (20,21). ChIP experiments performed with cells over-expressing LSD1 confirmed an increased recruitment of hSET1 and WDR5, two members of the hCOMPASS complex (Figure 7A, blue columns 4 and 5) to the HIV-1 promoter, together with an increased H3K4 trimethylation. Inversely, knocking-down endogenous LSD1 decreased hSET1 and WDR5 association to the viral promoter and H3K4 trimethylation (Figure 7A pink columns). ChIP experiments performed with the wt-LTR-Luc or the Sp1-mutated LTR-Luc reporter constructs further confirmed that hSET1, WDR5 and LSD1 are recruited concomitantly to the proximal Sp1-binding

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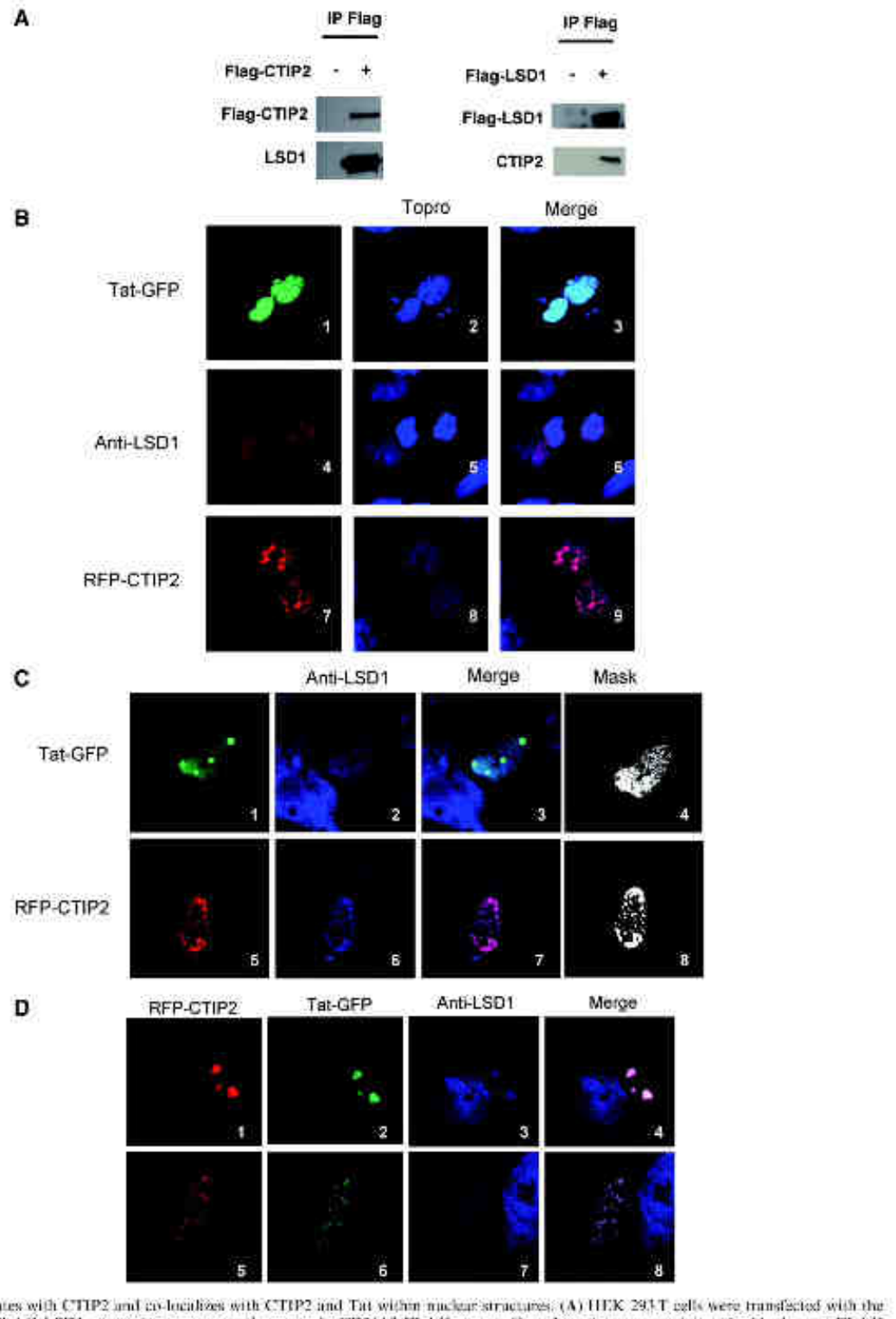

Figure 5. LSD1 associates with CTIP2 and co-localizes with CTIP2 and Tat within nuclear structures. (**A**) HEK 293T cells were transfected with the pFLAG-CTIP2, the pFLAG-LSD1 expression vectors or the control pCDNA3-FLAG vector. Complexes immunoprecipitated with the anti-FLAG antibody were immunodetected for the presence of FLAG-CTIP2, FLAG-LSD1, endogenous LSD1 and CTIP2 proteins by western blot as indicated. (**B–D**) Microglial cells were transfected with pTat-GFP or/and pRFP-CTIP2 as indicated and accessed for endogenous LSD1 immunodetection with primary anti-LSD1 antibodies. The primary immunocomplexes were revealed by CY3- or CY5-labeled secondary antibodies. Mask columns show the co-localized staining.

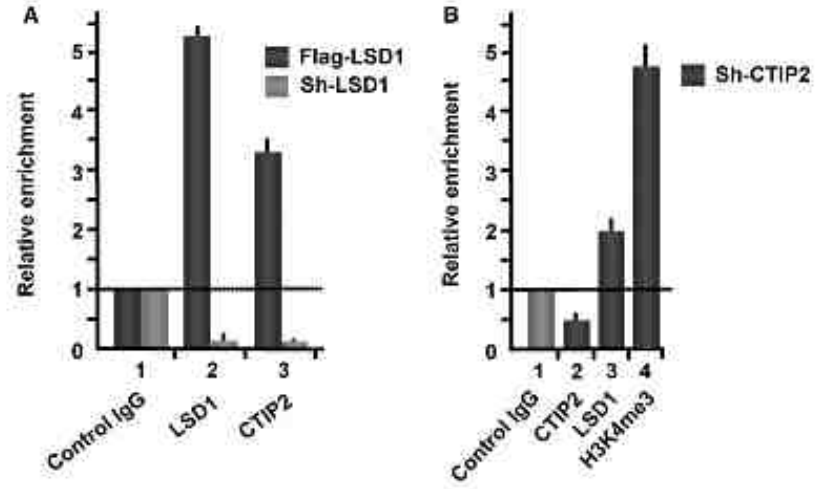

Figure 6. CTIP2 recruitment on the HIV-1 proximal promoter requires LSD1. (**A**) ChIP experiments were performed on HEK 293 T cells transfected with the HIV-1 LTR-LUC episomal vector in the presence of the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Sp1-binding sites. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or LSD1 depletion were expressed relative to the value obtained with the pcDNA3-FLAG and the pshRNA-control vectors, respectively. Results were expressed relative to the value obtained with the episomal LTR-LUC plasmid co-transfected with the pcDNA3-FLAG or the pshRNA-control vectors. (**B**) Control and CTIP2 knocked-down microglial cells were infected with the VSV-pseudotyped pNL-4.3-Env virus 24 h before being subjected to ChIP experiments with the indicated antibodies. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of CTIP2 depletion were expressed relative to the value obtained with the control cells. Specific enrichments at the HIV-1 proximal promoter were quantified by real-time PCR targeting the LTR-Sp1-binding sites region.

sites of the viral promoter. Indeed, the abrogated association of LSD1 to the Sp1-mutated LTR (Figure 7B lane 2 column versus column) correlated with a reduced recruitment of both hSET1 and WDR5 (Figure 7B, columns 3 and 4). From these results, we hypothesized that HIV-1 reactivation in microglial cells could be associated with a release of LSD1 and an alongside reduced recruitment of hSET1 and WDR5 to the HIV-1 promoter. In agreement with these results, PMA treatment released LSD1, WDR5 and hSET1 from the viral promoter of HIV-1 infected cells (Figure 7C, columns 2, 4 and 5) and decreased H3K4 trimethylation (Figure 7C column 3). Taken together, our data suggest that LSD1-associated increase of H3K4 trimethylation at the HIV-1 proximal promoter region might be linked to hSET1 and WDR5 recruitment.

DISCUSSION

The introduction of HAART in 1996 has raised hopes for curing patients infected with HIV-1. Unfortunately, long-term suppression of HIV-1 replication has unveiled the existence of latent HIV-1 reservoirs such as resting $CD4^+$ T cells and monocytes/macrophages (25,38). Microglial cells, the CNS-resident macrophages, are the brain major targets for HIV-1 and constitute latently infected cells (39). Understanding the molecular mechanisms of establishment, maintenance and reactivation of HIV-1 latency in microglial cells is therefore crucial for efficient therapeutic intervention (26,27). In recent papers, we reported that CTIP2 inhibits HIV-1 replication and transcription in microglial cells (29,30) by recruiting a chromatin-modifying complex which contains histone deacetylases (HDAC1 and HDAC2) and a histone methyltransferase (SUV39H1) (13). A better comprehension of the molecular mechanisms involved in establishment and maintenance of HIV-1 latency would be achieved by the identification of additional factors able to induce heterochromatin formation at the viral promoter (26,31). Since histone and DNA methylations have been implicated in the silencing of the integrated provirus (12,13,40,41), we further investigated the influence of specific demethylase enzymes. LSD1, a demethylase first identified by Shi *et al.* (14) as a transcriptional repressor, constituted therefore a potential candidate to play a role in HIV-1 silencing. In this report, we first showed that LSD1 represses HIV-1 expression by inhibiting the transcription step of the viral life cycle. ChIP experiments revealed that LSD1 is recruited to the HIV-1 proximal promoter. In addition, the repressive epigenetic mark H3K9me3 was linked to LSD1 recruitment at the viral promoter. Since LSD1 was initially characterized as a repressor associated with demethylation of H3K4 (42), we focused our investigation on this point. Surprisingly, we observed an increased H3K4 trimethylation level in the HIV-1 proximal promoter region upon LSD1 recruitment. Such a pattern has previously been described in a previous work in the context of the HSV promoter by Liang *et al.* (21). However, these authors have further shown that this H3K4 trimethylation correlated with a demethylation of H3K9 and with transcriptional activation (21). Here, we demonstrate for the first time that LSD1-induced

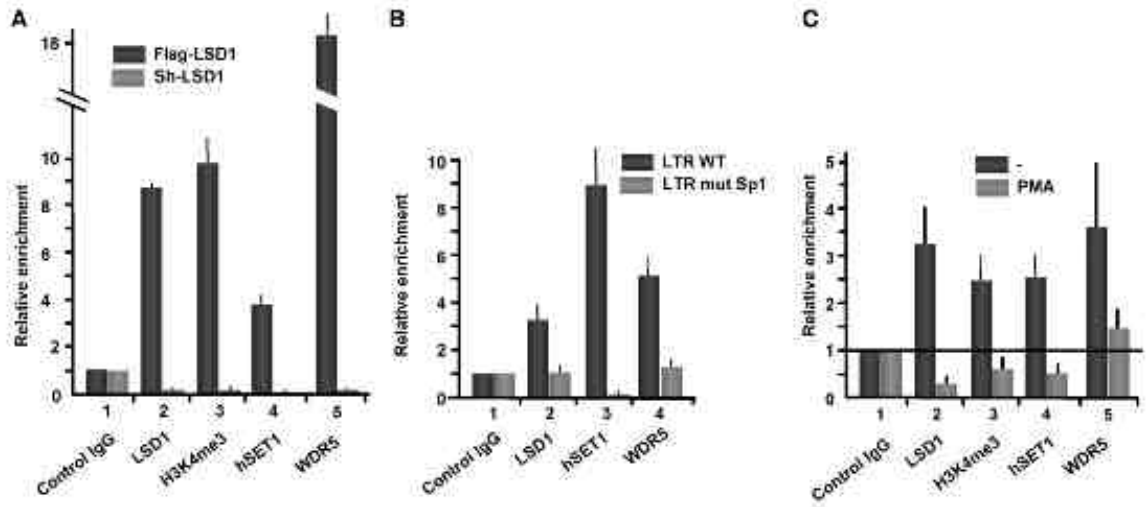

Figure 7. LSD1 favours hSET1 and WDR5 recruitment at the HIV-1 proximal promoter. (**A**) ChIP experiments were performed on HEK 293 T cells transfected with the HIV-1 LTR-LUC episomal vector in the presence the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or depletion were expressed relative to the value obtained with the pcDNA3-FLAG or the pshRNA-control vectors, respectively. Specific enrichments in the HIV-1 proximal promoter region were quantified by real-time PCR. (**B**) HEK 293T cells were transfected with the pLTR-LUC (1 789) or with the pLTR-LUC (1 789) mut Sp1 vector 48 h before being subjected to ChIP experiments with the indicated antibodies. Input and immunoprecipitated DNAs were quantified by real-time PCR using primers targeting the Sp1-binding sites region of the HIV-1 promoter. The amounts of immunoprecipitated material were normalized to the input DNA and presented relative to the non specific control IgG. (**C**) Microglial cells were infected with the VSV-pseudotyped pNL4.3-Env virus 24 h before being subjected to ChIP experiments with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR. Enrichments are presented relative to the non specific IgG values set at 1.

transcriptional repression can be associated with both H3K4me3 and H3K9me3 epigenetic marks. We further show that these epigenetic marks are linked to LSD1 recruitment at the HIV-1 proximal promoter through the Sp1-binding sites as previously demonstrated for CTIP2 (13). Our results are in agreement with the model presented by the David Margolis group who demonstrated that HDAC1 is released from the HIV-1 promoter upon mutation of the Sp1-binding sites (37). Indeed, we demonstrated that LSD1 and HDAC1 are associated with the wild-type but not with the Sp1 mutated promoter. New functional and biochemical investigations will be needed to determine if these enzymes compete or cooperate for their binding to the HIV-1 promoter. However, it is now clear that both enzymes are involved in the silencing of HIV-1 gene transcription.

We next evaluated whether two epigenetic marks, H3K9me3 and H3K4me3, could result from LSD1 recruitment onto the HIV-1 promoter. Regarding H3K9me3, we speculated that LSD1 could cooperate with CTIP2 as we had previously shown that this factor recruits SUV39H1, enzymes which specifically methylates H3K9 (13). In agreement with this hypothesis, we showed here that LSD1 cooperates functionally with CTIP2 to repress HIV-1 replication and transcription in a synergistic manner. We further demonstrated that this cooperation correlates with a physical interaction between CTIP2 and LSD1. ChIP assays showed that LSD1 and CTIP2 interact physically, and confocal microscopy experiments suggested an *in vivo* interaction between these two proteins, which was found to occur in previously characterized CTIP2-induced nuclear structures (30). In addition, the transactivator Tat relocated LSD1 in the nucleus. Since we believe that these ball-like nuclear structures reflect a heterochromatin environment, the co-localization of LSD1 with both CTIP2 and Tat strongly suggests that LSD1 is involved in promoting local heterochromatin environment to repress HIV-1 gene transcription in microglial cells. Moreover, LSD1 seems to have a more critical role in promoting HIV-1 silencing than CTIP2. Indeed, we showed that LSD1 is required for CTIP2 recruitment onto the HIV-1 proximal promoter whereas the reverse is not observed.

Mechanisms underlying LSD1-mediated increase of H3K4 trimethylation might rather rely on LSD1 ability to anchor other factors at the promoter than to its own enzymatic activity. Indeed, LSD1 is also known to be associated with the recruitment of hSET1 and WDR5, two members of the hCOMPASS methyltransferase complex, which is believed to induce the trimethylation of H3K4 (43–46). In this context, LSD1, through its interaction with HCF-1, recruits this methyltransferase complex containing WDR5 and hSET1 to the HSV promoter (21). Here, we showed that LSD1 favours the recruitment of such a complex to the HIV-1 promoter. H3K4 trimethylation was associated with the recruitment of LSD1, hSET1 and WDR5 at the Sp1-binding sites of the HIV-1 LTR. Moreover, reactivation of HIV-1

proviruses correlated with the release of LSD1, hSET1 and WDR5 from the viral promoter and with a reduced H3K4 trimethylation. All together, our results strongly suggest that LSD1 is involved in the establishment and the maintenance of HIV-1 latency in microglial cells by favouring a local heterochromatin structure.

Association of both H3K4me3 and H3K9me3 epigenetic marks with LSD1 recruitment may thus constitute a new level of eukaryotic gene regulation. These observations are consistent with the discovery that H3K4 methylation at certain chromatin loci may prevent gene expression (11). Interestingly, such a gene repression linked to H3K4me3 has been proposed to prevent the expression of cryptic promoters (5,11). This is strengthened by the findings that HIV-1 preferentially integrates in active genes and therefore could be considered as a cryptic gene. Additional mechanisms including transcriptional interference are believed to prevent expression of such cryptic promoters (47). It could be argued that H3K4me3 is already present on the HIV-1 promoter upon integration into the host cell genome as it integrates into active genes enriched with euchromatic histone modifications such as H3K4 methylation and histones acetylation (48). The subsequent silencing characterized by H3K9me3 would then occur through LSD1-mediated recruitment of the CTIP2-HDAC-SUV39H1 complex. However, the fact that both H3K4me3 and H3K9me3 were lost following reactivation in association with the release of LSD1 from the HIV-1 promoter argues that, at least in the context of HIV-1 promoter, these two epigenetic marks are associated with transcriptional repression and favour the establishment and the maintenance of latency.

The exact mechanisms underlying LSD1 function in HIV-1 repression are complex and far to be elucidated. Although we showed in this report that LSD1 interacts physically and cooperates functionally with CTIP2, it remains to be determined whether all the components recruited to the HIV-1 promoter are in the same complex or whether they interact with LSD1 independently. Even if the exact mechanisms by which LSD1 promotes a local heterochromatin structure remain unknown, LSD1 constitutes a new target for potential therapeutic strategies aiming at purging the HIV-1 latent reservoirs.

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Publication 2 – En préparation

HIC1 and SIRT1 cooperate to repress TAT-mediated HIV-1 transcription.

Le Douce V, Rohr O, Schwartz C.

Depuis l'introduction des multi-thérapies, l'infection au VIH-1 est maintenue sous contrôle dans la plupart des patients. Malheureusement, les multi-thérapies ne permettent pas l'éradication du virus. En effet, le virus peut rester hors de porté des traitements dans des sanctuaires anatomiques, où la biodisponibilité des antirétroviraux est moindre, ou dans des réservoirs cellulaires, où le virus est intégré de façon latente. Les cellules microgliales sont les macrophages résidents du système nerveux central (SNC) et la cible privilégiée du VIH-1 dans le cerveau. Le SNC est un des sanctuaires viraux les plus étudié, tandis que les cellules microgliales ont été décrites comme un réservoir cellulaire avec une longue durée de vie. Ce genre de cellule, infectée de façon latente, apparaît comme un des principaux obstacles à l'éradication virale et la guérison complète des patients. Ainsi, la compréhension des mécanismes sous-jacents impliqués dans l'extinction de la transcription virale semble une étape cruciale afin d'arriver à une cure. Nous avons précédemment démontré que le facteur transcriptionnel CTIP2 coopère avec la deméthylase LSD1 afin d'établir un environnement hétérochromatinien au niveau du promoteur viral. Dans le présent rapport, nous indiquons comment le suppresseur de tumeur HIC1 est en mesure de réprimer la transcription virale sous gouvernance du transactivateur viral TAT en interagissant avec la déacétylase de classe III SIRT1. HIC1 peut en effet neutraliser l'activité transactivatrice de TAT, favorisant ainsi l'établissement et le maintien de la latence. Il est intéressant de noter qu'une mutation ponctuelle dans le motif de SUMOylation/Acetylation de HIC1 est suffisante pour abroger sa capacité à réprimer la transcription virale.

J'ai effectué l'intégralité des expériences présentées dans ce compte-rendu.

Since the introduction of combination antiretroviral therapy (cART) HIV-1 has been kept under control in the majority of patients. Unfortunately cARTs do not lead to total eradication of the virus. The virus can indeed stay out of reach of the cART in sanctuaries where drugs bioavailability is poor or in cell reservoir where the provirus is silently integrated. Microglial cells are resident macrophages of the central nervous system (CNS), and also the primary target of HIV-1 in brain. CNS is one of the most studied reservoir and microglial cells have been described as a long lived HIV-1 cell reservoir. Such latently infected cells constitute the main obstacle to virus eradication. Therefore understanding of the underlying mechanisms implicated in the viral transcriptional silencing appears to be a critical step towards an HIV cure. We previously demonstrated that the cellular transcriptional factor CTIP2 cooperates with the demethylase LSD1 to establish heterochromatin in the vicinity of the viral promoter. In this report we show how the tumor suppressor HIC1 is able to repress TAT-mediated viral transcription by interacting with the class III deacetylase SIRT1. HIC1 can indeed counteract the transactivating activity of the viral protein TAT thus favouring latency establishment. Interestingly a single mutation in the HIC1 acetylation/SUMOylation switch is sufficient to render HIC1 unable to repress the viral transcription.

For over two decades combination antiretroviral therapy (cART) helped to successfully maintain the immune system and control viral load in HIV-1 infected patients. Unfortunately cART cannot act as a sterilizing cure due to the existence of latent proviruses lying in cellular reservoirs (Harrigan et al., 1999). Silenced proviruses in cellular reservoirs are virtually insensitive to cART drugs which can only target productive infection (Redel et al., 2009 , Le Douce et al., 2010). Microglial cells are resident macrophages in the central nervous system and the main HIV-1 target in brain (He et al,1997 ; Jordan et al., 1991 ; Suh et al., 2005). Microglial cells are long-lived cells that can be latently infected by HIV-1 (Davis et al., 1992 ; Barber et al., 2006 ; Le Douce et al, 2010). In the majority of cases, latency is achieved by

transcriptional extinction of the viral genome (Colin and Van Lint, 2009). This event can occur through viral promoter DNA methylation, chromatin remodelling or TAT-mediated transactivation inhibition (Redel et al., 2009 ; Weinberger et al., 2008). TAT is a viral protein synthetized during the early events of transcription. TAT then undergoes sequential post-translationnal modifications allowing it to achieve the transactivation of the viral genome transcription (Hetzer et al, 2005). TAT is so able to promote its own production while increasing drastically the overall viral transcription by recruiting the positive transcription elongation factor (P-TEFb) (d'Orso et Frankel, 2009 ; Krueger et al., 2010). However for a lapse of time, TAT concentration is low enough to make the system evolve either toward full transcriptional activation or toward latency (Weinberger et al, 2005 ; Weinberger et al, 2008). The provirus cannot sustain full TAT-mediated transcription indefinitely and will eventually be reverted to latency at some point. This means that an alteration of TAT-transactivation cycle can lead to an early abortion of the full transcription process, resulting in latency establishment. Our team previously demonstrated that the cellular transcription repressor COUP-TF Interacting Protein 2 (CTIP2) is able to cooperate with the lysine specific demethylase 1 (LSD1) to induce histone post-translationnal modifications in microglial cells (Le Douce et al, 2012). CTIP2 recruits histones deacetylases 1 and 2 (HDAC1&2) and methyltransferases (SUV39h1) activities while LSD1 recruits the methyltransferase complex hCompass (Marban et al, 2007 ; Le Douce et al 2012). The chromatine remodelling complexe associated to LSD1/CTIP2 promotes the establishment of heterochromatin at the viral promoter, and thus latency. More recently we identified CTIP2 as a new member of the inactive pool of P-TEFb (Cherrier et al, unpublished). CTIP2 can decrease TAT-mediated transactivation by reducing the stock of active P-TEFb available for TAT. Moreover, CTIP2 can also directly interact with TAT and P-TEFb to decrease initiation and elongation of viral transcription. CTIP2, by impairing TAT-driven transactivation cycle is able to induce latency establishment.

HIC1 is a cellular tumor suppressor involved in DNA damage response, cell survival, and neural development (Grimm et al., 1999 ; Carter et al, 2000 ; Dehennaut

and Leprince, 2009). Loss of heterozygosity (LOH) of *Hic1* has been implicated in numerous cancers, such as medulloblastoma or leukemia and is associated with increased malignancy and poor prognosis (Rood et al., 2002 ; Britschgi et al., 2008 ; Fleuriel et al, 2008). LOH of HIC1 can occur through hypermethylation or deletion of the 17p13.3 region, where the *Hic1* gene is located (Wales et al, 1995). HIC1 is a transcriptional repressor divided in three main regions. A BTB/POZ protein-protein interaction domain (Broad complex,Tramtrack and Bric à brac/Pox viruses and Zinc finger) located in the amino-terminal part (Deltour and Leprince, 1999). This region has an autonomous transcriptional repressive activity through its interaction with the NAD-dependant class-III histone deacetylase SIRT1 (Chen et al., 2005). The central region contains two phylogenetically conserved sequences. A GLDL^{225}SKK conserved motif, to which the C-terminal binding protein (CtBP) binds. Interaction with CtBP grants HIC1 with a class-I and –II HDAC-dependant transcriptional repression activity (Deltour et al., 2002). The second motif is a SUMOylation/acetylation switch MK^{314}HEP. Acetylation of lysine 314 by CBP/P300 decreases HIC1 interaction with its co-repressor. Acetylation and SUMOylation compete for the same lysine residue (Stankovic-Valentin et al., 2007). SUMOylation on the other hand has been described to activate and potentiate HIC1-mediated transcriptional repression (Van Rechem et al., 2010). Finally, the C-terminal part of HIC1 contains five krueppel-like C_2H_2 zinc fingers. A cluster with the last four zinc fingers allows HIC1 to interact with a 5'C/GNGC/GG**GGCA**C/ACC-3' consensus sequence in HIC1 target genes, the HIC1 responsive element (HiRe) (Pinte et al., 2004). Recently the zinc fingers have been described to be implicated in the recruitment of SIRT1 in order to promote HIC1 lysine deacetylation followed by SUMOylation favoured by HDAC4 (Stankovic-Valentin et al., 2007 ; Dehennaut et al., 2012).

Herein we report for the first time that tumor suppressor HIC1 is able to promote TAT-dependent HIV-1 transcription silencing in microglial cells. HIC1 induces nicotinamide-sensitive HIV-1 transcription extinction by cooperating with the deacetylase SIRT1. SIRT1 is known to deacetylate the lysine lying in the MKEHP

motif of HIC1. Interestingly a single amino-acid mutation in the MKEHP acetylation/SUMOylation switch, which mimics a constitutive acetylation of the lysine 314 is sufficient to abrogate HIC1-driven transcriptional repression. Moreover, a constitutively acetylated HIC1 mutant loses its ability to interact with TAT. We propose in this report that SIRT1-driven HIC1 acetylation regulation is a critical step which occurs upstream of HIC1/TAT interaction.

Materials and methods.

Plasmids

Most of the constructs used in our assays have been previously described: pcDNA3, pFlag-TAT, pHA-TAT (Kiernan et al, 1999), pGFP-TAT (Rohr et al., 2003), pFlag-HIC1, pFlag-HIC1 $K^{314}Q$, pFlag-HIC1 $K^{314}R$, pFlag-HIC1 $E^{316}A$ (Stankovic-Valentin et al., 2007), pFlag-HIC1 $L^{225}A$ (Stankovic-Valentin et al., 2006), pFlag-HIC1 $C^{521}S$, pFlag-BTB/POZ, pFlag 5Znfs (Dehennaut et al., 2012), the episomal pLTR-Luc (Marban et al., 2007), pNL-4.3 (Marban et al., 2005). pshHIC1 and pshscramble control were manufactured by Suresilencing® (Quiagen).

pCBP/P300 et pTAR ; pFlag-SIRT1 et pFlag-SIRT1 $H^{363}Y$

Cell culture

Human microglial cells (Janabi et al., 1995) and HEK293T cells were cultivated in Dubelcco's Modified Eagle's Medium (DMEM) supplied with 10% fetal calf serum and 100U/ml penicillin-streptomycin.

Luciferase Assay

Microglial cells were cultivated in 48-well plate and transfected with fixed amount of plasmid DNA using calcium phosphate precipitation technique. Trichostatine A (TSA) and nicotinamide (NA) treatment were performed 24-hours after transfection. Cells were incubated with either 450nM TSA or 10mM NA or were mock treated with the proper dilutant. 48 hours post-transfection cells were subjected to Dual-Glo® Luciferase Assay System (Promega) and luciferase activity was measured.

Values are representative of at least three independent experiments in at least duplicates. Basal activity which corresponds to transfection with only control vectors, were adjusted to one.

P24 ELISA assay

Microglial cells were cultivated in 48-well plate and transfected with 1µg/well of plasmid DNA using calcium phosphate precipitation technique. 48 hours post-transfection cell supernatants were harvested and subjected to Innotest® p24 ELISA Assay (Innogenetics). Values are representative of at least three independent experiments in at least duplicates. Basal activity which corresponds to transfection with only control vectors, were setted at one.

Co-immunoprecipitation assays

HEK293T cells were cultivated in 144mm petri-dishes and transfected with 75µg of plasmid DNA using calcium phosphate precipitation method. 48-hours post-transfection cells were harvested and lysed to recover nuclear proteins. Nuclear extracts (1mg) were incubated overnight at 4°C with M2 anti-Flag antibody (SIGMA), anti-TAT antibody (abcam) or anti-SIRT1 antibody (Millipore). Complex proteins/antibody were washed with both high-salt (500mM NaCl) and low salt (125mM NaCl) buffers. Complexes were studied either by SDS-PAGE and Western blot or RT-PCR and RT-qPCR.

SDS-PAGE and Western blot analysis

SDS-PAGE were performed using standard procedures. Proteins were western-blotted with M2 anti-flag antibody (Sigma), anti-TAT antibody (abcam), anti-HIC1 anitbody (abcam) and anti-SIRT1 antibody (Millipore). Proteins were detected using the Super Signal West Dura® Chemiluminesence Detection System (Pierce biotech).

RT-PCR and RT-qPCR

Protein/RNA complexes from co-immunoprecipitation experiments were released from capture antibody by heating and incubation in an elution buffer. Recovered RNAs were purified using RNeasy plus kit® (Quiagen) and then subjected to reverse transcription chain polymerase reaction (RT-PCR). TAR region and GAPDH were quantified among the cDNAs using primers flanking these regions. GAPDH quantification has been used to control the specificity of the TAR enrichments.

Immunocytochemistry and confocal microscopy

Microglial cells were cultivated on glass coverslips in 24-well plate and transfected with Jetprime® (polyplus transfection) according to manufacturer protocol. 48-hours post-transfection cell were fixed with PFA 4% and permeabalized with PFA4%/triton1%. Cells were incubated in BSA blocking buffer, then incubated 1 hour with primary mouse M2 anti-Flag antibody (Sigma) followed by a 1 hour incubation with cyanine 3-labelled anti-mouse antibody. Finally cells were incubated 30 minutes with TOPRO-3 to stained cell nucleus. Cells were washed with PBS inbetween each step. Fluorescences were recorded by confocal microscopy using a Zeiss laser scanning microscope (model 510 invert) equipped with a Planapo oil (63x) immersion lens (numerical aperture=1.4).

HIC1 represses HIV-1 p24 production and TAT-mediated transcription.

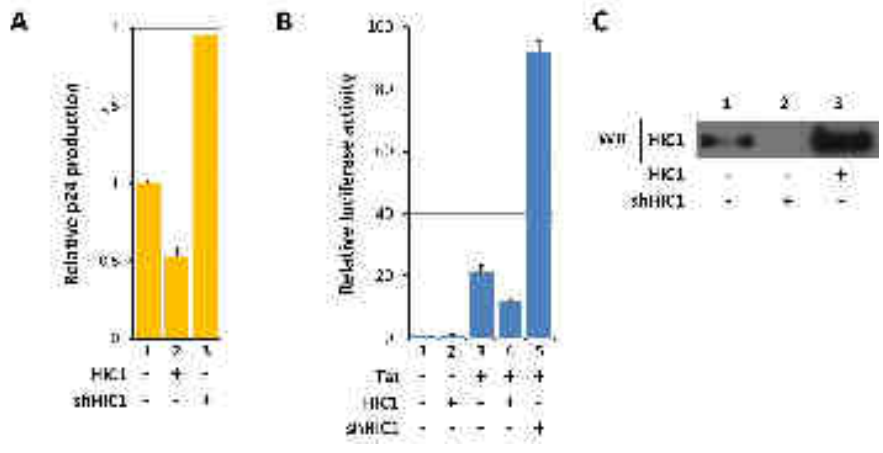

Figure 1: Effect of HIC1 over-expression or knock-down on HIV-1 in microglial cells. Microglial cells were transfected either with the episomal pLTR-luciferase reporter (A) or the pNL-4.3 provirus (B) and the indicated plasmids. 48-hours later, cells were lysed (A) or supernatants were harvested (B). Cell lysate were subjected to luciferase assay, normalized with the renilla luciferase system and expressed as relative value with their respective mock control (A). Viral p24 concentrations were titrated in supernatant and normalized relatively to their appropriate empty vector control (B). Nuclear HEK293T cell extracts were obtained after 48-hours transfection of the indicated vectors and subjected to western-blot experiments to detect endogenous and over-expressed HIC1 (respectively lane 1&2 and lane 3)(C).

To decipher the role of HIC1 on HIV-1, microglial cells were transfected with a pNL-4.3 provirus in the context or not of HIC1 over-expression and knock-down. We assessed the efficiency of the shHIC1 by western blot (Fig1.C. lane1 vs 2). In the context of viral infection, we observed that a knock-down of HIC1 is linked with a 2-fold increase in p24 production in microglial cells (Fig1.B lane1 vs 3). Concomitantly, an over-expression of HIC1 is associated with a 2-fold decrease of p24 production (Fig1.B lane1 vs 2). These results argue in a HIC1 repressive activity on HIV-1 p24 synthesis.

Since HIC1 is described as a transcriptional repressor, we next studied whether the HIC1 inhibitory activity could occur at the transcriptionnal level. To do so, microglial cells were transfected with the episomal viral LTR-promoter fused to the luciferase reporter (pLTR-Luc). pLTR-luc was co-transfected either with or without over-expression or knock-down of HIC1. In addition, the impact of the over-expression of

HIC1 has been investigated on either TAT-dependent and –independent transcription steps, by including or not a TAT expressing vector. While an over-expression of HIC1 has no effect in absence of TAT (Fig1.A lane1 vs 2), the same over-expression is able to repress tat-dependant viral transcription up to 2-fold (Fig1.A lane3 vs 4). In presence of Tat, a knock-down of HIC1 is associated with a 4-fold increase of LTR activity (Fig1.A lane3 vs 5).

These results suggest that HIC1 inhibits p24 production by repressing TAT-dependent viral transcription in microglial cells.

HIC1 re-locates and interacts with TAT in dense nuclear sub-structures.

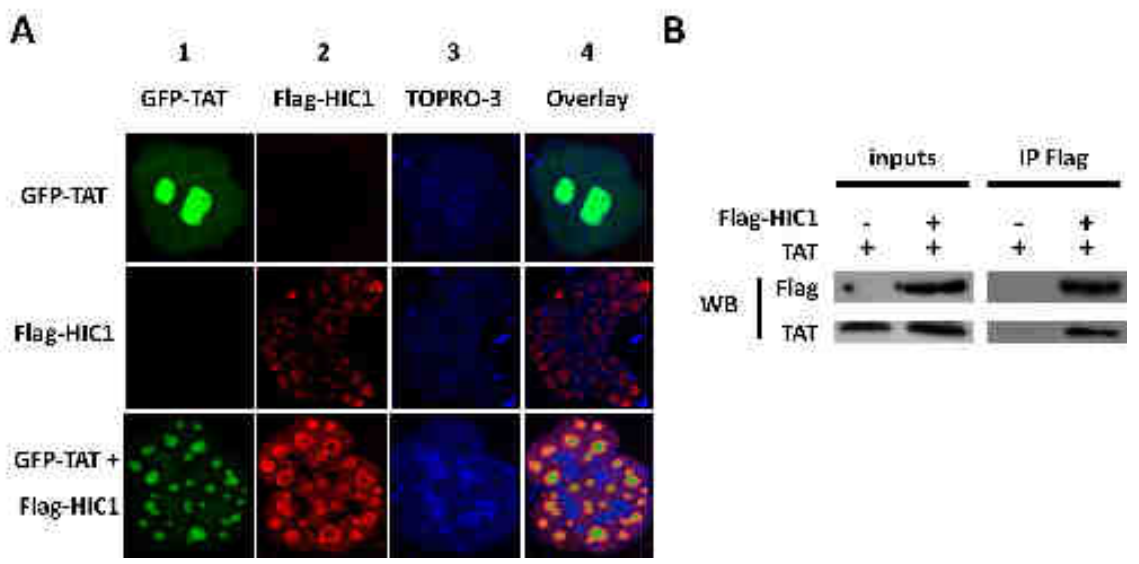

Figure 2: HIC1 relocates TAT in sub-nuclear ball like structures in which they interact. (A) Microglial cells were transfected with pGFP-TAT (row 1), pFlag-HIC1 (row 2) or both (row 3). 48-hours post-transfection cells were fixed and stained with TOPRO-3 (column 3) to detect the nucleus. Flag-HIC1 was detected by incubating cells with an anti-flag antibody and and immunostained with a cyanine 3-labelled secondary antibody (column2). Localization of Flag-HIC1 and GFP-TAT has been acquired by confocal microscopy. (B) HEK293T cells were transfected with pTAT alone or in combination with pFlag-HIC1. 48-hours later cells were lysed and nuclear extracts have been subjected to immunoprecipitation with an anti-Flag tag antibody. TAT and HIC1 were detected by western blot with respectively anti-TAT and anti-Flag antibodies.

Our results indicate that HIC1 is only able to repress tat-mediated transcription, which strongly suggest a critical role of the viral protein in the inhibition process. We first investigated the localization of the two proteins by confocal microscopy in

microglial cells. Cells were transfected with pGFP-TAT, pFlag-HIC1 or both expression vectors (Fig2.A row 1 to 3). Both proteins displayed a nuclear localization when expressed alone (Fig2.A row 1&2, lane 3). Although TAT was found as well in the nucleoplasm as in the nucleoli (Fig2.A row1 lane1), Flag-tag immunostaining revealed that HIC1 was found in the outskirts of nuclear ball-like structures (Fig2.A row 2 lane 2). When co-transfected TAT is relocated and co-localizes with HIC1 in the HIC1-induced ball-like structures (Fig2.A row1 lane1 vs row3 lane1). Our team already observed such a phenomenon with the transcriptional repressor CTIP2. In our previous report, immunostaining of Flag-CTIP2 failed to reveal the presence of the protein in the center of the ball-like structure due to poor antibody penetration (Marban et al., 2005). These previous observations may explain the absence of HIC1 detection into these nuclear sub-structures. Moreover HIC1 has already been described to induce the formation of "HIC1 bodies" in the nucleus, where it relocates TCF-4/β-catenin to prevent transcriptional activation of their target genes (Valenta et al., 2006). HIC1 may also render TAT-mediated transactivation inefficient by sequestering TAT in the ball-like structures.

Since HIC1 relocates and co-localizes with TAT, we investigated whether both protein could physically interact. We performed immunoprecipitation on nuclear extract from cell transfected with pFlag-HIC1 and/or pTAT vectors (Fig2.B). Flag-targeted immunoprecipitation of HIC1 was able to co-immunoprecipitate TAT, arguing that both proteins could be found in at least one same complex.

HIC1 is so able to physically recruit Tat and can relocate it into dense ball-like nuclear sub-structures.

HIC1-mediated repression of HIV1 transcription is TSA-insensitive and CtBP independent.

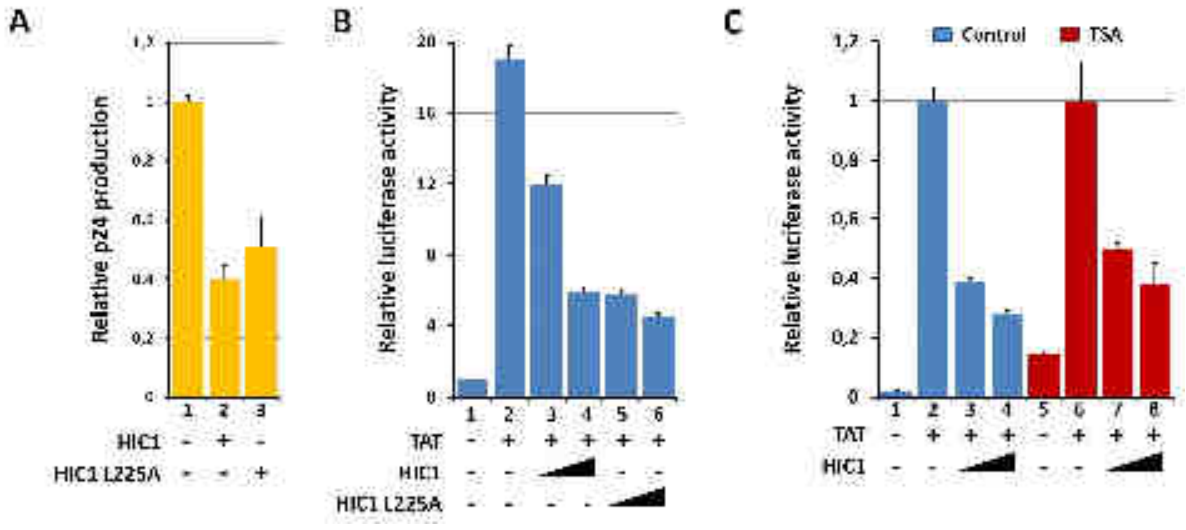

Figure 3: L[225]A mutation and TSA treatment have no effect on HIC1-mediated HIV-1 transcription repression. Microglial cells were co-transfected with the indicated constructs and either pNL-4.3 viral genome (A) or episomal pLTR-Luc (B&C). 24-hours after transfection microglial cells were either mock treated (C. blue bars) or incubated with 450nM TSA (C. red bars). Increasing amount of vector transfected is indicated with a solid black triangle. 48-hours post-transfection, cells were lysed and subjected to luciferase assay, while normalized with renilla luciferase system (B&C) or supernatants were harvested and p24 concentration titrated (A). Values are normalized relatively to basal level corresponding to empty vector (A lane1, B lane1) or relatively to empty vector + pTAT (C lane1 and lane5).

A previous study indicated that a single mutation, L[225]A, in the GLDL[225]SKK is sufficient to abolish the HIC1/CtBP interaction (Stankovic-Valentin et al., 2006). CtBP acts as a co-repressor for HIC1 by recruiting class-I and II HDACs, granting an autonomous repressive activity to the HIC1 central domain (Deltour et al., 2002). Moreover, the interaction between HIC1 and CtBP has been shown to be necessary for re-location and sequestration of TF4/β-catenin in nuclear "HIC1 bodies" (Valenta et al., 2006). HIC1 is able to induce similar ball-like structures in microglial cell nucleus and to re-locate TAT into them.

Hence we decided to investigate whether CtBP was implicated in the process of HIC1-mediated HIV-1 transcription inhibition. Microglial cells were co-transfected with the pNL-4.3 provirus or the episomal pLTR-Luc in presence of TAT with or without an over-expression of L[225]A singly mutated HIC1. Although this HIC1

mutant is unable to interact with CtBP, HIC1 $L^{225}A$ is surprisingly still able to repress p24 production (Fig3.A lane1 vs 3) and HIV-1 TAT-dependant transcription (Fig3.B lane2 vs 5&6). To corroborate these observations, we investigated the impact of trichostatin A (TSA), a class-I and-II HDACs inhibitor, on HIC1 repressive activity. Wherefore, microglial cells were co-transfected with the pLTR-Luc with or without an over-expression of HIC1 in the presence of TAT. As expected, over-expression of HIC1 is associated with a severe decrease of luciferase production (Fig3.C lane2 vs 3&4). In addition, 24-hours incubation of TSA failed to counteract HIC1-mediated transfection repression (Fig3.C compare lane 2 vs 3&4 and lane6 vs 7&8). According to prior reports, the absence of effect from TSA and the $L^{225}A$ HIC1 mutant being still effective suggest that the inhibition process requires neither the recruitment of CtBP nor class-I and class-II HDACs in microglial cells.

HIC1-mediated repression of HIV1 transcription is neither dependent on class-I and –II HDACs nor on its CtBP interaction.

BTB/POZ domain and zinc fingers are alone not sufficient but *sine qua none* for HIC1-mediated inhibition.

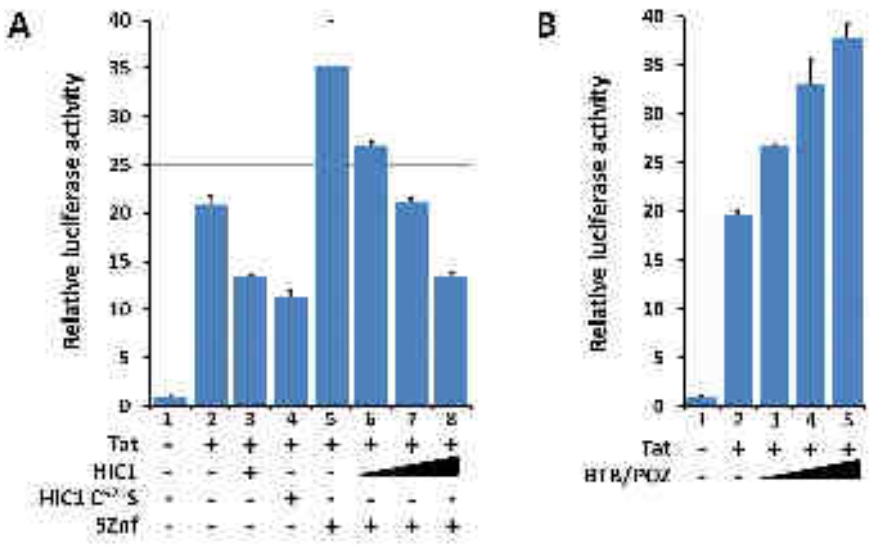

Figure 4: $C^{521}S$ HIC1 mutant is able to repress HIV-1 transcription while HIC1 BTB/POZ domain and 5Znfs cannot. Microglial cells were co-transfected with pLTR-Luc and the indicated constructs (Fig4.A&B). Increasing amount of vector transfected are indicated with a solid black triangle: increasing amount of HIC1 are used to compete with the 5Znfs construct over-expression (A lane 5 vs 6to8) or increasing amount of BTB/POZ constructs are transfected to compete with endogeneous HIC1 (B lane2 vs 3to4). Cells are subjected to luciferase assay 48-hours post-transfection and are normalized

with the renilla luciferase system. Values are normalized relatively to the mock transfected cells (A lane1, B lane 1).

There are currently only a few cellular gene that have been described to be targeted and regulated by HIC1. Among them, *Cxcr7*, *E2f1*, *Sirt1* and *Atoh1* display in their promoter the consensus HiRe (Dehennaut and Leprince 2009)). Interaction between HIC1 and a target promoter occurs through the interaction of the four last C-terminal krüppel-like zinc fingers with the HiRe (Pinte et al., 2004). Moreover, HIC1 needs to oligomerize through its BTB/POZ domain for an efficient binding of the repressor onto its target gene promoters. HIC1 multimers are critical in order to bind multiple HiRes simultaneously (Ahmad et al., 1998 ; Pinte et al., 2004). A closer look at the HIV-1 LTR indicates that 4 copies of the HiRe core motif GGCA can be found in the LTR sequence. To examine whether HIC1 could directly bind the viral promoter, we used a construct containing only for the five zinc fingers (5Znfs). EMSA (Electrophoretic Mobility Shift Assay) experiments, using probes with the HIV-1 LTR HiRe sequences incubated with either HIC1 or the 5Znfs construct, were negative (data not shown). Mutation of a single cysteine residue from the third zinc finger is sufficient to alter the capability of HIC1 to bind its target genes. Thus, we compared the transcriptional effect of HIC1 $C^{521}S$, unable to bind to the HiRes, with the repressive abilitiy of HIC1 wild type on TAT-mediated HIV1 transcription. Microglial cells were co-transfected with the episomal LTR-luc with or without an over-expression of either HIC1 or HIC1 $C^{521}S$, both in presence of TAT. Our results indicated that the $C^{521}S$ mutant is as efficient as the HIC1 wild type in repressing TAT-mediated HIV-1 transcription (Fig.A Lane2 vs 3&4). This means that the inhibition process does not necessitate HIC1 to directly bind the GGCA sites in the HIV-1 LTR. However, over-expression of 5Znfs in presence of TAT in LTR-Luc co-transfected microglial cells was able to reactivate the viral transcription up to 2-fold (Fig4.A Lane2 vs 5). Since HIC1 is endogenously produced in microglial cells, we hypothesized that the over-expression of the 5Znfs construct could impair the repressive activity of endogenous HIC1. When microglial cells were subjected to the same 5Znfs over-expression with increasing amount of HIC1 in presence of TAT, we

were able to compensate for the 5Znfs effect in a dose dependant-manner (Fig.A lane5 vs 6to8). The 5Znfs domain appears to be necessary to promote HIC1-mediated repression, but is not a stand-alone HIV-1 transcription repressor.

The HIC1 BTB/POZ domain has previously been described as an autonomous TSA-insensitive transcriptional repressor (Deltour et al., 1999). Since we demonstrated that HIC1-mediated HIV1 repression was TSA insensitive and CtBP-independant, we investigated whether the BTB/POZ domain could contain the inhibitory activity associated with HIV1 transcription decrease. To establish the potential involvement of this domain, we used a vector expressing the HIC1 BTB/POZ domain only. Microglial cells were co-transfected with LTR-Luc and with or without increasing amount of BTB/POZ construct in presence of TAT (Fig4.B). Similarly to what we observed in response to the over-expression of the 5Znfs, the BTB/POZ alone was not able to repress the viral transcription, but was even able to increase the TAT-mediated viral transcription in a dose dependant-manner (Fig4.B lane 2 vs 3to5) as seen with the 5Znfs (data not shown). The BTB/POZ domain displays a HIV-1 transcription activator role, most likely by competing with the endogenous HIC1.

Altogether these results suggest that HIC1 do not bind directly the viral promoter, since a $C^{521}S$ mutant is still able to inhibit TAT-mediated transcription. Nevertheless, the 5 zinc fingers are still required, even though they are not sufficient to promote the transcription repression in microglial cells. Finally the BTB/POZ domain appears also to be critical in HIC1-driven TAT-transactivated transcription inhibition. However its ability to reactivate viral transcription lead us to think that this domain is most likely required for its protein-protein binding properties.

HIC1-mediated viral transcription repression requires SIRT1 deacetylase activity.

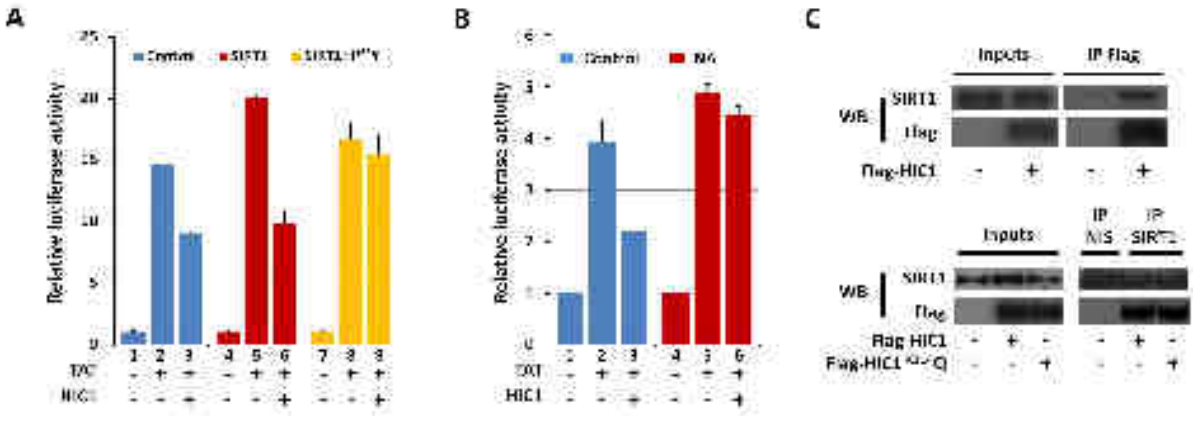

Figure 5: HIC1-mediated TAT-dependent HIV-1 transcription repression process requires SIRT1 deacetylase activity. Microglial cells were transfected with pLTR-Luc and the indicated expression vector. (A) TAT mediated luciferase production was studied in absence (A. blue bars) or presence of over-expression of either wild-type SIRT1 (A. red bars) or $H^{363}Y$ SIRT1 mutant (A. yellow bars) 24-hours after transfection cells were either untreated (B. blue bars) or incubated with 10mM nicotinamide (NA) (B. red bars). 48-hours post-transfection cells were lysed and luciferase activity measured. Values were normalized using renilla luciferase system. Empty vector transfection of each condition has been set to one and other transfection points were normalized accordingly. HEK293T cells were transfected with pFlag-HIC1 wild-type or $K^{314}Q$ (C). 48-hours later cell were lysed and nuclear extracts have been subjected to immunoprecipitation with an anti-flag tag antibody(C upper part, IP flag), a non-immune serum (C lower part, IP NIS) or anti-SIRT1 antibody (C lower part, IP SIRT1). Endogenous SIRT1 as well as Flagged HIC1 wild type and $K^{314}Q$ mutant are detected by western-blot with respectively anti-SIRT1 and anti-flag antibodies. It is to note that transactivation level in panel B is 3-4 times lower than panel A due to changes in TAT-expressing vector quantity transfected.

According to our previous results, the five krüppel-like zinc fingers and the BTB/POZ domain are both required for a HIC1 repressive activity. The class-III deacetylase SIRT1 has already been described as a co-repressor of HIC1, by interacting with the BTB/POZ domain (Chen et al, 2005). The resulting SIRT1/HIC1 association is one of the numerous HIC1/co-repressor transcription repressor complexes. The most characterized target gene is *Sirt1*, the promoter of which is bound by HIC1/SIRT1 during the negative feedback loop Sirt1/p53/Hic1 in response to DNA damages (Chen et al., 2005). Recently, the region 610-677 of SIRT1 has been shown to interact with the 5 zinc fingers of HIC1 (Dehennaut et al., 2012). This interaction can happen even despite punctual mutations disrupting any of the zinc fingers, as the previously mentioned HIC1 $C^{521}S$ mutant.

While results from previous studies indicate that SIRT1 can interact with the BTB/POZ domain and the 5 zinc fingers, we showed that both HIC1 structural elements were necessary for functional transcription inhibition of HIV-1.

We so hypothesized that SIRT1 could be part of the repression mechanism involving HIC1. Microglial cells were co-transfected with pLTR-Luc in presence of TAT and with or without an over-expression of HIC1. We compared the impact of HIC1 on TAT-mediated HIV1 transcription repression alone (Fig5.A lane2 vs 3) with an additional over-expression of either SIRT1 (Fig5.A lane5 vs 6) or SIRT1 $H^{363}Y$ mutant (Fig5.A lane8 vs 9). The single amino-acid mutated $H^{363}Y$ SIRT1 variant is catalytically inactive, thus lacks deacetylase activity (Brunet et al., 2004). As previously observed, HIC1 over-expression decreases TAT-mediated viral transcription. However, while co-over-expression of HIC1 and SIRT1 still induces viral transcription inhibition (compare Fig5.A lane2 vs 3 with lane5 vs6), co-over-expression of HIC1 and $H^{363}Y$ SIRT1 mutant displayed no effect anymore (compare Fig5.A lane2 vs 3 with lane8 vs 9). To validate that SIRT1 loss of activity was detrimental to HIC1-mediated repression mechanism, we studied the effect of 24-hours treatment with nicotinamide (NA) on HIC1 inhibitory activity (Fig5.B). NA behaves as an end production inhibitor of the NAD+-dependant SIRT1 deacetylase. We compared HIC1-mediated transcription repression with no or NA treatment in microglial cells. Cells were beforehand co-transfected with pLTR-Luc in presence of TAT with or without an over-expression of HIC1.

NA treatment abolished HIC1 inhibitory effect (compare Fig5.b lane 2 vs 3 with lane 5 vs 6), which again linked SIRT1 to the HIC1-dependant inhibition of TAT-mediated HIV1 transcription mechanism. In the end this results indicate that HIC1 repressive activity is NA-sensitive and so relies on the ability of SIRT1 to deacetylate its substrate.

HIC1-mediated inhibition is SUMOylation independant and is impaired by acetylation.

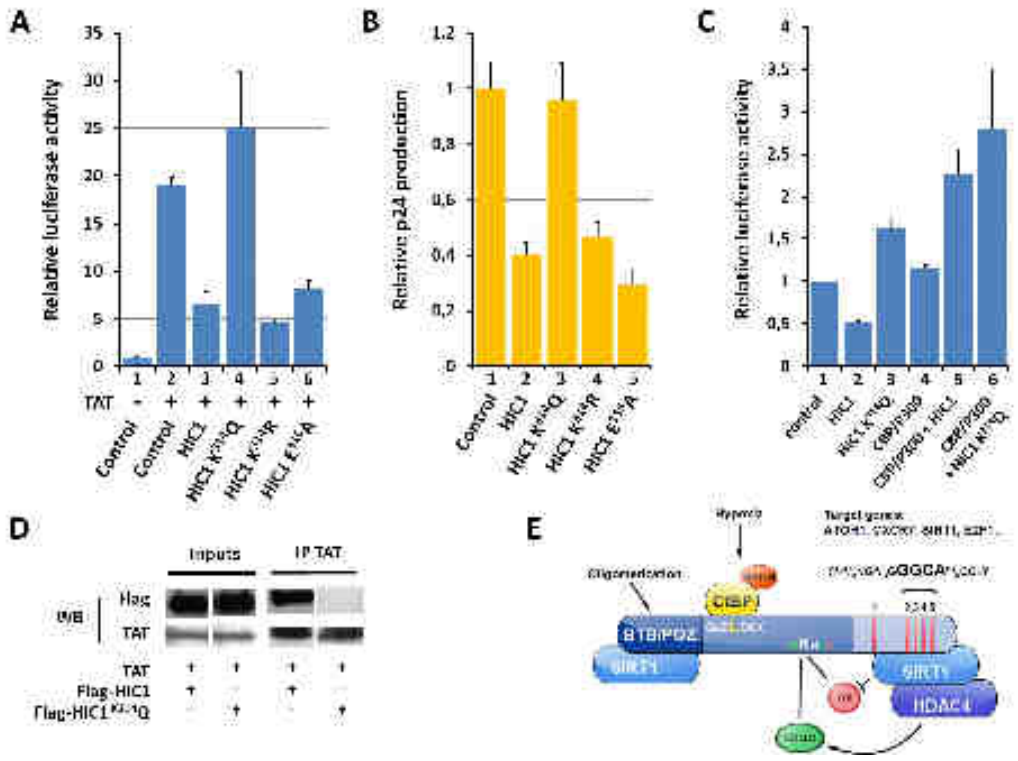

Figure 6: Acetylation of lysine 314 is detrimental to HIC1 inhibitory activity and disrupts HIC1/TAT assocation. HIC1 central domain contains a MK^{314}HEP conserved sequence, in which the lysine 314 (K^{314}) acts as an acetylation/SUMOylation switch (E). SIRT1 interacts with the HIC1 5 krueppel-like zinc fingers and deacetylates K^{314}. SIRT1 recruits also HDAC4 which facilitate SUMOylation of K^{314} (E). Microglial cells were co-transfected with the indicated constructs and either episomal pLTR-Luc (A) or pNL-4.3 viral genome (B&C). 48-hours post-transfection, cells were lysed and subjected to luciferase assay, while normalized with renilla luciferase system (A) or supernatants were harvested and p24 concentration titrated (B&C). Values are normalized relatively to basal level corresponding to empty vector (A, B, C lane1). HEK293T cells were transfected with pFlag-HIC1 wild-type or K^{314}Q in presence of TAT (C). 48-hours later cell were lysed and nuclear extracts have been subjected to immunoprecipitation with an anti-TAT antibody (D). Co-immunoprecipitations of Flagged HIC1 wild type and K^{314}Q mutant with TAT were compared by western-blot with respectively anti-flag and anti-TAT antibodies.

The central region of HIC1 contains a MK^{314}HEP conserved sequence, in which the lysine 314 (K^{314}) acts as an acetylation/SUMOylation switch. Acetylation and deacetylation of HIC1 K^{314} is respectively under control of the cellular acetyl-transferase CBP/P300 and SIRT1 (Stankovic-Valentin et al., 2007). SIRT1 binds the krueppel-like C_2H_2 zinc fingers in the carboxy-terminal end of HIC1 to promote its

non-histone protein deacetylase activity onto K^{314} (Dehennaut et al., 2012). At that time, SIRT1 also recruits HDAC4, which facilitates the SUMOylation of K^{314} by a still unknown mechanism (Fig6.E). SUMOylation of HIC1 has previously been associated with recruitment of the co-repressor NuRD through the interaction between HIC1 and the subunit MTA1 of NuRD (Van Rechem et al., 2010). Single amino-acid modifications in the $MK^{314}HE^{316}P$ sequence modify the post-translationnal pattern of HIC1. Thus mutation of the glutamic acid 316 ($E^{316}A$) disrupts the SUMOylation ψKxEx motif and abolishes HIC1 SUMOylation. The K^{314} is the target of both SUMOylation and acetylation post-translationnal marks. When K^{314} is mutated into an arginine, HIC1 can neither be acetylated nor SUMOylated (Stankovic-Valentin et al., 2007). However mutation of K^{314} into a glutamine mimics a constitutively acetylated HIC1, since the side chain of glutamine is similar in charge and structure to an acetylated lysine.

We demonstrated that SIRT1 cooperated with HIC1 to repress viral transcription. The two proteins interact (Fig5.C) and it may be thanks to both BTB/POZ and 5Znfs as it has been described in the mechanism of HIC1 deacetylation by SIRT1. Since SIRT1 can deacetylate and facilitates SUMOylation of HIC1, we investigated whether specific post-translationnal modifications could be involved in the repression process. Microglial cells were co-transfected with the episomal pLTR-Luc with or without an over-expression of the $K^{314}R$ or $K^{314}Q$ or $E^{316}A$ mutants in presence of TAT. $E^{316}A$ and $K^{314}R$ mutants are still able to repress the TAT-mediated transcription in a HIC1 wild-type manner (Fig6.A lane 2 vs 3,5&6). Yet the "constitutively acetylated" $K^{314}Q$ mutant displayed no inhibitory activity (Fig6.A compare lane 2 vs 3 and 2 vs 4). Microglial cells co-transfected with a pNL-4.3 provirus and the same over-expressions exhibit a p24 production profile similar to the TAT-mediated transcription profile, the $K^{314}Q$ HIC1 mutant being also unable to decrease viral p24 production (Fig6.B compare lane 1 vs 2,4&5 and 1 vs 3).

It appears so that lacks of SUMOylation and acetylation marks on K^{314} are not detrimental to HIC1 inhibitory activity in on HIV-1 transcription. However, mimicry

of an acetylation mark on K^{314} impairs all effect of HIC1 on p24 production through viral transcription inhibition.

CBP/P300 abrogates HIC1-mediated HIV1 p24 production decrease.

Constitutively acetylated HIC1 mutant $K^{314}Q$ is unable to promote p24 production and TAT-mediated HIV-1 transcription repression. Our results suggest that K^{314} acetylation is deleterious for HIC1 inhibitory activity on viral transcription and p24 production.

Acetylation of HIC1 is driven by the acetyl-transferase CBP/P300 (Stankovic-Valentin et al., 2007). We postulated that over-expression of CBP would destabilize the equilibrium toward HIC1 K^{314} acetylation and in fine could alter HIC1 repressive activity. Microglial cells were co-transfected with a pNL-4.3 provirus and an over-expression of HIC1 or HIC1 $K^{314}Q$ alone or simultaneously with an over-expression of CBP/P300 (Fig6.C). When expressed alone HIC1 and HIC1 $K^{314}Q$ displayed the previously observed effects on p24 production (Fig6.C lane1 vs 2&3). However when over-expressed together with CBP/P300, HIC1 wild type and mutant $K^{314}Q$ induce a similar 2-fold increase (Fig6.C lane4 vs 5&6). It appears that CBP/P300 is able to abrogate the HIC1 repressive effect, which mimics the absence of inhibitory effect of the $K^{314}Q$ mutant.

HIC1 acetylation leaves SIRT1/HIC1 interaction unchanged while is associated with loss of HIC1/TAT interaction.

So far we demonstrated that HIC1 requires SIRT1 to promote TAT-mediated HIV-1 transcription repression and that HIC1 physically interacts with both SIRT1 and TAT. Acetylation of HIC1 appears to be deleterious to HIC1 activity and thus could also be detrimental to HIC1/SIRT1 and/or HIC1/TAT interaction.

Since the Q^{314} side chain is not a removable acetyl-group, SIRT1 cannot deacetylate HIC1. We performed SIRT1-immunoprecipitation experiments of nuclear extract co-transfected with a SIRT1 and Flag-HIC1 $K^{314}Q$ expressing vectors. The $K^{314}Q$ mutant was still co-immunoprecipitated with SIRT1 (Fig5.C bottom panel).

We next investigated whether constitutive acetylation of K^{314} could modify TAT/HIC1 interaction. We compared the ability of TAT to co-immunoprecipitate with Flag-HIC1 or Flag-HIC1 $K^{314}Q$ in HEK cell nuclear extracts (Fig6.D). It appears that, unlike the HIC1 wild-type, the Flag-HIC1 $K^{314}Q$ mutant is not recovered by TAT immunoprecipitation. Since the inactive $K^{314}Q$ mutant is still able to interact with SIRT1, a default of SIRT1 recruitment is most likely not responsible for the loss of repressive activity. In the mean time, the absence of repressive effect of HIC1 $K^{314}Q$ is concomitant with a lack of TAT/HIC1 interaction. Altogether these results allow us to hypothesized that prior deacetylation of HIC1 K^{314} by SIRT1 could be an essential upstream step that allows TAT/HIC1 interaction.

RNA TAR element is a critical component of HIC1 inhibitory activity.

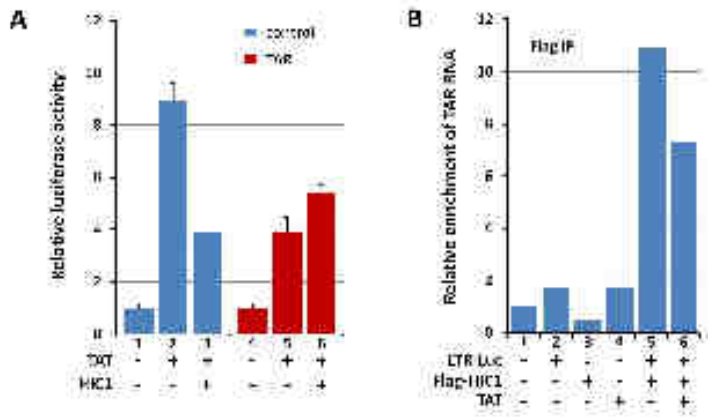

Figure 7: HIC1 is recruited to the TAR RNA. Microglial cells were co-transfected with pLTR-luc in presence of TAT with or without HIC1 (A). The implication of the TAR element in the HIC1-mediated HIV-1 transcription was assessed by comparing HIC1 repressive activity in absence (A, lane 1,2&3) or presence of free TAR RNA(A, lane 4,5&6). The interaction of HIC1 and the TAR element was investigated with immunoprecipitation followed by reverse transcription PCR and RT-qPCR. HEK293T cells were co-transfected with combination of empty vectors, pLTR-luc, pTAT and/or pFlag-HIC1. 48-hours post-transfection nuclear cell extracts were harvested and sudjected to flag immunoprecipitation overnight. Following immunoprecipitation, HIC1-containing complexes were eluted and RNAs have then been purified and reverse-transcribed. cDNAs were finally amplified by real-time qPCR with primers flanking the TAR region (B). Results are presented in relative enrichment after double normalization with initial TAR and GAPDH quantities engaged in the experiments.

Early after initiation of HIV-1 transcription, the viral RNA trans-activation element (TAR) is synthesized and structures itself into a RNA hairpin. The complex TAT/P-TEFb is recruited to viral promoter after initiation of transcription through the interaction between TAT and the TAR element (Kim et al., 2002 ; D'Orso and Frankel, 2009). Since HIC1-mediated HIV-1 transcription is dependent on TAT and that HIC1 and TAT can interact, we investigated whether HIC1 repressive activity requires the TAR element. We assumed that free TAR could bind to its partner in the cell and would impair mechanism requiring to be addressed in the LTR vicinity through TAR/protein interaction. Microglial cells were co-transfected with LTR-Luc in presence of TAT with or without HIC1 over-expression (Fig7.A lane 1,2&3) and we compared this situation with the same experimental set-up with the addition of free TAR (Fig7.a lane 4,5&6). It is to note that TAT-transactivation in presence of free TAR is twice as a less as basal transactivation. Free TAR seems to compete with LTR-produced TAR for TAT recruitment (Fig7.A compare lane 2 and lane5). However, while HIC1 represses TAT-mediated HIV-1 transcription up to 50% in the first case

scenario, the presence of free TAR in the cells in the second case lead to an abolishment of HIC1-driven repression (Fig7.A compare lane 2vs3 and lane5vs6). Free TAR may hijack some proteins involved in HIC1 inhibitory activity, and in that way cannot be addressed to the HIV-1 LTR anymore.

We demonstrated that HIC1-mediated HIV-1 transcription is independent of HiRe binding and that TAR is required for HIC1 repressive action. HIC1 could then be recruited at the LTR through interaction with the TAR element. To assess whether HIC1 could be part of a TAR containing complex, we performed flag immunoprecipitation on nuclear extracts from cells co-transfected with pFlag-HIC1, the episomal pLTR-Luc and/or pTAT. The immunoprecipitations were followed by a reverse transcription step to convert all RNA molecules retrieved by co-immunoprecipitation with HIC1 into DNA. The cDNAs thus produced were then quantified by quantitative real-time PCR with primer flanking the TAR element (Fig7.B). We observed a 6-fold and 4-fold enrichment of TAR RNA in flag-immunoprecipitations from respectively flag-HIC1/LTR_Luc and flag-HIC1/TAT/LTR-luc co-transfections (Fig7.B lane2 vs 5&6). HIC1 so exists in at least one complex in which TAR can be found. Surprisingly TAR/HIC1 complex exists as well with TAT as in absence of TAT. TAR may act as a HIC1 reservoir in the proximity of the transciption initiation site of the HIV-1 LTR promoter.

Discussion

HIV-1 latency establishment is a complex process tuned by viral and cellular proteins. TAT-mediated transcription is a pivotal process for productive HIV-1 infection. TAT recruits cellular transcription activator thus up-regulates viral transcription. Therefore destabilization of TAT transactivation cycle is associated with a heavy drop of proviral transcriptional activity leading to HIV-1 silencing (Weinberger et al, 2005 ; Weinberger et al., 2008). During the transactivation cycle, TAT competes with HEXIM1 to increase the stock of active P-TEFb (Barboric et al, 2003). When acetylatyted on lysine 28, TAT interacts with P-TEFb and recruits Cdk9

kinase activity at the viral promoter to increase RNApolII stability (Zhu et al., 1997 ; Majello et al., 1999). Loss of interaction with the positive elongation factor P-TEFb has been shown to impair drastically transactivation (Kiernan et al., 1999). Our team identified CTIP2 as a HIV-1 transcriptionnal repressor able to engage a multi-enzymatic complex to the viral promoter which favours heterochromatin formation (Marban et al., 2007). More recently our team observed that CTIP2 was also able to counteract TAT-mediated transactivation through CdK9 kinase activity inhibition in the TAR/TAT/CTIP2/P-TEFb complex (Cherrier et al., unpublished). In this work we present the tumor suppressor HIC1 as a new TAT transactivation cycle repressor. HIC1 undergoes post-translational modifications on its lysine 314. K314 acts as a SUMOylation/acetylation switch which enable or impair recruitment of co-repressor such as the NuRD complex (Van Rechem et al., 2010). HIC1 and its co-repressors are recruited at the promoter of their target genes to repress their transcriptional activity (Dehennaut and Leprince., 2009). The class-III NAD-dependent deacetylase SIRT1 and HIC1 are tightly associated in negative feedback loop. SIRT1 is able to interact with both the HIC1 BTB/POZ domain and the five krueppel-like zinc fingers to respectively inhibit *Sirt1* transcription and deacetylate lysine 314 of HIC1 (Chen et al., 2005 ; Dehennaut et al., 2012). In our results we demonstrated that HIC1 repressive activity requires SIRT1 deacetylase activity in microglial cells. Moreover, a single mutation in the $MK^{314}HEP$ acetylation rendering HIC1 constitutively acetylated is sufficient to abolishe HIC1 inhibitory effect. Constitutively acetylated HIC1, unlike HIC1 wild type, is neither able to repress TAT-mediated HIV-1 transcription nor to interact with TAT, although the SIRT1/HIC1 interaction still remains. It seems that SIRT1/HIC1 interaction may promote deacetylation of HIC1 to facilitate HIC1/TAT interaction, but need further investigations to validate (Fig8). We also found that HIC1 is member of at least one TAR-containing complex independently from TAT. Our results suggest that the TAR element may serve as HIC1 reservoir at the viral promoter to facilitate HIC1/TAT interaction. The transciption inhibition mechanism is not elucidated yet and may involve a complex interplay between HIC1, SIRT1 and TAT. SIRT1 and TAT are indeed known to

interact at the end of the transactivation cycle. SIRT1 removes acetylation marks of lysine 28 and 50 of TAT allowing the recycling of the viral transactivor (Blazek and Peterlin, 2008). Additionnaly, preliminary results suggest that the acetylation status of TAT modulates its interaction with HIC1 (data not shown). Deciphering the sequential process happening between HIC1, SIRT1 and TAT would allow us to understand the modalities of HIC1-mediated TAT-transactivation repression to prevent HIV-1 silencing. Indeed, even though class-III deacetylase inhibitors did not successfuly reactivate viral production yet, combination of class-I and –II deacetylase inhibitors and methyltransferases inhibitors showed promising reactivator effects on latently infected quiescent CD4+ T cells (Reuse et al., 2009). SIRT1 as well as HIC1 appears as a potential target for reactivation strategies..

Figure 8: Model of SIRT1-mediated HIC1 deacetylation promoting HIC1/TAT interaction. Deacetylation of HIC1 lysine 314 is a critical step in order to achieve TAT-mediated HIV-1 transcription repression. We propose that SIRT1 deacetylates HIC1 K^{314} prior to TAT recruitment. HIC1 belongs to at least one TAR RNA-containing complex in absence of TAT. The possibility that deacetylation process and TAT/HIC1 occur at the TAR RNA remains unclear (dotted shapes). Similarly it is not yet clear if SIRT1, TAT and HIC1 interact altogether in one same complex.

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Projet HIC1 : Résultats supplémentaires.

Dans cette partie nous traiterons de résultats obtenus sur le projet HIC1, mais qui ne s'intègre pour l'instant pas dans le mécanisme TAT/SIRT1/HIC1 précédemment présenté.
Ces résultats n'ayant pu être liés les uns aux autres dans un mécanisme, ces résultats seront discutés au cas par cas et seront également traités dans la partie de discussion finale de ce manuscrit.

Je suis à l'origine de l'intégralité de données exposées dans cette partie.

HIC1 interacts with CTIP2 and LSD1.

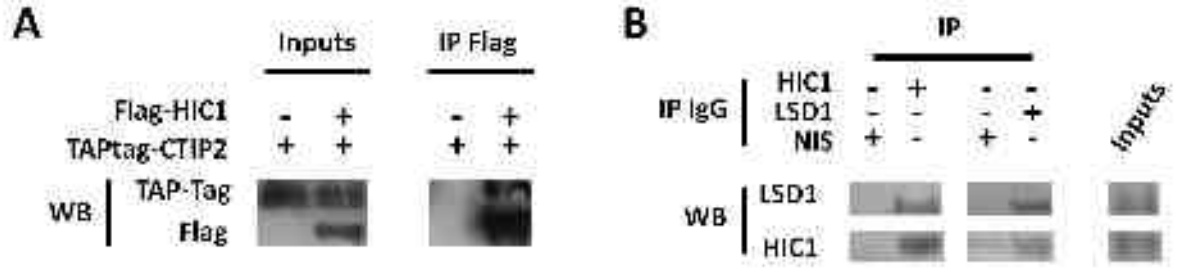

Figure 9 : CTIP2 and LSD1 co-immunoprecipitate with HIC1. HEK293T cells either were transfected with the expressing vectors coding for the indicated proteins (A) or untransfected (B). 48 hours post-transfection, cells were lysed and nuclear cell extracts were incubated with the capture antibody anti-flag (A), or a non-imune serum (NIS), or ant- HIC1, or anti-LSD1 (B). Immunoprecipitated complexes were subjected to western-blot with the indicated antibodies (A&B).

Knowing that LSD1 and CTIP2 interact in a same multi-enzymatic chromatin remodeling complex, we investigated whether HIC1 could also be a component of a CTIP2 and/or LSD1 repressive complex (Le Douce et al., 2012). HEK293T cells were transfected with either pTAPtag-CTIP2 with or without pFlag-HIC1 or untransfected. Nuclear extracts from transfected cells were subjected to Flag-targeted immunoprecipitation (Fig9A), while nuclear extracts from untransfected cells were subjected to immunoprecipitation with a non-immune serum (NIS), an anti-HIC1 antibody or an anti-LSD1 antibody (Fig9.B). TAPtag-CTIP2 and endogenous LSD1 were able to co-immunoprecipate with respectively Flag-HIC1 and endogenous HIC1 (Fig9.A&B). Reciprocally endogenous HIC1 was able to co-immunoprecipitate with endogenous LSD1 (Fig9.B). So far, HIC1 is able to interact with both LSD1 and CTIP2 *in-vitro*. To decipher the functional significance of these interactions, luciferase assay performed on microglial cells co-transfected with pLTR-luc and combination of pshHIC1 and/or pshCTIP2 or pshLSD1. Simultaneous knock-down experiments did not produce any synergistic effects, with the combination of shCTIP2/shHIC1 as with shLSD1/shHIC1 (data not shown).

HIC1 belongs to a complex containing CTIP2 and a complex containing LSD1. It is to note that the possibility that CTIP2/HIC1 and LSD1/HIC1 may be one same multi-proteic complex cannot be excluded, especially since CTIP2 and LSD1 are found in

one same complex (Le Douce et al., 2012). Even so CTIP2 and LSD1 cooperate to mediate HIV-1 transcription inhibition, double knock-downs experiments failed to show a partnership between HIC1 and CTIP2 or HIC1 and LSD1 in the HIV-1 transcription inhibition. The relevance of these two HIC1 interactions has yet to be elucidated. Nonetheless they may not be related to HIV-1 transcription or may not be occuring in microglial cells.

HIC1-mediated inhibition requires component of the 7sk snRNP complex.

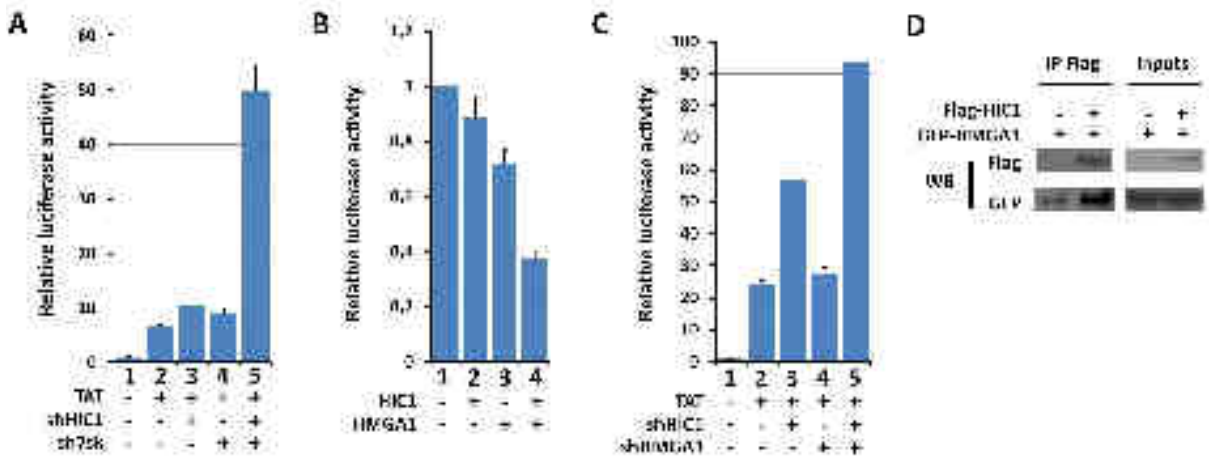

Figure 10: HIC1 cooperates with components of the 7skRNP complex. Microglial cells were transfected with either pLTR-Luc (A, C) or pNL-4.3 env-Luc (B) with the expressing vector coding for the indicated proteins or shRNA. HEK293T cells were transfected with pGF-HMGA1 with or without pFlag-HIC1 (D). 48 hours after transfection cells were lysed and either luciferase activities were measured (A,B,C) or nuclear cell extracts were subjected to flag-targeted immunoprecipitation (D).

During the transactivation cycle, TAT and P-TEFb interaction is a critical step which allows viral transcription initiation (Kao et al., 1987 ; Krueger et al., 2010). However, previous teams reported indicated that the 7sk RNA inactivates the positive elongation factor P-TEFb by sequestrating it in the 7sk snRNP complex (small nuclear RiboNucleoProtein) (Yik et al., 2003). Therefore the 7sk RNA can negatively regulate TAT-driven HIV-1 transcription. HIC1 represses only HIV-1 transcriptionnal activity in presence of TAT. We so investigated the potential involvement of the 7sksnRNP complex and P-TEFb in the HIC1 inhibitory process implicated in HIV-1 silencing. While immunoprecipitation of HIC1 failed to co-immunoprecipitate any of the P-TEFb subunits in our experiments (data not shown), luciferase experiments involving HIC1 and the 7sk RNA in microglial cells suggested

that they cooperate to inhibit HIV-1 transcription in presence of TAT. Microglial cells were transfected with pLTR-luc with or without pshHIC1 and/or psh7sk. Single knock-downs only led to an about 2-fold viral transcription increase (Fig10.A lane2vs3&4), whereas a simultaneous knock-down of HIC1 and the 7sk RNA induced a heavy 10-fold reactivation of TAT-driven viral transcription (Fig10.A lane2vs5).

As HIC1 could not interact with P-TEFb it seems unlikely that HIC1-mediated transcription inhibition originates from P-TEFb sequestration in the 7sksnRNP through HIC1. However the 7skRNA appears to be an important actor of the HIC1 inhibition process.

7sk snRNP is known to modify gene transcription by down-regulating either P-TEFb and/or HMGA1 (High-Mobility Group Protein AT-hook 1). The HIV-1 LTR promoter contains several binding sites for HMGA1 and notably could bind to AP1 sites in the vicinity of Nuc-1 in jurkat cells (Henderson et al., 2000). Moreover, HMGA1 is associated with transcriptional activation of HIV-1 in lymphocytes. HMGA1 recruits the chromatine remodelling complex SWI/SNF, by interacting with the subunit BRG1, to promote Nuc-1 destabilization and so facilitate transcription initiation (Henderson et al., 2004). Since the 7sk RNA appears important for HIC1 inhibitory activity and P-TEFb seems to be unrelated to HIC1-driven HIV-1 repression, we investigated whether HMGA1 could be targeted by HIC1 and the 7skRNA.

Surprisingly, an overexpression of HMGA1 in microglial cells transfected with pLTR-luc and TAT had no effect on viral transcription. We so decided to study the impact of a simultaneous knock-down of HIC1 and HMGA1. We co-transfected microglial cells with pLTR-luc with pshHIC1 or pshHMGA1 or both, in presence of TAT. In our experimental condition HIC1 knock down produced a 2-fold increase of viral transcription while HMGA1 knock-down displayed no effect. However, simultaneous knock-down allowed a synergistic HIV1 transcription reactivation of about 5-fold.

We next assessed whether HIC1 and HMGA1 could interact together. We co-transfected HEK293T cells with pGFP-HMGA1 with or without pFlag-HIC1. Nuclear

cell extracts were subjected to Flag-targeted immunoprecipitation. When immunoprecipitated Flag-HIC1 was able to co-immunoprecipitate HMGA1.

These results first indicate that, HIC1 cooperates with the 7sk RNA to repress viral transcription in a P-TEFb independent manner. Moreover, HIC1 and HMGA1 are present in at least one common complex and they are both part of the HIV-1 transcription repression mechanism in presence of TAT.

We report here for the first time a negative impact of HMGA1 on HIV-1 transcription. Previous studies presented HMGA1 as a positive regulator of viral transcription. However the activator role of HMGA1 on HIV-1 transcription has always been reported in lymphocytes. We already demonstrated that transcription factors can have ambivalent effects on HIV-1 transcription depending on the cell type. Thus CTIP2 which operates as a transcriptional repressor on HIV-1 in microglial cells, is an activator of viral transcription in activated lymphocytes (Redel et al., unpublished).

HMGA1 has been shown to promote its HIV-1 transcription activator role through recruitment of the SWI/SNF complex. SWI/SNF is a chromatin remodelling complex which can act as an activator or a repressor of transcription depending on the subunits engaged in the process. Thus the ARID1B subunit is found in transcriptional activator SWI/SNF complexes, while ARID1A is associated with transcriptional repression (Nagl et al., 2007 ; Flowers et al, 2009). Previous reports demonstrated that HIC1 interacts with the subunit ARID1A and so recruits the SWI/SNF complex. Recruitment of ARID1A-containing SWI/SNF allows HIC1 to down-regulate its target gene *E2f1* (Van Rechem et al., 2009). The possibility that HIC1 and HMGA1 cooperates to recruit SWI/SNF at the viral promoter has to be investigated.

Finally, we demonstrated that HIC1 was not directly bound to the HIV-1 LTR, but was recruited in a TAR-containing complex. A recent study indicated that HMGA1 interacts with TAR RNA Binding Protein (TRBP) (Chi et al., 2011). HMGA1 could be the link between HIC1 and HIV-1 TAR RNA through interaction with TRBP. This also needs further investigation to be validated.

Discussion

L'infection au VIH-1 reste à l'heure actuelle un problème sanitaire majeur au niveau mondial. Les traitements à base d'antirétroviraux (ARVs) permettent le contrôle de la maladie et le maintien des défenses immunitaires, mais ne guérissent pas les patients. En effet, des provirus latents restent tapis dans des réservoirs cellulaires, empêchant les ARVs d'aboutir à l'éradication complète du virus. Malheureusement, le contrôle de la maladie à long terme grâce aux traitements ARVs n'est pas exempt de défauts pour les patients. Les effets indésirables, l'observance des protocoles, le coût, voir même l'accès aux traitements dans certaines zones géographiques, font que les régimes ARVs actuels ne peuvent pas rester à eux seuls le moyen de traiter la maladie.

Une des stratégies serait d'associer aux multi-thérapies des molécules visant à la purge des réservoirs viraux. Le partenariat entre l'efficacité des ARVs sur le virus productif et la liquidation des stocks de virus archivés semble être une stratégie prometteuse.

Cependant, pour développer les médicaments de demain, il est d'abord essentiel de connaître l'éventail de cibles thérapeutiques disponibles.

En partant de ce postulat, notre équipe de recherche étudie les événements moléculaires conduisant à l'établissement et au maintien de la latence virale dans le réservoir cellulaire cérébral principal, les cellules microgliales.

Notre laboratoire a précédemment établi le mécanisme d'action par lequel le facteur transcriptionnel cellulaire CTIP2 était en mesure d'établir l'entrée en latence du provirus (Marban et al., 2007). CTIP2, en recrutant des activités HDACs et méthyl-transférases au niveau du promoteur viral, permet l'établissement de marques associées à la formation d'hétérochromatine et donc d'induire un environnement réfractaire à la transcription des gènes viraux.

Dans le cadre de cette thèse, nous démontrons maintenant que la déméthylase LSD1 est aussi engagée dans ce processus et favorise l'établissement de marques épigénétiques supplémentaires en recrutant à la fois la machinerie multienzymatique associée à CTIP2 et le complexe multiprotéique COMPASS (*publication 1*).

De plus, nous présentons à l'occasion de ces travaux, un nouvel acteur de la restriction de l'expression transcriptionnelle des gènes viraux, le suppresseur de tumeur HIC1. HIC1, contrairement à LSD1, n'est pas au cœur d'un système de remodelage de la chromatine, mais un perturbateur de la transactivation virale (*publication 2*). Le dérèglement de la transactivation est un événement critique qui peut tout aussi bien mener un provirus faiblement productif vers l'extinction transcriptionnelle, ou empêcher la sortie de la latence en interférant avec les systèmes de réactivation (Weinberger et al., 2005).

CTIP2, LSD1 et HIC1 sont donc trois acteurs de l'établissement et du maintien de la latence dans les cellules microgliales et donc trois cibles potentielles, dans la logique de purge des réservoirs. Ils interviennent à des stades différents, l'établissement et le maintient de la latence.

CTIP2 et LSD1 favorisent L'établissement de la latence dans les cellules microgliales.

CTIP2 est au cœur de la thématique de notre équipe. Par la passé, nos travaux ont permis de montrer que le facteur transcriptionnel CTIP2 est en mesure de réprimer l'expression des gènes viraux durant la phase précoce et tardive de la transcription virale, dans les cellules microgliales. Lors de la phase précoce de la transcription, CTIP2 va participer à l'établissement d'un environnement hétérochromatinien compact au niveau du promoteur viral, afin d'empêcher l'initiation de la transcription du VIH-1. Au voisinage du nucléosome Nuc-1, CTIP2 engage les histones déacétylases de classe I HDAC1 et HDAC2, qui déacétylent la lysine 9 de l'histone H3. Une fois cette marque, associée à l'activation transcriptionnelle, retirée, la méthyltransferase SUV39h1 est à son tour recrutée par CTIP2 et triméthyle Nuc-1 sur la H3K9. Séquentiellement, CTIP2 induit la perte d'une marque associée à l'euchromatine (acétylation) au profit d'une associée à l'extinction transcriptionnelle (H3K9me3) (Marban et al., 2007).

Ici, nous proposons la déméthylase LSD1 comme nouveau répresseur de la transcription du VIH-1 dans les cellules microgliales. Au même titre que CTIP2, LSD1 est en mesure de réguler négativement la transcription des gènes viraux aussi bien pendant la phase précoce que dans la phase tardive de la transcription (*Publication 1*).

Les résultats présentés ici permettent de poursuivre le travail déjà entrepris avec CTIP2, en ajoutant de nouveaux partenaires au complexe multienzymatique et rajoutant des étapes au modèle mécanistique précédemment proposé (Figure A).

Ainsi, CTIP2 est recruté au promoteur viral par l'intermédiaire de la protéine LSD1, elle-même fixée au promoteur en se liant à SP1 (Specificity Protein 1). Une fois recruté au promoteur, LSD1 intervient en tant que plateforme d'ancrage pour CTIP2, qui à son tour engage le complexe de remodelage de la chromatine décrit auparavant. De plus, LSD1 recrute également le complexe méthyltransferase COMPASS, vraisemblablement en charge de la triméthylation de la lysine 4 de l'histone H3, en adéquation avec les observations précédemment réalisées dans la littérature (Krogan et al., 2002 ; Schneider et al., 2005)).

Actuellement, nous ne sommes pas en mesure de définir une cinétique de recrutement des complexes CTIP2/HDACs/SUV39h1 et hCOMPASS par LSD1. Ce mécanisme peut faire intervenir l'association séquentielle ou simultanée des différents complexes. Cet aspect du mécanisme reste à élucider pour mieux définir les modalités d'établissement des marques épigénétiques.

LSD1 et CTIP2 appartiennent à un même complexe de remodelage de la chromatine et induisent un profil épigénétique atypique.

LSD1 est connue pour déméthyler spécifiquement des lysines de l'histone H3, K9 et K4, pour promouvoir une régulation respectivement soit positive soit négative de la transcription de ses gènes cibles (Shy et al., 2004 ; Metzger et al., 2005). Dans notre cas, à la suite de la suppression du groupement acétyle sur la lysine 9, marque

activatrice, par les HDACs, la méthyltransferase SUV39h1 triméthyle ce même résidu. La H3K9me3 est une marque répressive sur laquelle la protéine HP1 (Heterochromatin Protein 1) se fixe et induit la compaction en cascade du provirus.

Parallèlement, LSD1 recrute les sous-unités SET1 et WDR5 du complexe méthyltransferase COMPASS au promoteur viral, ce qui va permettre l'adjonction d'une marque triméthyle sur la lysine 4 de l'histone H3 (*Publication 1*). Cette constatation est en adéquation avec les précédentes observations où LSD1, plutôt que d'user de son activité déméthylase pour réguler les gènes, va recruter une machinerie méthyltransferase, et plus particulièrement WDR5 (Wang et al., 2007). WDR5 est associé à une augmentation de la marque H3K4me3, notamment dans le développement des vertébrés (Wysocka et al., 2005). Ainsi, comme décrit dans le cas du virus de l'herpès simplex, LSD1 peut favoriser l'établissement de marques triméthyle au niveau de la lysine K4 (Liang et al, 2009). Cependant, contrairement à ce qui a été précédemment décrit, Nous observons dans le cas du VIH-1 que l'établissement de la marque activatrice H3K4me3 est concomitant de l'établissement de la marque H3K9me3 inhibitrice. Dans le cas du HSV, la marque H3K4me3 est associée à la diminution de la marque inhibitrice H3K9me3 et à une levée de la latence virale par réactivation de l'expression du génome viral (Huang et al., 2006 ; Liang et al., 2009). Nous décrivons ici pour la première fois l'association de ces deux marques, qui habituellement débouchent sur des modifications opposées de la transcription génique, dans le cadre d'une répression transcriptionnelle chez les eucaryotes. Cette observation rejoint la récente association de la marque H3K4me3 à l'inhibition de l'expression de gènes, notamment au niveau de promoteurs cryptiques chez la levure (Pinskaya et al, 2009, 2009b). Les gènes cryptiques sont des séquences géniques silencieuses, faiblement transcrites durant le cycle cellulaire. Le promoteur du VIH-1 peut être aisément considéré comme cryptique étant donné la propension du provirus à intégrer des zones introniques (Han et al., 2004).

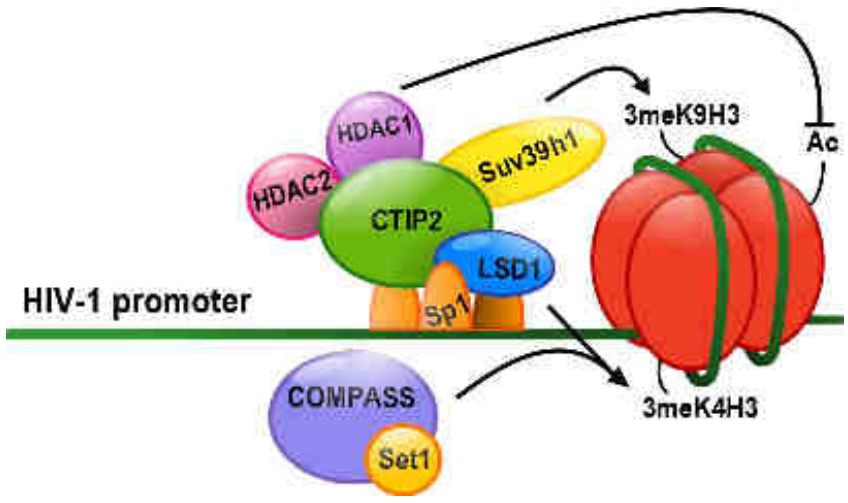

Figure A : Modèle d'établissement de la latence induit par LSD1 et CTIP2

HIC1 et SIRT1 participent au maintien de la latence virale dans les microglies.

Hic1 est un gène codant pour un suppresseur de tumeur et dont l'activité transcriptionnelle est éteinte dans de nombreux cas de cancers (Rood et al., 2002 ; Pieretti et al., 1995 ; Kanai et al., 1998 ; Fujii et al., 1998 ; Yamamakam et al., 2003). En effet, l'absence de production de HIC1 est associée à dérégulation de boucles de rétro-contrôle intervenant dans la réparation des dommages à l'ADN, l'hypoxie et la privation calorique (pour revue Dehenaut et Leprince, 2009 ; Fleuriel et al., 2009). Contrairement à LSD1 et CTIP2, HIC1 a été associé à un mécanisme d'extinction transcriptionnelle du VIH-1 où le transactivateur viral TAT est la cible de ce répresseur transcriptionnel et non le nucléosome Nuc-1 (*publication 2*). Néanmoins, HIC1 a été décrit comme un répresseur transcriptionnel capable de recruter des complexes corépresseurs tels que SWI/SNF, NuRD et Polycomb (Van Rechem et al, 2009 ; Van rechem et al., 2010 ; Boulay et al, 2012). Il est essentiel d'étudier la possibilité de modifications épigénétiques induites par HIC1.

Pour l'heure, nous avons clairement pu lier activité répressive de HIC1 à l'activité déacétylase de SIRT1 (*publication 2*). Etant donné que l'acétylation de HIC1 sur sa lysine 314 du motif MKHEP est une condition suffisante pour empêcher HIC1

d'exercer son inhibition, l'hypothèse la plus simple serait que la déacétylation préalable de HIC1 par SIRT1 amène HIC1 à interagir et inhiber TAT (Figure B).

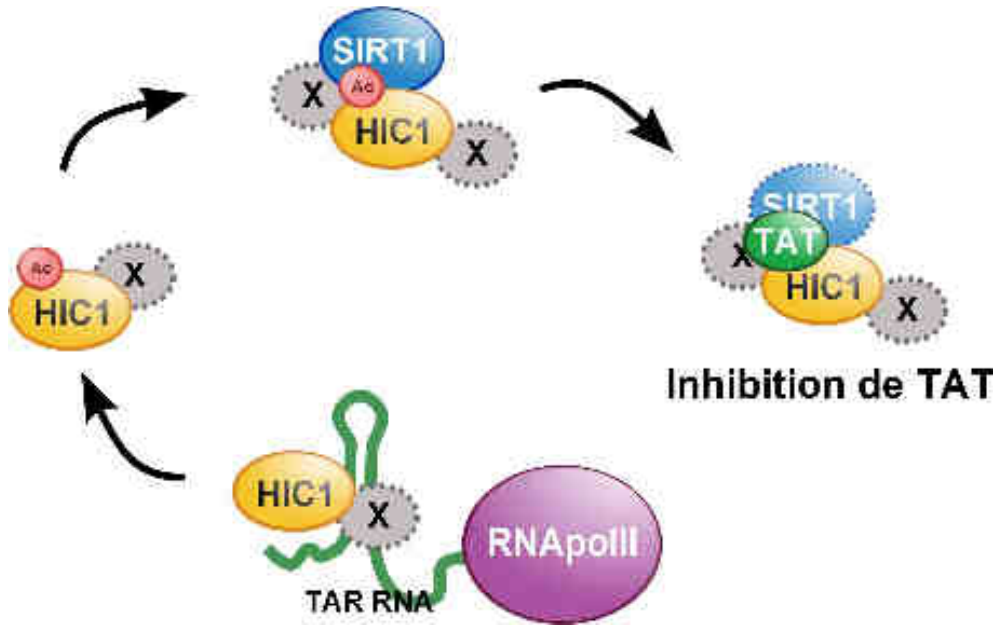

Figure B : Modèle de l'inhibition par HIC1

Nous avons pu établir que HIC1 et la TAR interagissent, mais sans réussir à définir pour l'instant la signification de cette interaction dans le circuit de répression. La TAR pourrait servir de réservoir de HIC1 à proximité du lieu même de l'initiation de la transactivation de l'élongation de la transcription du génome viral. Il est tout à fait possible que l'interaction entre HIC1 et la TAR ne soit pas directe et requiert des partenaires supplémentaires. HMGA1 pourrait faire office d'interface entre HIC1 et la TAR, en effet, nous avons montré que HIC1 et HMGA1 pouvait interagir et que ces deux protéines interviennent coopérativement dans le processus de répression (*HIC1 – Résultats supplémentaires*). Cette hypothèse vient du fait que la publication récente de l'interactome de la TRBP (TAR-RNA Binding Protein) révèle que cette protéine est en mesure d'interagir avec HMGA1 (Chi et al., 2011).

Ces hypothèses feront l'objet d'une étude approfondie.

Un rôle inédit de SIRT1 dans la régulation du VIH-1.

Bien qu'impliqué dans le recyclage de TAT en fin de cycle de transactivation en déacétylant TAT, et permettant une « remise à zéro » du transactivateur viral, SIRT1 est associé à la restriction de réplication virale dans la littérature (Blazek et Paterlin, 2008 ; Weinberger et al., 2008). SIRT1 réduit l'activité de NF-κB, limitant l'expression génique du provirus, mais aussi de gènes impliqués dans l'activation des lymphocytes, comme le gène de l'IL-2 (Kwon et al, 2008). Même si SIRT1 est requis pour un cycle de transactivation complet, TAT induit une diminution de l'activité de SIRT1 par différents moyens. TAT est associé à une augmentation du miARN-217, ciblant le mRNA de SIRT1, une déplétion du NAD+ disponible, indispensable à l'activité de SIRT1 et serait un compétiteur fort de SIRT1 pour ses autres substrats (Kwon et al, 2008 ; Zhang et al., 2009 ; Zhang et al., 2012).

Une hypothèse propose que pendant la phase tardive de la transcription du provirus, au moment où les taux de TAT sont très élevés dans la cellule, TAT bloque la cavité catalytique de SIRT1 après y avoir été déacétylé, car TAT apparait comme un puissant inhibiteur de SIRT1 *in-vitro* (Kwon et al, 2008). Ce mécanisme empêcherait SIRT1 de déacétyler des protéines essentielles à la réplication virale, comme NF-κB.

Il est encore difficile de définir si l'activité de SIRT1 se limite uniquement à la déacétylation de HIC1 dans le processus de répression. Il est tout à fait possible que SIRT1 soit amenée à déacétyler précocement TAT ce qui pourrait mettre à mal le cycle de transactivation. En effet, nos travaux indiquent également que l'activité répressive de HIC1 requiert l'élément TAR, indiquant donc que les phénomènes interviennent tôt dans la transactivation. Dans des travaux précédents, des mutations sur les lysines acétylables de TAT ont été décrites comme délétères pour la transactivation virale (Weinberger et Shenk, 2007 ; kwon et al., 2008).

Ainsi, en marge de l'étude de l'éventuel impact de HIC1 au niveau épigénétique, il est primordial de définir si les complexes HIC1/TAT et HIC1/SIRT1 sont un seul et même complexe, ou s'ils interviennent de façon séquentielle. De

même les niveaux d'acétylation de TAT en présence et absence de HIC1 doivent être examinés pour répondre à ces questions. Des résultats préliminaires semblent indiquer que l'état d'acétylation de TAT agit sur l'interaction du transactivateur viral avec HIC1. Si cela s'avère exact, le rôle de SIRT1 dans ce mécanisme deviendrait d'autant plus primordial.

Il reste également à savoir si l'acétylation de HIC1 est à l'origine d'un défaut d'interaction avec TAT de manière directe, ou par l'absence de recrutement d'une protéine permettant de faire le lien entre TAT et HIC1. Des expériences complémentaires seront requises pour répondre à cette question. Dans tout les cas, les modifications post-traductionnelles de HIC1 sont un pan important de la mécanistique derrière l'inhibition de la transcription virale, comme elles le sont dans la régulation des gènes cibles de HIC1. Le Dr. Leprince et son équipe ont déjà montré l'importance des modifications de la lysine 314 dans la potentialisation de HIC1 et sa capacité à recruter ou nom ses corepresseurs (Stankovic-Valentin et al., 2007, Van Rechem et al, 2010). Contrairement aux modèles déjà proposés, où la SUMOylation est toujours une marque profitable à l'activité répressive de HIC1, nous montrons ici que la SUMOylation n'intervient pas dans le mécanisme de répression de TAT. De plus, nous observons que l'acetylation de HIC1 est nuisible à son activité et son intéraction avec TAT, d'une manière comparable à la perte d'interaction entre HIC1 et le complexe NuRD décrite dans la littérature lorsque HIC1 porte cette marque post-traductionnelle (Van Rechem et al., 2010).

LSD1, CTIP2 et HIC1, des cibles thérapeutiques délicates.

Même s'il apparait évident que la perturbation des mécanismes d'établissement de marques épigénétiques répressives du complexe multienzymatique LSD1/CTIP2/HDACs/Méthyle-transferases s'inscrit parfaitement dans la logique de purge des réservoirs, en limitant la taille des réservoirs viraux, ce mécanisme d'entrée en latence n'est validé que dans les cellules microgliales.

L'élimination, ou tout au moins la réduction de la taille du réservoir viral cérébral fait parti des buts à atteindre en vu d'un traitement curatif. Malheureusement, nos propres travaux, mais également ceux d'autres équipes, révèlent que CTIP2 et LSD1 ont des activités ambivalentes suivant le type cellulaire. L'équipe du Dr. Ott a récemment démontré que LSD1, dans les lymphocytes, participait au déroulement du cycle de transactivation de TAT (Sakane et al., 2011). LSD1 déméthyle la lysine 51 de TAT dans les premières étapes de la transcription, ouvrant le bal des modifications post-traductionnelles du facteur de transactivation viral. Ainsi, la déplétion de LSD1 aurait un rôle activateur dans les macrophages cérébraux, tandis qu'elle favoriserait la latence dans les lymphocytes, et donc l'augmentation de la taille de ce réservoir. Cibler LSD1 semble dès lors contre-productif. Cependant, à l'heure actuelle le mécanisme activateur de LSD1 dans les lymphocytes intervient au niveau de TAT et non de la modification d'histones, comme nous l'observons dans les cellules microgliales. La possibilité de cibler des interactants de LSD1 n'intervenant pas dans le mécanisme de déméthylation de TAT, comme COMPASS, semble plus avisée. De cette manière, il serait possible d'agir sur le réservoir macrophagique sans potentialiser la mise en place du réservoir lymphocytaire.

Le cas de CTIP2 est plus controversé dans les lymphocytes. Une équipe décrit CTIP2 en tant que répresseur du VIH-1, notamment en recrutant le complexe NuRD, mais le décrit également comme un activateur du gène de l'IL-2, cytokines associé à une facilitation de l'infection et dont le gène est également up-régulé en présence de TAT (Cismasiu et al., 2005 ; Kumar et al., 2005 ; Cismasiu et al., 2006 ; Cismasiu et al ; 2008). CTIP2 serait aussi capable, toujours dans les lymphocytes, d'augmenter la translocation de NF-κB dans le noyau en provoquant la dégradation de IκB par l'augmentation de l'activité de IKK (Cismasiu et al., 2009). Dans nos mains, CTIP2 a un rôle activateur dans la lignée lymphocytaire Jurkat et aucune activité répressive dans les lymphocytes primaires (Redel et al., unpublished). La mécanistique derrière cette activité de CTIP2 reste néanmoins à approfondir et à corréler avec les données bibliographiques, notamment de Cismasiu et al.

Le cas de HIC1 est encore différent. Son impact dans les cellules T n'a pas encore été établit, mais, au vue des effets opposés de CTIP2 et LSD1 dans ce type cellulaire vis-à-vis de la lignée monocyte-macrophage, il est primordial d'étudier l'effet de HIC1 dans ces cellules. De surcroit, il faut garder en mémoire qu'HIC1 intervient dans des boucles de rétro-contrôle finement contrôlées et que la perte d'hétérozygotie, et donc la diminution intracellulaire de HIC1, est associé à des pathologies graves (Chen et al, 2003 ; pour revue Chopin et Leprince, 2006 ; Dehennaut et al., 2009). Ainsi, cibler et contrecarrer HIC1 peut sembler, à juste titre, complexe et demandera des ajustements minutieux. On pourra alors lui préférer SIRT1, qui apparait déjà comme un facteur de restriction à bien des niveaux, comme cible de drogues visant à la réactivation virale. En limitant l'action de SIRT1, il serait possible à la fois de réduire son action anti NF-κB, décrit dans la littérature et anti TAT par son association à HIC1, que nous suggérons ici.

La compréhension de ces mécanismes s'inscrit dans la dynamique de réactivation virale

Finalement, même si LSD1, CTIP2 et HIC1 ne sont pas les cibles thérapeutiques idéales, décortiquer les mécanismes par lesquels ils permettent de favoriser la latence virale nous permet de mettre en avant ceux qui deviendront les prochaines cibles des thérapies antirétrovirales.

Les mécanismes impliquant LSD1 et CTIP2 font intervenir des HDACs de classe I et des méthyletransférases, comme SUV39h1 et COMPASS. Les HADCs de classe I, ainsi que SUV39h1 sont justement sous le feu des projecteurs dans l'optique de réactiver les réservoirs viraux. En effet, la chaetocine, un inhibiteur spécifique de SUV39h1 et le vorinostat, un inhibiteur de HDACs de classe I et II ont très récemment été proposés comme réactivateur de la latence virale (Bernhard et al., 2011 ; Bouchat et al., 2012. ; Archin et al., 2012 ; conférence AIDS 2012). Ils ont avec succès, permis la réactivation de provirus latents dans des cellules T CD4+ quiescents (vorinostat et chaetocine) et des PBMCs (chaetocine) de patients (Bouchat et al.,

2012. ; Archin et al., 2012). Cette preuve de concept renforce l'intérêt des recherches fondamentales sur la latence virale.

Ce travail de thèse a pour vocation non seulement d'indiquer de nouvelles cibles, comme SIRT1 ou COMPASS/WDR5/SET1, mais aussi de comprendre les effets de molécules ciblant des effecteurs pléiotropes comme la chaetocine et le vorinostat plus précisément.

Des inhibiteurs de SIRT1 actuellement en cours d'essai clinique, comme l'EX-527 dans le cadre de la maladie de Huntington (Solomon et al., 2006 ; phase clinique I, Elixir Pharmaceutical, 2010), pourraient être, au vu des résultats présentés ici, de nouvelles molécules à joindre aux traitements antirétroviraux en combinaison avec d'autres drogues de réactivation comme le vorinostat et la chaetocine. De plus, le vorinostat et le cambinol, un autre inhibiteur de SIRT1, ont récemment été mis en évidence pour leur forte efficacité *in-vitro* sur les neuroblastomes, laissant présager une utilisation des inhibiteurs de HDACs de classe I, I et III chez l'humain (Lautz et al., 2012). Un autre rapport fait état de combinaisons de différents inhibteurs d'HDACs de classe I et II (acide valproique, butyrate et vorinosat) avec des inhibiteurs de sirtuines (EX 527, cambinol, sirtinol) et de leurs efficacités synergiques dans le cadre de pathologies leucémiques (Cea et al., 2011). L'utilisation, combinée ou non, d'inhibiteurs ciblant différentes classes d'HDACs, est en cours d'analyse et de validation dans le cadre des cancers, et pourrait être transposée à l'infection au VIH-1. Ce qui concrètement permettrait de lever à la fois la latence induite par LSD1/CTIP2 au niveau épigénétique, et celle induite par l'interférence de HIC1 sur l'activité transactivatrice de TAT, dans les cellules microgliales.

C'est sur cette note d'optimisme que je clôturerai cette partie.

Annexes

Publication 3 – En preparation

The cellular cofactor CTIP2 is a negative P-TEFb regulator.

Cherrier T, et al.

Le complexe positif d'elongation (P-TEFb) est impliqué dans des évènements physiologiques et pathologiques majeurs, tels que le cancer, l'inflammation, l'hypertrophie cardiaque et le SIDA. L'équilibre entre sa forme active et inactive est étroitement contrôlé afin d'assurer l'intégrité de la cellule.
Ici nous démontrons que le répresseur transcriptionnel CTIP2 est un régulateur majeur de l'activité du complexe P-TEFb. CTIP2 est co-purifié et interagit avec la forme inactive du complexe P-TEFb, contenant le 7SKsnRNA et HEXIM1. CTIP2 s'associe directement à HEXIM1 et, via la boucle 2 du 7SKsnRNA, à P-TEFb. Dans ce complexe protéine/acide nucléique, CTIP2 réprime considérablement l'activité kinase de la sous-unité CDK9 de P-TEFb. De plus, nous montrons que CTIP2 inhibe également la transcription des gènes régulés par P-TEFb ainsi que des gènes viraux. En conséquence, CTIP2 contrôle de nombreux gènes sensibles au 7SKsnRNA. En marge de ces observations, nous indiquons aussi que CTIP2 réprime les voies de signalisation dépendante de P-TEFb dans le coeur de souris présentant une cardiomyopathie hypertrophique. Avec le SIDA, la cardiomyopathie hypertrophique constitue une pathologie majeure associée à P-TEFb. Ainsi, en tant que nouvelle composante du complexe P-TEFb inactif, CTIP2 se pose comme une nouvelle cible pharmaceutique dans le cadre des pathologies dépendantes de P-TEFb.

J'ai effectué une partie des expériences de ces travaux et serait assigné au travail de reviewing.

The cellular cofactor CTIP2 is a negative P-TEFb regulator

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ABSTRACT

The positive elongation complex (P-TEFb) is involved major physiological and pathological events such as cancer, inflammation, cardiac hypertrophy and AIDS. The balance between its active and inactive forms is tightly controlled to ensure the cellular integrity.

Here we show that the transcriptional repressor CTIP2 is a major modulator of the P-TEFb complex activity. CTIP2 copurifies and interacts with an inactive P-TEFb complex containing 7SKsnRNA and HEXIM1. CTIP2 associates directly with HEXIM1 and, via the loop 2 of the 7SKsnRNA, with P-TEFb. In this nucleo-proteic complex, CTIP2 dramatically represses the CDK9 kinase activity of P-TEFb. Moreover, we show that CTIP2 inhibits the P-TEFb-sensitive cellular and viral gene transcriptions. Accordingly, CTIP2 controls a large set of 7SKsnRNA sensitive genes. Beside to these observations, we show that CTIP2 represses the P-TEFb sensitive pathways in heart of hypertrophic cardiomyopathic mouse. Together with AIDS, hypertrophic cardiomyopathy constitutes a major P-TEFb dependent pathology. Thereby, as a key component of a new inactive P-TEFb complex, CTIP2 constitute a new pharmaceutical target for P-TEFb-dependent pathologies.

Discovered in 1995 (Marshall and Price, 1995), the elongation complex P-TEFb (CyclinT1/CDK9) is involved in physiological and pathological transcriptionally regulated events such as growth, differentiation, cancer and AIDS (for review see (Brasier, 2008; Zhou and Yik, 2006)). It has been suggested to be required for transcription of most RNA polymerase II-dependent genes. However, a recent study suggests that a subset of cellular genes are distinctively sensitive to CDK9 inhibition (Garriga et al.). P-TEFb is dynamically regulated by both positive and negative regulators. In contrast to Brd4, which is associated with active P-TEFb forms (Jang et al., 2005; Yang et al., 2005), the small nuclear RNA 7SK (7SK snRNA) and HEXIM1 inhibit CDK9 activity in the inactive P-TEFb complex (Michels et al., 2004; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). P-TEFb elongation complexes are crucial for HIV-1 gene transactivation and for viral replication. Recently new P-TEFb complexes containing the HIV-1 Tat protein were characterized (He et al., ; Sobhian et al.) providing evidence for the recruitment of an inactive Tat-P-TEFb complex at the HIV-1 promoter (D'Orso and Frankel). However, defining the diverse nature and the functions of the P-TEFb complexes will need more investigations. The cellular cofactor CTIP2 (Bcl11b) has been highlighted as a key transcription factor for thymocyte (Ikawa et al., ; Li et al.) and neurone (Arlotta et al., 2005) development, odontogenesis (Golonzhka et al., 2009), cancer evolution (Ganguli-Indra et al., 2009) and HIV-1 gene silencing (Marban et al., 2007).

Here, we report that CTIP2 represses P-TEFb function as part of an inactive P-TEFb/7SK/HEXIM1 complex.

RESULTS

CTIP2 is associated with the inactive P-TEFb complex.

First we asked whether CTIP2 was associated with one of the previously described form of the P-TEFb complex. To do so, we performed immunoprecipitations experiments. We found that CTIP2 coprecipitated with the CyclinT1 and the CDK9 components of P-TEFb complex (Figure 1A). To define the CTIP2-containing

complexes, we then separated the previously described P-TEFb complexes by gel-filtration chromatography (Figure 1B). As shown in figure 1B, CDK9 was detected in the low molecular weight (LMW) complexes ("free" P-TEFb complexes) and in the high molecular weight (HMW) complexes that co-eluted with HEXIM1 and the 7SK snRNA. Interestingly, we found CTIP2 in these earlier fractions containing the HMW P-TEFb complexes (Figure 1B). These observations suggested that CTIP2 may be part of an inactive HEXIM1/7SK-including P-TEFb complex. To confirm this hypothesis, we performed additional coimmunoprecipitation experiments targeting the active and the inactive forms of the P-TEFb complexes. We found that CTIP2 co-purified with CyclinT1, CDK9, HEXIM1 and 7SK but not with Brd4, making CTIP2 a new component of the inactive P-TEFb complex. Confocal observations further confirmed the co-localization of CTIP2, P-TEFb and HEXIM1 in the previously described CTIP2-induced nuclear structures (Rohr et al., 2003) (supplemental figure1). As suggested by the gel filtration elution profile, CTIP2 was not found in the active Brd4/P-TEFb complexes. The 7SK snRNA functions as a scaffold RNA facilitating the interaction between HEXIM1 and P-TEFb. To investigate how CTIP2 associates with the inactive P-TEFb complex, we performed coimmunoprecipitation experiments following RNase treatments to degrade the 7SK snRNA (Figure 1D). As previously described, degradation of the 7SK snRNA (Figure 1D right panel) induced the dissociation of P-TEFb and HEXIM1 (Figure 1D left panel column 5 vs 3). However, CTIP2 remained associated with HEXIM1 upon RNase treatment suggesting that CTIP2 interacts directly with HEXIM1 and that the 7SK RNA is required to associate with P-TEFb. These biochemical observations were supplemented by functional experiments. Since the HIV-1 LTR is a *bonafide* P-TEFb sensitive promoter, we quantified its activity upon CTIP2 knock-down, Tat transactivation and P-TEFb inhibition. We observed that knocking down CTIP2 increased the Tat-dependent transcriptional activity of the HIV-1 promoter suggesting that endogenous CTIP2 might contribute to the repression of Tat by repressing P-TEFb functions (Figure 1E). In support of this hypothesis, treatments with Flavopyridol, a potent P-TEFb inhibitor, abrogated this synergistic transcriptional activation. Therefore, we conclude that CTIP2 may

contribute to P-TEFb repression in the inactive P-TEFb complex. Along this line, we further investigated on CTIP2 function in this P-TEFb, HEXIM1 and 7SKsnRNA including nucleo-proteic complex (Figure 2).

CTIP2 interacts with the 7SK snRNA

First, gel shift experiments were performed to define if CTIP2 interacts with the 7SKsnRNA *in vitro* (Figure 2A). As shown, increasing amounts of purified GST-CTIP2 protein shifted increasing amounts of ^{32}P-labelled 7SKsnRNA indicating that CTIP2 interacts with the 7SK *in vitro*. To define whether this interaction persist *in vivo*, we performed immunoprecipitations of CTIP2-associated complexes and look for the presence of the 7SK by RT-PCR. We found that the 7SKsnRNA copurified with CTIP2 suggesting that this association exists *in vivo* (Figure 2B). Micro Scale Thermoforesis experiments confirmed this result but also allow us to precise the domain of the 7SK involved in this interaction. CTIP2 associated with the Loop 2 domain of the 7SK snRNA (Figure 2C). We then asked which domain of CTIP2 was involved in the interaction with the 7SK and P-TEFb. To do so, we performed immunoprecipitation experiments with truncated forms of CTIP2 (Figure 2D). This mapping confirmed the need of the 7SK snRNA for interaction with P-TEFb but not with HEXIM1 (figure 2D and supplemental figure 2). Indeed, a CTIP2 mutant lacking amino-acid 355-813 was unable to associate with 7SK snRNA and P-TEFb but still associated with HEXIM1 (Figure 2D column 3). Moreover, we identified a domain centered on the amino-acid 350 as the potential interface for the 7SKsnRNA interaaction. Surprizingly, deletion of the 717 (out of 813) first amino-acids of CTIP2 was needed to release the interaction with HEXIM1 (Figure 2D column 1). Next, we tested whether CTIP2 may regulate P-TEFb activity.

CTIP2 inhibits CDK9-mediated phosphorylations

To explain CTIP2-mediated repression of the P-TEFb function, we first hypothesized that CTIP2 may favor P-TEFb recruitment within the inactive complex. CyclinT1 immunoprecipitation experiments were performed to confirm this hypothesis.

Surprisingly, CTIP2 overexpression did not favor recruitment of P-TEFb into the inactive, 7SKsnRNA containing complexes (figure 2E). Since the amount of P-TEFb in the inactive complex was not impacted by CTIP2, we next asked for the inhibition of the P-TEFb kinase activity. To do so, we first quantified the CDK9 kinase activity in CyclinT1 immunoprecipitated complex in the presence or not of CTIP2 overexpression. As shown in figure 2F, CTIP2 overexpression dramatically inhibited CDK9 activity (Figure 2F and supplemental figure 3). To confirm that this repression also occurs in more physiological conditions, we looked for the global level of RNApolII serine 2 phophorylation in CTIP2 knocked down cells. Accordingly, higher levels of RNApolII serine 2 phophorylation were observed in CTIP2-depleted cells (Figure 2G). These observations suggested that CTIP2 represses P-TEFb functions by inhibiting CDK9 activity.

The P-TEFb- and the Chromatin modifying enzymes- complexes are exclusive CTIP2-including complexes

We have reported that CTIP2 silences HIV-1 gene expression by recruitment of HDAC1, HDAC2 and SUV39H1 at the viral promoter (Marban et al., 2007). To examine the existence of multiple CTIP2 complexes, we performed sequential immunoprecipiations experiments. For this purpose, CTIP2 associated complexes (IP1) were further immunoprecipitated with anti-HEXIM1 or anti-HDAC2 to discriminate between the P-TEFb and the chromatin modifying complexes, respectively (IP2). As shown in Figure 3, HDAC2 and Suv39H1 were excluded from the P-TEFb complex while CDK9, CyclinT1 nor Hexim1 were found in the chromatin modifying complex. These data demonstrate that CTIP2 associates at least with two distinct nuclear complexes.

CTIP2 regulates P-TEFb sensitive genes

To validate the model emerging from our observations and establishing CTIP2 as a negative component of the P-TEFb complex, we examined the genome-wide transcriptional consequences of knocking-down CTIP2 in microglial cells and HEK

cells. Using DNA microarray analysis, genes whose expression was modulated by siRNA-mediated deletion of CTIP2 (supplemental table 1) were compared to those regulated by expression of a dominant negative mutant of CDK9 (dnCDK9) (Garriga et al.). Hierarchical clustering of the genes revealed a strong anti-correlation between the two gene expression profiles (Figure 4A). Clustering of highly coregulated genes (-1> Log2 values >1 in both gene profilings) revealed more precisely that 86% of CDK9 sensitive genes are inversely regulated by knocking-down CTIP2 (figure 4B and supplemental figure 4). The comparisons of the genes significantly ($p<0.05$) regulated by CTIP2 overexpression, knock down and dnCDK9 expression in HEK 293 cells confirmed the observations made in the microglial cells (Figure 4C, D, E). Twenty-five percents of the genes whose expression was significantly ($p<0.05$) modified following CTIP2 knock down or overexpression were also sensitive to dnCDK9 and among them 76% were regulated consistently with a CDK9-inhibitory activity of CTIP2. Note, that the vast majority of these consistently regulated genes is repressed upon dnCdk9 expression, correlating with the elongation-activating role of P-TEFb. Besides, we identified 14% (microglial cells) and 24% (HEK293 cells) of the CTIP2 target genes to be divergently regulated upon dnCdk9 expression, pointing at a different mechanism, where CTIP2 can potentially also contribute to Cdk9 activation (Figure 4D). Whether this mode of action is direct or indirect, needs to be further investigated.

CTIP2 regulates 7SKsnRNA sensitive genes

Since the 7SK snRNA has been described as a key inhibitor of the P-TEFb complex, we further compared the 7SK-dependent and the CTIP2-dependent transcriptome (Figure 4F , G). About 48% of the genes were similarly affected by CTIP2 overexpression or 7SK knockdown. This is consistent with a repressive role for CTIP2 in the P-TEFb complex and coincides with our model, in which both 7SK snRNA and CTIP2 contribute to the inactivation of Cdk9. Surprisingly, 52% of the genes were found to be inversely regulated following CTIP2 overexpression or 7SK knock-down

suggesting that CTIP2 would regulate a subset of 7SK-sensitive genes by still unknown, P-TEFb independent, mechanism (Figure 4F).

CTIP2 regulates CDK9 sensitive genes from heart of hypertrophic cardiomyopathic mouse

As a member of the an inactive P-TEFb complex, CTIP2 should contribute to regulate P-TEFb related physiopathological events. To investigate the physiological relevance of our observations, we focused on one of the most characterized P-TEFb associated pathology: the hypertrophic cardiomyopathy (HCM). Gene expression profiles from HCM mouses were compared to those obtained upon CDK9 overexpression and CTIP2 knock-down (Figure 6). CDK9 overexpression regulated half of the HCM modulated genes confirming the influence of P-TEFb on this specific pathological gene expression patern (Figure 6A). We observed a significant correlation between the gene expression levels from both conditions (Figure 6B). By comparing this cluster of genes with CTIP2 sensitive genes, we observed that 50% of the CDK9-sensitive HCM genes were consistantly regulated by a CTIP2 knock-down (Figure 6C and 6D). Interestingly, key HCM pathways are enriched in the CTIP2/CDK9 cluster of modulated genes (Figure 6E). Moreover, a network analysis of the HCM CDK9- and CTIP2- sensitive genes highlighted the major impact of CTIP2 on the regulation of the cardiac hypertrophy (Figure 6F). Indeed, key HCM pathways, such as the MAPK, the Ca2+/Calmodulin, the NF-kB/NFAT and the PI3K/AkT pathways, are regulated by CTIP2 (Figure 6F). As a final result, we found that the gene of the sarcomeric b-myosin heavy chain was regulated by CTIP2 consistantly with a repression of the CDK9 function (figure 6 G). This observation further argue for a major influence of CTIP2 in the control of the heart sarcomeres sizes and thereby on the HCM.

DISCUSSION

The cellular cofactor CTIP2 is a key transcriptional repressor involved in development (Albu et al., ; Arlotta et al., 2005; Golonzhka et al., 2009; Ikawa et al., ; Li et al.), T lineage commitment (Li et al., ; Li et al.), cancers (De Keersmaecker et al., ;

Ganguli-Indra et al., 2009) and HIV-1 gene silencing (Marban et al., 2007). In association with chromatin modifying enzymes, CTIP2 promotes the establishment of a local heterochromatin environment at target promoters such as the cellular p21 gene- (Cherrier et al., 2009) and the viral HIV-1 gene- promoters (Marban et al., 2005; Marban et al., 2007; Rohr et al., 2003). Interestingly, both genes have been reported to be highly sensitive to the P-TEFb elongation complex (Giraud et al., 2004; Gomes et al., 2006; Wei et al., 1998).

To secure the cell integrity, P-TEFb is dynamically and physiologically regulated. The small nuclear RNA 7SK and HEXIM1 inhibit CDK9 activity in an inactive HEXIM-1/7SK/P-TEFb complex (Michels et al., 2004; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). We show here that CTIP2 associates with the HEXIM1/7SK/P-TEFb inactive complex, that is distinct from the previously described, CTIP2-associated, chromatin modifying complex (Marban et al., 2007). Our genome-wide comparison of the CDK9-sensitive and CTIP2-sensitive cellular genes are consistent with CTIP2 being a repressive component of the P-TEFb complex. Mechanistically, we observed that RNA pol II Serin 2 phosphorylation level increases in CTIP2-knocked down cells. In addition, *in vitro* kinase assays identified CTIP2 as a direct inhibitor of CDK9 enzymatic activity. Based on these observations, we propose that when associated with HEXIM-1/7SK/P-TEFb, CTIP2 strongly represses the CDK9-associated kinase activity and leads to the inhibition of P-TEFb function. Interestingly, the presence of the 7SKsnRNA is crucial in recruiting CTIP2 to P-TEFb. CTIP2 is able to bind the 7SKsnRNA *in vitro* and *in vivo* assays suggesting a direct interaction between both components. The 7SK snRNA has been shown to have at least two major functions in gene expression: it negatively regulates P-TEFb activity and it acts as a negative regulator of the architectural transcription factor HMGA1 (Eilebrecht et al.). Interestingly, CTIP2 and HMGA1 both associate with the loop 2 region of the 7SK. Moreover, the results that we present in the same issue (Authors to be defined) suggest that CTIP2 collaborates with HMGA1 to inhibit HIV-1 gene transcription and the viral replication. Since we have also observed that endogenous CTIP2 repressed the Tat/P-TEFb- mediated transactivation of the HIV-1 promoter

(figure 1E), the repressive activity of CTIP2 and HMGA1 on this P-TEFb sensitive promoter is now attested. In addition, this control P-TEFb might contribute to counteract the viral reactivations in silent HIV-1 reservoirs.

While approximately 50% of the genes were similarly modified upon CTIP2 overexpression or 7SK knockdown, the remaining half showed an inverse correlation. This subset of genes was identified as activated by CTIP2, but repressed by 7SK. These genes are likely regulated by CTIP2 in a CDK9-independent manner. D'Orso and collaborators showed the recruitment of the inactive P-TEFb complex at the Sp1 binding sites of HIV-1 promoter. However, no mechanistic evidence was provided on the way of this recruitment. Since CTIP2 is anchored to the Sp1 region via an association with Sp1 protein, it is more than expectable that CTIP2 constitutes a platform for P-TEFb recruitment at target promoters.

Together with AIDS, hypertrophic cardiomyopathy is a well described P-TEFb dependent pathology (for review see (Harvey and Leinwand, ; McKinsey and Kass, 2007)). Upon hypertrophic cardiomyopathy (HCM), the ◎-myosin heavy chain overexpression induces an increasing size of the sarcomeres and a pathological thickening of the heart muscle. This final target gene expression is controled by multiple pathways all contributing to HCM (Harvey and Leinwand). Our results suggest that CTIP2 contributes to the control of the MAPK-, the Ca2+/Calmodulin-, the NF-kB/NFAT- and the PI3K/AkT- pathways. In addition, we show that CTIP2 inhibits the expression of the ◎-myosin heavy chain (MYH7) consistantly with repression of the CDK9 activity. Taken together, our results suggest that CTIP2 is a potent inhibitor of P-TEFb functions in twoo of the most charaterized P-TEFb sensitive pathologies that are AIDS and HCM. As part of a 7SK/HEXIM1/P-TEFb complex, CTIP2 controls P-TEFb-sensitive gene expression and thereby may constitute a new pharmaceutical target for the P-TEFb-dependent pathologies.

EXPERIMENTAL PROCEDURES

Plasmids

The constructs used in our assays have been described previously: pcDNA3, pshRNA-CTIP2, pshRNA-control, RFP-CTIP2, pFLAG-CTIP2 and pFLAG-CTIP2 deletion constructs in (Marban et al., 2007).

Cell culture

The human microglial cell line (provided by M. Tardieu, Paris, France) (Janabi et al., 1995), and HEK293T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum and 100 U/ml penicillin–streptomycin.

Co-immunoprecipitation assays

HEK 293T cells cultured in 100-mm-diameter dishes were transfected using the calcium phosphate co-precipitation method with the indicated plasmids (30μg). Two days post-transfection, immunoprecipitations were performed using the standard technique with M2 anti-FLAG (Sigma), anti-Hexim1 (Abcam) or anti-cyclinT1 (Santa Cruz) antibodies overnight at 4°C with or without RNAse or HMBA treatments. Finally, the immunoprecipitated complexes were processed for SDS–PAGE and Western blot analysis, real time RT-Q-PCR or RT-PCR assay. For real time RT-Q-PCR experiments, 7SK snRNA was quantified for each immunoprecipitation and results were normalized to the EEF1A1 copies. The presence of 7SK in immunoprecipitated-complexes was also access by agarose gel electrophoresis after RT-PCR amplification.

SDS–PAGE and Western blot analysis

SDS–PAGE were performed using standard techniques. Proteins were detected using antibodies directed against the FLAG epitope (M2 mouse monoclonal from Sigma), CyclinT1 and CDK9 (Santa Cruz), CTIP2, Brd4 and Hexim1 proteins (Abcam). Proteins were visualized by chemiluminescence using the Super Signal Chemiluminescence Detection System (Pierce).

Gel filtration experiments

3mg of microglial cells nuclear extracts were concentrated by using a Microcon YM-10 Centrifugal Filter Unit (3,000 Nominal Molecular Weight Limit) and separated by gel filtration on a Superose 6 PC 3.2/30 column (Amersham Biosciences) that has been previously calibrated with protein standards (dextran blue, 2000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa) from Amersham Biosciences, as previously described (Topark-Ngarm et al., 2006). The 29 fractions collected were accessed by SDS–PAGE and Western blot analysis. PCR experiments were also performed on each fraction after RT-PCR amplification, and the presence of the 7SK was visualized after agarose gel electrophoresis.

Micro Scale Thermophoresis (MST)

Total cellular extracts from Flag-CTIP2 expressing HEK293 cells were diluted with total cellular extract from non-transfected cells. For MST experiments a series of 16 successive dilutions was used ranging from 1:0 to $1:2^{14}$. 5 µl of each dilution were incubated with 60 ng of Fam6-labeled 7SK L2 RNA (5'-GGG CGU CCC UCC CGA AGC UGC GCG CUC GGU CGA AGA GGA CGA CCU UCC CCG AAU AGA GGA GGA C-3') for 10 minutes on ice in EMSA buffer (10mM HEPES/KOH, pH 7.9, 100mM KCl, 5mM MgCl2, 0.25mM DTT, 0.1mM EDTA, 10% glycerol and 2 mg yeast tRNA). MST experiments were conducted using a Monolith NT.115 (Nanotemper Technologies) as described previously [3,4] (Laser-power 12%, Laser-on time 60s, LED-power 30%).

Transcriptome analyses

For microarray analyses, RNA amplification, labeling, hybridization and detection were performed following the protocols supplied by Applied Biosystems using the corresponding kits (Applied Biosystems, ProdNo: 4339628 and 4336875). The data obtained were analyzed as described previously (Eilebrecht et al., ; Eilebrecht et al.)

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FIGURE LEGENDS

Figure 1 *CTIP2 is associated with the inactive P-TEFb complex*.

(A) HEK 293T cells expressing Flag-CTIP2 were lysed and immunoprecipitated with anti-CyclinT1 antibodies or with Non Immune Serum IgG (NIS). Input and immunoprecipitated proteins were probed with anti-Flag (f-CTIP2), anti-CyclinT1, anti-CDK9 antibodies. **(B)** Protein complexes from microglial cells nuclear extracts were separated in 29 fractions by HPLC gel filtration. Each collected fraction was submitted to Western blot analysis with the indicated antibodies and processed for 7SK detection by RT-PCR. Before all separations, the column was calibrated with proteins standards (dextran blue, 2000 kDa, fraction 9; thyroglobulin, 669 kDa, fraction 14; ferritin, 440 kDa, fraction 17). No proteins of interest or 7SK snRNA was detected in fractions from 1 to 5 and in fractions from 26 to 29 (data not shown). **(C)** HEK 293T cells expressing Flag-CTIP2 were lysed and immunoprecipitated with anti-Flag antibodies. Input (line 1 and 2) and immunoprecipitated proteins (line 3 and 4) were probed with anti-Flag, anti-BrD4, anti-CyclinT1, anti-CDK9 and anti-Hexim1 antibodies. The presence of the 7SK snRNA was accessed by RT-PCR amplification. **(D)** Cellular extracts were treated (+) or not (-) with RNAse before beeing subjected to immunoprecipitation with anti-Hexim1 antibodies or non immune serum as indicated. Inputs (line 1) and immunoprecipitated extracts (line 2, 3, 4 and 5) were probed with anti-CTIP2, anti-CyclinT1, anti-CDK9 and anti-Hexim1 antibodies. 7SKsnRNA degradation has been controled by RT-PCR. **(E)** Cells were transfected with the LTR-LUC (HIV-1 promoter) epizomal reporter construct in the presence of Tat and/or sh-CTIP2 plasmids or the respective control constructs. Cells were treated with 30 or 100nM of Flavopyridol 24h post-transfection and subjected to luciferase assays 48hours post transfection. Efficiencies of CTIP2 knock-down and Tat expression were controled by Western blot (data not shown). Results are representative to at least tree independent experiments perfomed in triplicate.

Figure 2 *Associated with the 7SK snRNA, CTIP2 inhibits CDK9-mediated phosphorylations*
(A) EMSA experiments were performed with a ^{32}P-7SK snRNA probe and GST or GST-CTIP2 proteins as indicated. Shifted complexes were visualized by autoradiography. The control GST EMSA has been performed with the same quantity of GST as the maximal input of GST-CTIP2 **(B)** HEK 293T cells were lysed and subjected to immunoprecipitation with indicated antibodies. The presence of 7SKsnRNA was quantified by RT-Q-PCR after complexes elution and RNA extraction. . **(C)** Micro Scale Thermoforesis (MST) of Fam6-labeled 7SK L2 RNA in exponential dilutions (2^{-n}, n=0..14) of FLAG-CTIP2 transfected in non-transfected HEK293 cell-lysates. All mixtures were verified for protein content and for every other mixture. Quantification of the relative expression rates of tagged proteins using ImageJ Software has been performed. Coomassie staining and ◎-FLAG immuno-blotting is shown. **(D)** Mock, Flag-CTIP2 wild-type or Flag-CTIP2 constructs transfected HEK293T cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies. The presence of 7SKsnRNA was quantified by RT-Q-PCR after complexes elution and RNA extraction. Detection of immunoprecipitated proteins was determined by Western blot analysis with indicated antibodies. **(E)** Mock or Flag-CTIP2 transfected cells were lysed and subjected to immunoprecipitation with indicated antibodies. The presence of 7SKsnRNA was quantified by RT-Q-PCR after complexes elution and RNA extraction. **(F)** Flag-CTIP2 or mock transfected cells were lysed and subjected to immunoprecipitation with anti-Cyclint1 antibodies. Immunoprecipitated proteins were incubated 1H at 30°C with GST-CTD and 1μCi of P32 labelled ATP. After SDS-PAGE electrophoresis, labelled GST-CTD and endogenous CDK9 were detected with radiosensitive film. Film quantification was performed using ImageJ software. Presence of indicated proteins was determined by Western blot analysis with the indicated antibodies. The level of GST-CTD was assessed by coomassie staining. Specificity of the GST-CTD substract has been controled by using mutated GST-CTD (supplemental figure 5). **(G)** Extracts from WT or CTIP2-knocked down cell lines were subjected to Western blot analysis with the indicated antibodies.

Figure 3 *P-TEFb- and Chromatin modifying enzymes- complexes associated with CTIP2 are exclusive*

Mock or Flag-CTIP2 transfected HEK293T cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies. After washing, antibodies-bound complexes were eluted with Flag peptide and immunoprecipitated with the indicated antibodies. Presence of indicated proteins was determined by Western blot analysis.

Figure 4 *CTIP2 regulates P-TEFb sensitive genes*

(A) Samples of 250 ng of RNA from CTIP2 knocked-down microglial cells and wt cells were amplified and labeled with Cy5 (e1a) and Cy3 (mock) (Perkin Elmer), respectively, using the Agilent Two Colors Low RNA Input Linear Amplification Labeling Kit according to manufacturer's instructions. Labeled RNA was hybridized to the Agilent Human whole-genome array (G2534-60011) and analyzed. Expression data were extracted using Agilent Feature Extraction software (version 9.1.3.1). Raw data were natural log (ln) transformed and signals from multiple probes for the same gene were averaged. Each array was normalized so that the mean was zero and standard deviation was one. Data from three independent replicate experiments were averaged. The profiles were clustered using spearman rank hierachical clustering using the Cluster software. Shades of yellow and blue represent relative activation and repression respectively. Genes modulated by the knock-down of CTIP2 were compared to the genes modulated by an inhibition of the CDK9 activity (expression of the dnCDK9 construct) (Garriga et al.). **(B)** Genes highly sensitive to both dnCDK9 expression and CTIP2 knock-down (modulation average: log2>1) were clusterized and identified. **(C)** Transcriptome heatmap of genes statistically significantly (at least $p<0.05$) opositely regulated genes by Cdk9 and CTIP2 : comparison of the expression of dominant negative (dn; kinase dead) Cdk9 with CTIP2 over-expression and knockdown in HEK cells. **(D)** As in (C), but for the statistically significantly co-regulated genes. **(E)** Table showing the number of target

genes differentially expressed in each of the comparisons from (C) and (D). **(F)** Scatter plot of logQ values for 112 genes significantly ($p<0.05$) co-regulated by CTIP2 overexpression (black arrow up) and 7SK knockdown (black arrow down). The logQ values of the same set of genes upon CTIP2 knockdown (grey arrow down) and 7SK overexpression (grey arrow up) are illustrated in grey. **(G)** Same as in (F), but for 105 genes anti-regulated in these conditions.

Figure 5 *Model for CTIP2-mediated repression of P-TEFb*

CTIP2 interacts with HEXIM1 and the 7SKsnRNA to repress P-TEFb kinase activity and P-TEFb sensitive genes.

Figure 6 *CTIP2 regulates P-TEFb-sensitive genes in heart of hypertrophic cardiomyopathic mouse*

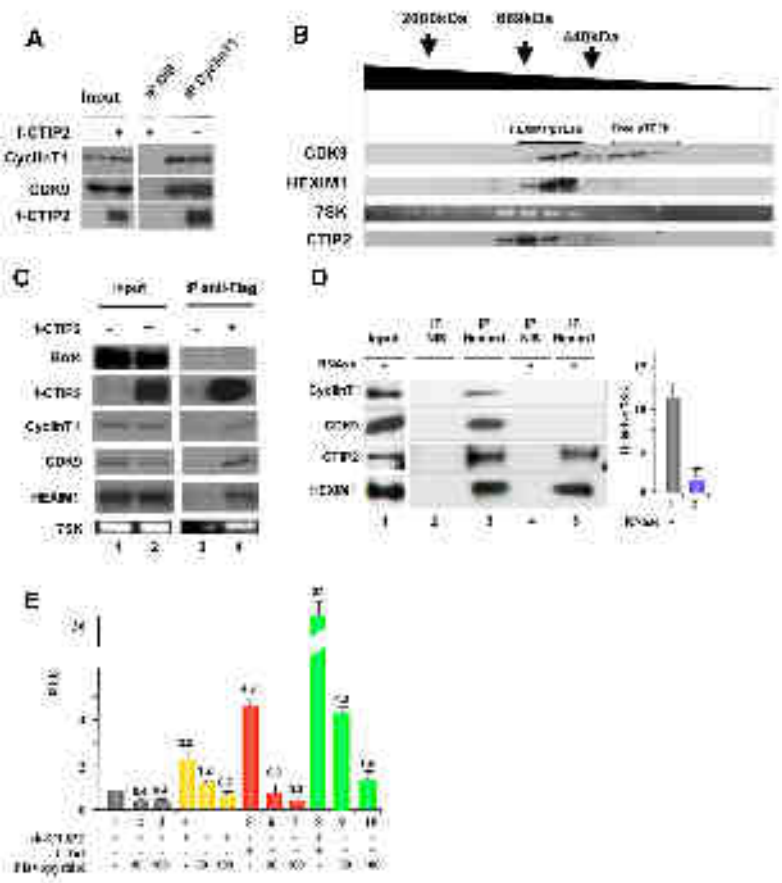

Figure 1

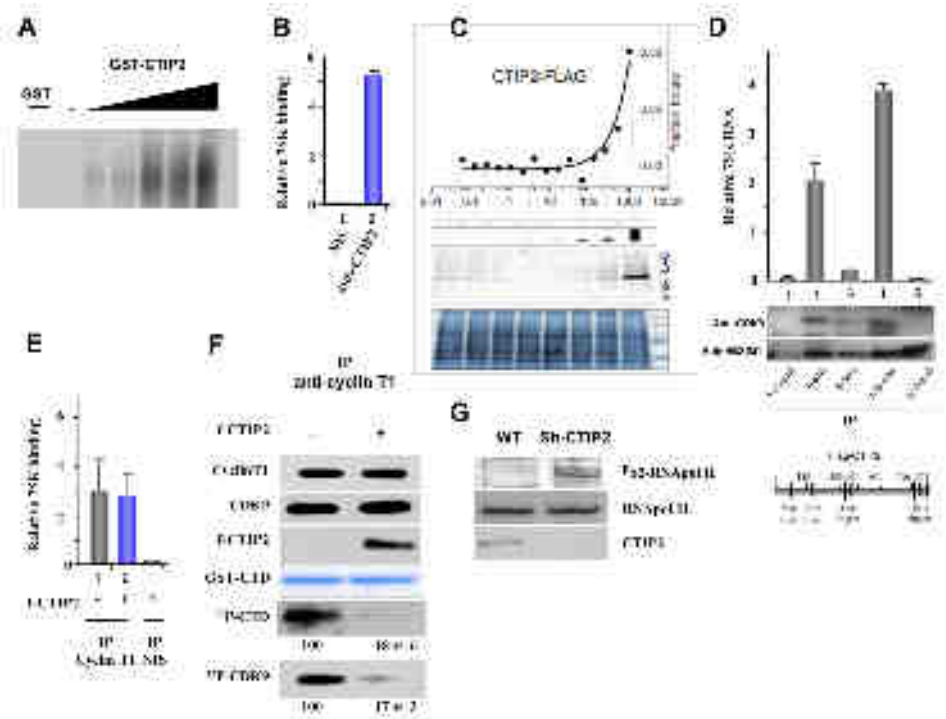

Figure 2

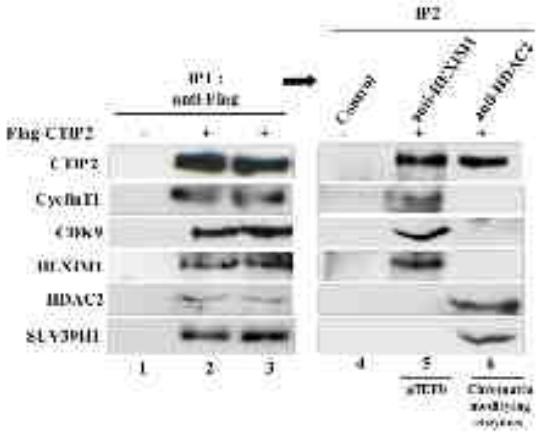
IP2
IP1 :
anti-Flag
Flag CTIP2
CTIP2
CycT1
CDK9
HEXIM1
HDAC2
SUV39H1
Control
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Figure 3

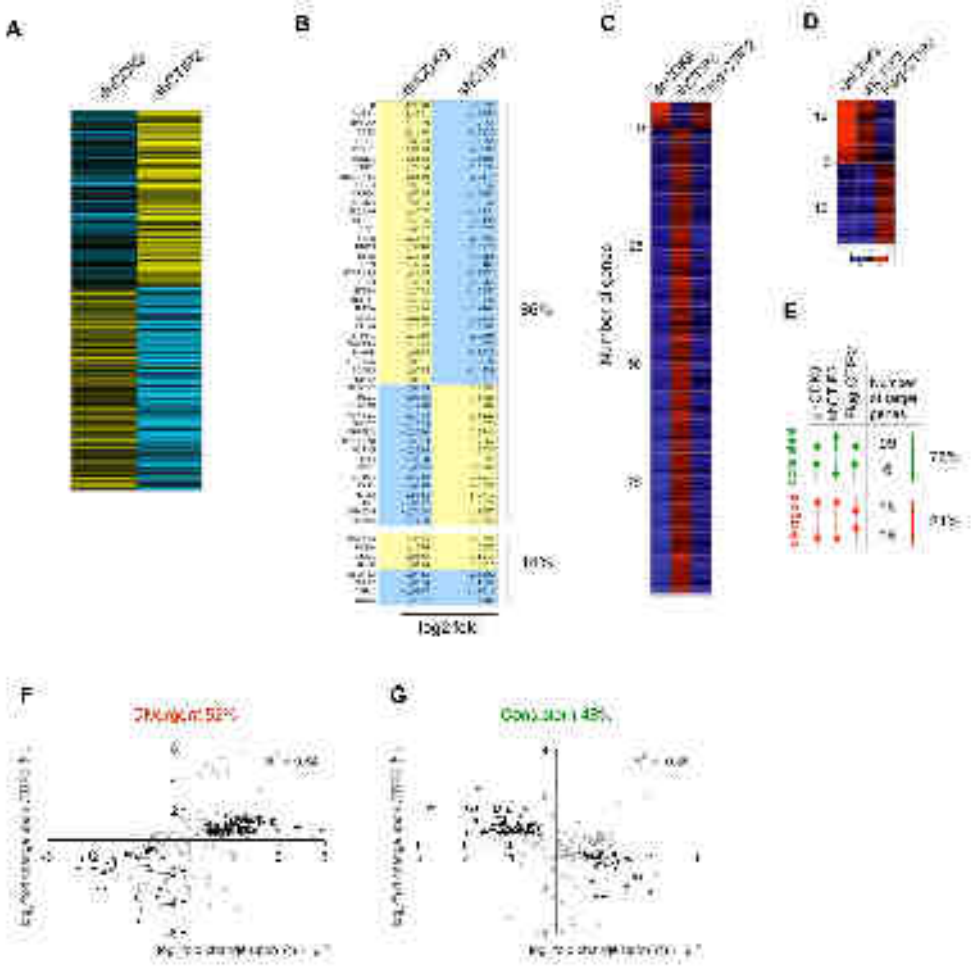


Figure 4

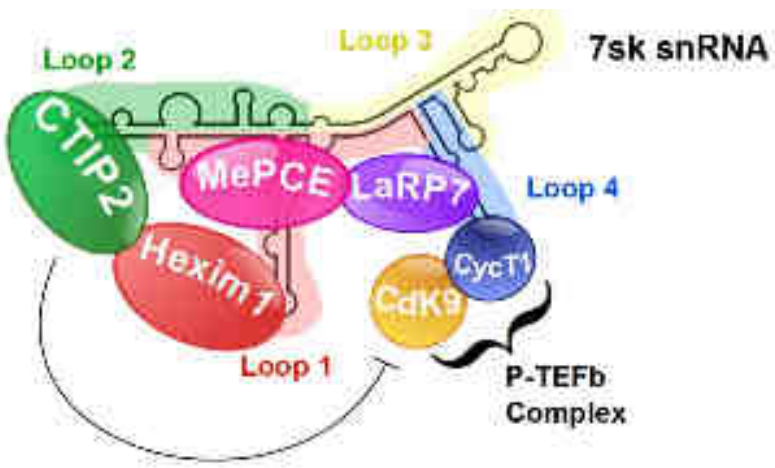


Figure 5

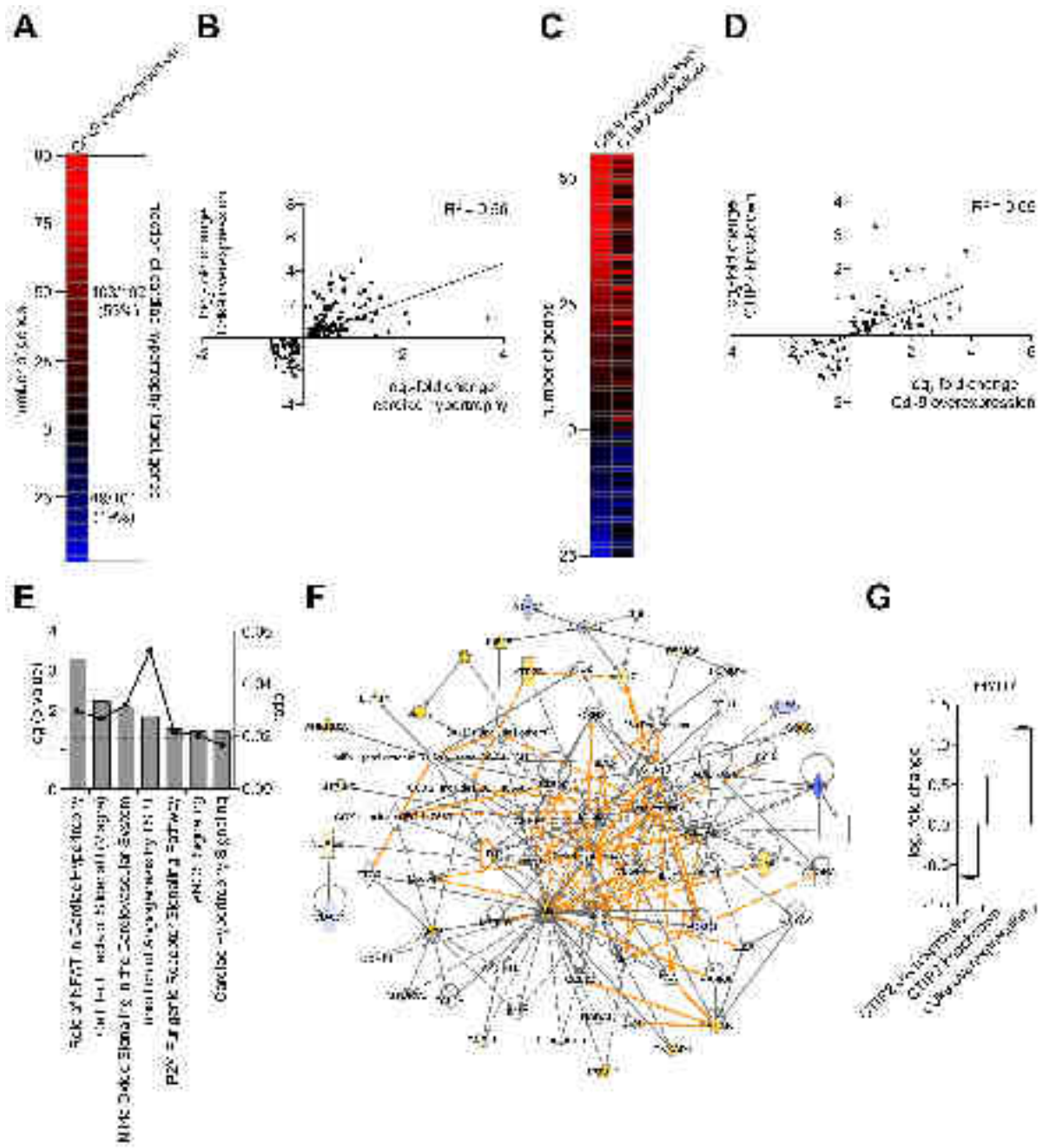


Figure 6

SUPPORTING ON LINE MATERIAL FOR

The cellular cofactor CTIP2 is a negative P-TEFb regulator

Thomas Cherrier[13], et al

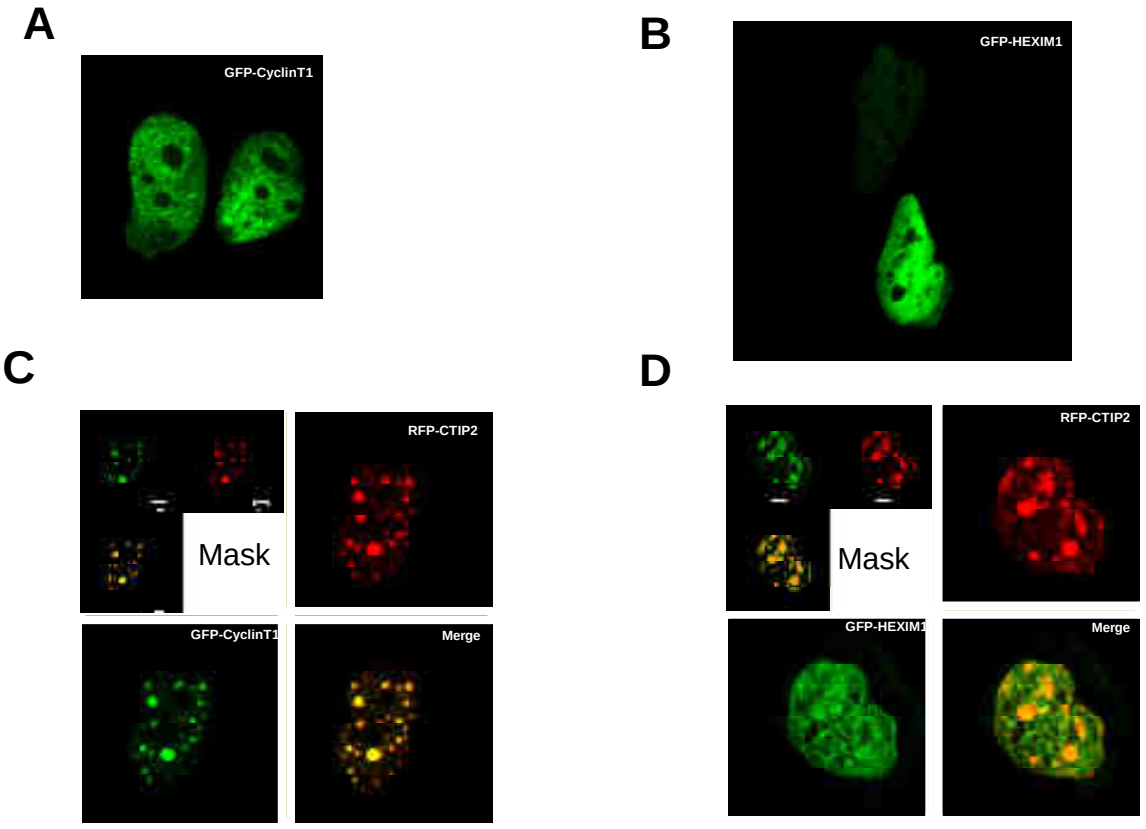
Files included :

Figures. S1 to S4

Table S1

Supplemental figure1, related to figure 1

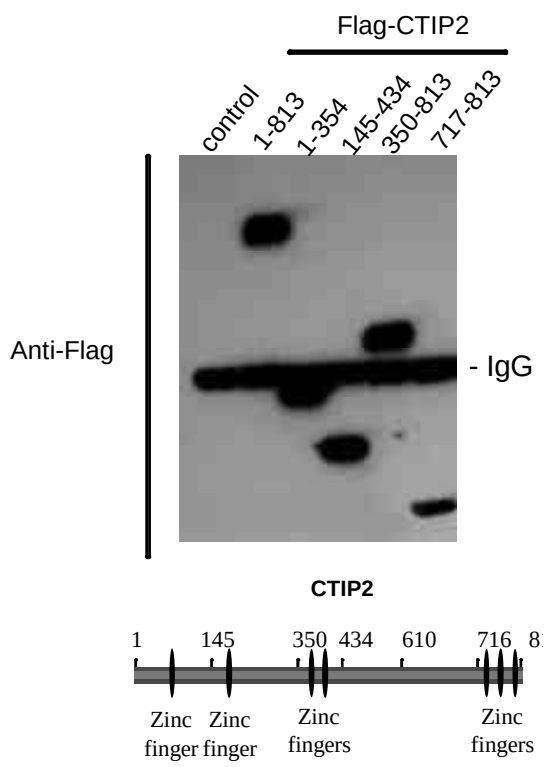
CTIP2, CyclinT1 and HEXIM1 colocalize in nuclei.



Cells were transfected with GFP-CyclinT1 (A) and GFP-Hexim1(B) alone or in the presence of RFP-CTIP2 (C and D). Subnuclear locations were observed by confocal microscopy using Zeiss laser scanning microscope (Zeiss, Jena, Germany; model 510 inverted) equipped with a Planapo oil (63x) immersion lens (numerical aperture = 1.4).

Supplemental figure 2, related to figure 2D

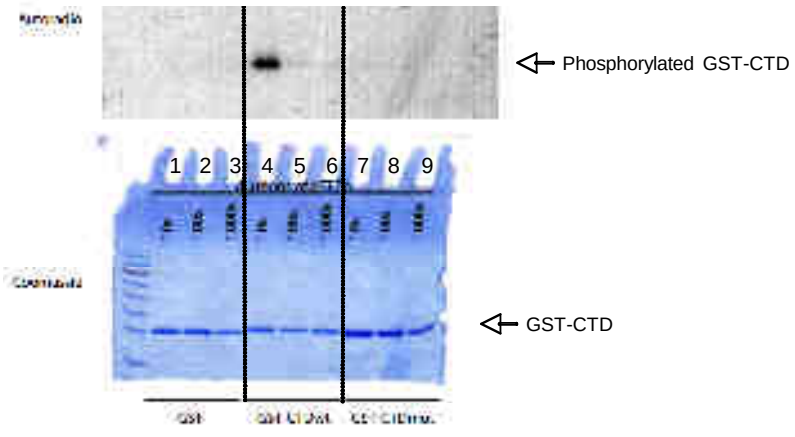
Immunoprecipitations of truncated CTIP2 constructs



Flag- immunoprecipitated CTIP2 fragments used in figure 2C were visualized by Western blot analysis with anti-Flag antibodies.

Supplemental figure 3, related to figure 2F

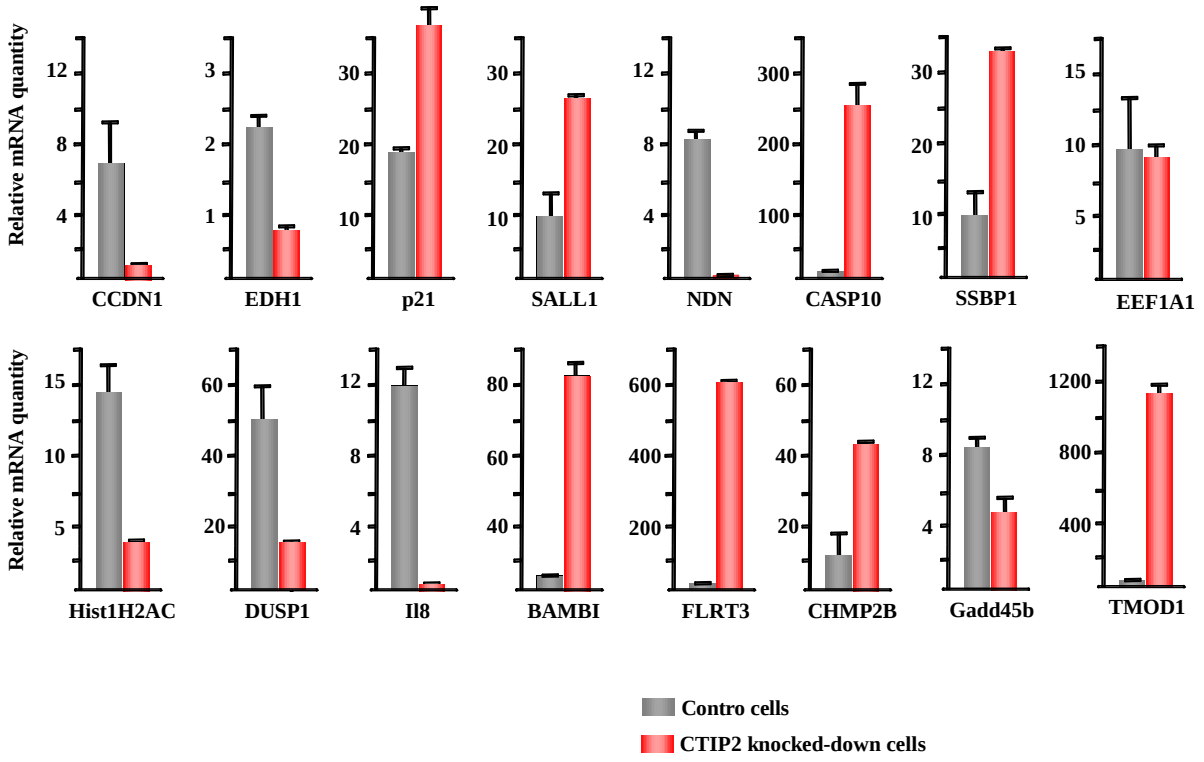
Specific phosphorylations of the CTD Serin 2 and Serin 5 by purified P-TEFb complexes



GST-, GST-CTD and S2/S5 mutated GST-CTD were subjected to phosphorylations assays with co-purified CDK9/CyclinT1 (Milipore ref 14-685).
(stock diluted 10X = 250ng , 100x = 25ng and 1000X = 2.5ng of purified proteins per assay)
GST proteins were separated by SDS-PAGE and phosphorylations were revealed by autoradiography.

Supplemental figure 4, related to figure 4 A,B

Quantifications of CTIP2 sensitive genes in control and CTIP2-knocked down cells.



Total RNA were extracted from control and CTIP2-knocked down cells to be subjected to RT-Q-PCR quantifications of the indicated mRNA. Results were presented relative to the house keeping EEF1A1 gene

Supplemental Table 1, related to figure 4A,B

Exel document available

Publication 4

Human-Phosphate-Binding-Protein inhibits HIV-1 gene transcription and replication.

Cherrier T, Elias M, Jeudy A, Gotthard G, Le Douce V, Hallay H, Masson P, Janossy A, Candolfi E, Rohr O, Chabrière E, Schwartz C.
Virology Journal 2011

La HPBP (Human Phosphate Binding Protein) est une lipoprotéine découverte fortuitement et capable de se lier au phosphate avec une haute affinité. HPBP appartient à la famille des protéines DING, protéines impliquées dans divers processus biologiques, comme par exemple la régulation du cycle cellulaire. Nous rapportons ici que HPBP est capabable de réprimer la trancription et la réplication virale dans une lignée de lymphocytes T, les lymphocytes primaire du sang périphérique et dans les macrophages primaires. Nous montrons également que HPBP est efficace aussi bien sur des souches naïves que des souches résistante à l'AZT. Dans nos résultats, HPBP se pose comme une nouvelle et puissante molecule anti-VIH inhibant la transcription virale, étape du cycle virale actuellement non ciblé lors des multi-thérapies. Ainsi, HPBP ouvre la voie à de nouvelles stratégies dans le traitement de l'infection au VIH.

Ma participation à ce travail a été modeste.

Cherrier et al. Virology Journal 2011, 8:352
http://www.virologyj.com/content/8/1/352

VIROLOGY JOURNAL

SHORT REPORT Open Access

Human-Phosphate-Binding-Protein inhibits HIV-1 gene transcription and replication

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Abstract

The Human Phosphate-Binding protein (HPBP) is a serendipitously discovered lipoprotein that binds phosphate with high affinity. HPBP belongs to the DING protein family, involved in various biological processes like cell cycle regulation. We report that HPBP inhibits HIV-1 gene transcription and replication in T cell line, primary peripherical blood lymphocytes and primary macrophages. We show that HPBP is efficient in naive and HIV-1 AZT-resistant strains. Our results revealed HPBP as a new and potent anti HIV molecule that inhibits transcription of the virus, which has not yet been targeted by HAART and therefore opens new strategies in the treatment of HIV infection.

Keywords: HIV-1, HPBP, transcription, HAART

Introduction

Human immunodeficiency 1 (HIV-1), identified in 1983 [1], remains a global health threat responsible for a world-wide pandemic. The introduction of the highly active antiretroviral therapy (HAART) in 1996 exhibited the potential of curing acquired immune deficiency syndrome (AIDS). Even though an effective AIDS vaccine is still lacking, HAART has greatly extended survival [2]. AIDS pandemic has stabilized on a global scale in 2008 with an estimated 33 million people infected worldwide (data from UN, 2008).

However, several problems have been encountered since the introduction of HAART, and improvements in the design of drugs for HIV-1 are needed. A drawback of HAART is that the treatment is very expensive with limitation of its use to western countries. HAART has also several serious side effects leading to treatment interruption. Another major concern is related to the emergence of multidrug resistant viruses which has been reported in patients receiving HAART [3-5]. Therefore, new antiviral drugs are needed with activities against both wild type and mutant viruses. Two major cellular targets for HIV-1 are currently known which have critical role in HIV pathogenesis, i.e. CD4+ T lymphocytes and monocytes/macrophages including microglial cells, which are the central nervous system resident macrophages [6-8]. However, several drugs being active in CD4+ T lymphocytes are ineffective in chronically infected macrophages (i.e. several reverse transcriptase inhibitors) [9], and protease inhibitors have significantly lower activities in macrophages compared to lymphocytes [10]. Finally, many observations strongly suggest that even long term suppression of HIV-1 replication by HAART cannot totally eliminate HIV-1. The virus persists in cellular reservoirs because of viral latency, cryptic ongoing replication or poor drug penetration [11-13]. Moreover, these cellular reservoirs are often found in tissue sanctuary sites where penetration of drugs is restricted, like in the brain [14-16]. All these considerations (existence of several reservoirs, tissue-sanctuary sites and multidrug resistance) urge the search for new and original anti HIV-1 treatment strategies. Currently there are seven classes of antiretroviral (ARV) drugs available in the treatment of HIV-1-infected patients: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry/fusion inhibitors (EIs), co-receptor inhibitors (CRIs) and integrase inhibitors (INIs) [17]. The therapy of HIV-1-infected patients is based on a combination of

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http://www.virologyj.com/content/8/1/352

three or more drugs from two or more classes [18]. There have been attempts without success to develop vaccines against HIV- 1 and this field of research needs new directions [19-21]. Improvement of HAART is therefore crucial.

We believe that new drugs should target other steps of the HIV-1 cycle such as transcription since there is no drug currently available targeting this step. An increasing number of studies suggest that inhibitors of cellular LTR-binding factors, such as NF-KB and Sp1 repress LTR-driven transcription [19,21-24]. Recently, it has been shown that proteins of the DING family are good candidates to repress HIV-1 gene transcription [25,26].

More than 40 DING proteins have now been purified, mostly from eukaryotes [[27] and personal communication] and most of them are associated with biological processes and some diseases [28]. The ubiquitous presence in eukaryotes of proteins structurally and functionally related to bacterial virulence factors is intriguing, as is the absence of eukaryotic genes encoding DING proteins in databases. However, theoretical arguments together with experimental evidences supported an eukaryotic origin for DING proteins [29,30]. A member of the DING family proteins, HPBP, was serendipitously discovered in human plasma while performing structural studies on another target, the HDL-associated human paraoxonase hPON1 [31-33]. The structure topology is similar to the one described for soluble phosphate carriers of the ABC transporter family [32-36] that makes HPBP the first potential phosphate transporter identified in human plasma. Moreover, the association with hPON1 has been hypothesized to be involved in inflammation and atherosclerosis processes [37]. Later, the *ab initio* sequencing of HPBP by tandem use of mass spectrometry and X-ray crystallography confirmed that its gene was missing from the sequenced human genome [38]. Immunohistochemistry studies performed in mouse tissues demonstrate that DING proteins are present in most of tissues, spanning from neurons to muscle cells and their cellular localization is largely variable, being exclusively nuclear in neurons, or nuclear and cytoplasmic in muscle cells [30]. Altogether, these localizations are consistent with the biological function that was associated to these proteins, especially the regulation/alteration of cell cycle.

To test whether HPBP is a potential HIV-1 repressor we carried out experiments in a lymphoblastoid cell line (Jurkat) and in primary cells (Peripherial Blood Lineage and macrophage cultures). We report that HPBP represses HIV-1 replication through the inhibition of its gene transcription. Furthermore, HPBP is also active against mutant viruses. Evidence that HPBP can block HIV-1 LTR promoted expression and replication should lead to the design of new drugs which target a not yet targeted step of the virus cycle i.e. transcription.

Materials and methods

Protein purification

HPBP/HPON1-containing fractions were obtained following previously described HPON1 purification protocol [39]. Then HPBP was purified from these fractions according to Renault *et al.* protocol [33]. HPBP/HPON1-containing fraction in 25 mM Tris buffer containing 0.1% Triton X-100, were injected on Bio-Gel HTP hydroxyapatite (BioRad Laboratories, Munich, Germany) equilibrated with 10 mM sodium phosphate pH 7.0. This step was followed by washing with the same buffer and elution by 400 mM sodium phosphate allowed to separate the two proteins. HPBP was not retained on hydroxyapatite equilibrated without $CaCl_2$ and was collected in the filtrate. On the contrary, HPON1 was retained and subsequently eluted by higher phosphate concentrations.

Cell culture

1G5 cells (a Jurkat stable cell line for LTR-luciferase) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and in the presence of penicillin and streptomycin (100 U/ml). Primary Macrophages were cultured and prepared as previously described [40].

Antiretroviral compounds

Stock of AZT (Glaxo Wellcome) was prepared as 0.1 mM solution in dimethylsulfoxide (Pierce) and stored at -70°C. Stock solutions were further diluted in culture medium immediately prior to use.

Luciferase assays

1G5 cells (a Jurkat stable cell line for LTR-luciferase) were transfected (5×10^6-10^7 cells/transfection) using DEAE-dextran transfection method with HIV-1 pNL4.3. Two days later, cells were collected and luciferase activity was determined using the Dual-GloTM Luciferase Assay System (Promega). Values correspond to an average of at least three independent experiments performed in duplicate.

HIV-1 infection and viral replication

1G5 cells (a Jurkat stable cell line for LTR-luciferase) were transfected (5×10^6-10^7 cells/transfection) using DEAE-dextran transfection method with HIV-1 pNL4.3. After 24 h indicated amount of HPBP was added to cell culture medium. HIV-1 replication was monitored as described previously [41].

Purified PBLs were prepared from peripheral blood of healthy donors as described previously [42]. For purified PBL preparation, Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)-isolated PBMCs were incubated for 2 h on 2% gelatincoated plates. Nonadherent cells, 98% that were PBLs, as assessed by CD45/CD14 detection by flow cytometry analysis (Simultest Leucogate, Becton

Dickinson, San Jose, CA, USA), were harvested after Ficoll-Hypaque isolation and adherence. PBLs were cultivated in RPMI with 10% (v/v) FBS supplemented with human recombinant IL-2 (20 IU/ml) following treatment with PHA (5 μg/ml) for 48 h. Cultured in 24-well plates, cells were electroporated (Biorad Gene Pulser X Cell) with the complete HIV-1 infectious molecular clone pNL4.3. For infection experiments, cells were infected (50 ng/million cells) with a wt lymphotropic strain pNL4.3 or an AZT resistant lymphotropic strain (purchased by NIH AIDS research and reference program (lot number 0014 A018-G910-6, post AZT isolates) [43]. HIV-1 replication was monitored as described previously [40].

Macrophages cells were cultured and prepared as previously described [40]. Cultured in 24-well plates, cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) with the complete HIV-1 infectious molecular clone pNL4.3. For infection experiments, cells were infected (50 ng/million cells) with the pseudo typed pNL4.3-VSV 1 virus. Vesicular stomatitis virus G protein (VSV-G) pseudotyped virions were produced by cotransfection of 293T cells with 500 ng of VSV-G expressed with plasmid pHCMVg along with 2 μg of the proviral clone. HIV-1 replication was monitored as described previously [40]. Values correspond to an average of at least three independent experiments carried out in duplicates.

MTT assay

Jurkat cells, as well primary cells, i.e PBL and macrophages, were seeded in 96-well plates and indicated amount of HPBP was added to cell culture medium. The possible cytotoxic effect of the antiretroviral compounds tested was examined using a 3- [4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [44]. Cells were grown at 37°C/5% CO2 for 6 days in the presence of antiretroviral compounds at individual concentrations of 100, 20, 5, or 0 nM, before removal of the supernatant and replacement with 0.25 mg/ml MTT (Sigma) in phenol red-free RPMI-1640 (Life Technologies). After incubation at 37°C/5% CO2 for 1 h, the MTT-containing supernatant was removed and the cells lysed with 5 ml of isopropanol:1M HCl (96:4 v/v). Triplicate 100 l volumes of dye-containing supernatant were transferred to a 96-well ELISA plate (Nunc) and the absorption measured at 570 nm, using background subtraction at 630 nm.

Statistical analysis

Values are the means and SDs of independent experiments. Statistical analysis was performed by Student's *t* test, and differences were considered significant at a value of $p < 0.05$.

Results

1. HPBP represses HIV-1 gene transcription and replication

In order to assess the anti HIV-1 activity of HPBP, we tested HPBP, HPON1 and the complex HPBP/HPON1, for their activities on HIV-1 gene transcription and replication. The complex HPBP-HPON (Figure 1.A and 1.B lane 2) and the purified HPON1 (Figure 1.A and 1.B lane 4) did not have significant impact neither on HIV-1 replication nor on HIV-1 gene transcription. However, purified HPBP strongly repressed HIV-1 replication and transcription (respectively 60 and 70% as shown in Figure 1.A and 1.B lane 3). AZT treatment (10 μM), used as a control, was efficient to repress HIV-1 replication but not HIV-1 gene transcription (Figure 1.A and 1.B lane 5). Heat-inactivated HPBP, used in another control experiment, had no effect on HIV-1 replication (data not shown).

2. Dose response and cytotoxicity assay for HPBP

Figure 2 shows the dose response effect of HPBP on the Jurkat cells with an IC50 (50% inhibitory concentration) equal to 5 nM. To measure the cytotoxic effect of HPBP on these cells we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity assay [44]. Results, shown in Figure 2 (green line), allowed us to calculate CC50 (50% toxicity concentration) to be equal to 526 nM. We next performed dose response experiments and MTT cytotoxicity assays in primary cells. In Peripherical Blood Cells (PBL), the IC50 is estimated to 5 nM and the CC50 is estimated to 200 nM. Comparable results were obtained in primary macrophages with an IC50 of 5 nM and a CC50 of 140 nM (see table 1).

3. HPBP is efficient against drug-resistant strain of HIV-1

To assess the anti HIV-1 activity of HPBP against mutant viruses, we performed a dose response effect of HPBP on PBL infected with an HIV-1 AZT-resistant strain (lot number 0014 A018-G910-6, post AZT isolates) [43]. As shown in Figure 3, HPBP is active against the mutant strain with an IC50 (5 nM) to the same extent as observed for the wild type strain.

Discussion

HPBP is a member of the DING protein family identified in eukaryotes for their implication in diverse biological processes [28,37]. Here, we show that the human phosphate binding protein has a potent anti HIV-1 activity. Previous observations suggested that $p27^{51}$, another member of the DING protein family isolated from the plant *Hypericum perforatum*, represses HIV-1 replication and transcription [25,45,46]. However, it is noteworthy to precise that this inhibitor effect is dose dependent. Indeed it was shown by the same group that

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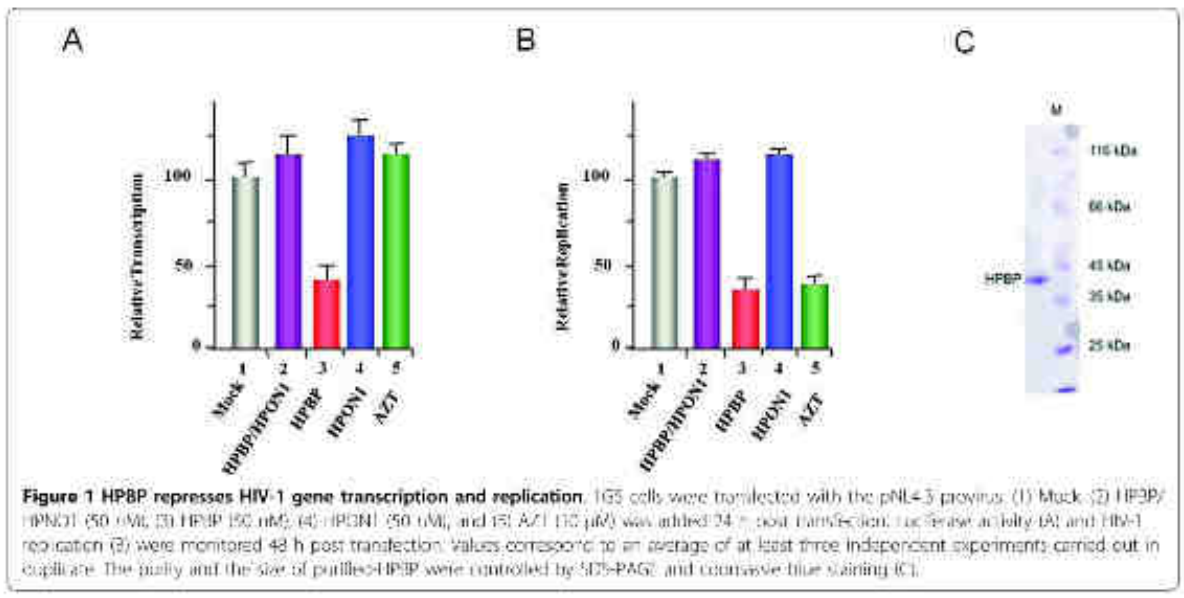

Figure 1 HPBP represses HIV-1 gene transcription and replication. IG5 cells were transfected with the pNL4-3 provirus (1) Mock, (2) HPBP/HPDN1 (50 nM), (3) HPBP (50 nM), (4) HPDN1 (50 nM), and (5) AZT (10 μM) was added 24 h post transfection. Luciferase activity (A) and HIV-1 replication (B) were monitored 48 h post transfection. Values correspond to an average of at least three independent experiments carried out in duplicate. The purity and the size of purified-HPBP were controlled by SDS-PAGE and coomassie blue staining (C).

p27[SI] has a dual role on MCP1(monocyte chemoattractant protein 1) gene transcription being an activator at low concentration and an inhibitor at high concentration [47]. This lead us to hypothesize that HPBP might also have antiviral activities. Since CD4+ T lymphocytes and cells from monocyte-macrophage lineages are major targets for HIV-1, we assessed the *in vitro* antiviral activity of HPBP in lymphoblastoid cell lines (Jurkat), primary monocyte/macrophage cells and peripheral blood lymphocytes presenting laboratory and clinical isolates of HIV-1. The inhibitory effect of HPBP on HIV-1 replication is very strong, the IC50 value being in the range of 5 nM to 10 nM, and also compared to other canonical drugs currently used in HAART (15 nM to 6.7 μM for AZT and 40 nM to 8.5 μM for tenofivir) [48]. At this concentration HPBP is also a potent anti HIV-1 drug in PBL and in primary macrophages, which is not true for several other anti HIV-1 drugs. For example, 3 RT inhibitors, i.e. Lamivudine, entricitabine and AZT have different IC50 values when assessed for their antiviral activity in PBL and macrophages [49]. Furthermore, the CC50 values for HPBP were in the range of 140 nM to 200 nM and the selectivity index CC50/IC50 (ratio between the toxic dose and the inhibitory dose) of HPBP was in the range of 28 to 40. This high ratio indicates that the therapeutical index should therefore be high enough for use in *in vivo studies*.

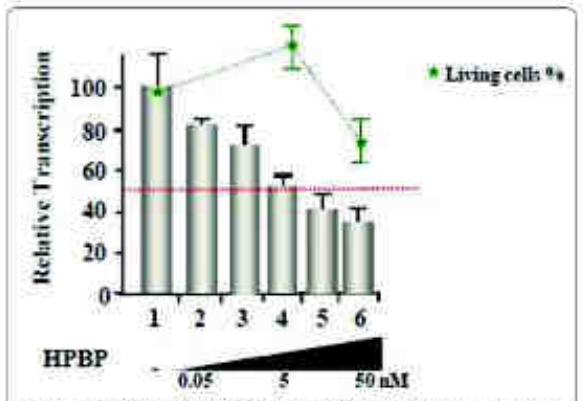

Figure 2 Dose response and cytotoxicity test. IG5 cells were infected with NL4-3 and treated with increasing amount of HPBP. 24 h post transfection, luciferase activity was assessed 48 h post transfection. MTT test were performed in the same conditions than the dose response experiment. HIV-1 inhibition (grey columns) and percentage of living cell (green line) are shown relative to the mock treated conditions as 100%. Values correspond to an average of at least three independent experiments performed in duplicate.

HPBP also emerged as a promising candidate for drug development as it targets HIV-1 transcription, a phase of the HIV-1 cycle not yet targeted by other drugs. In productive cells, the transcription of the provirus DNA is regulated by the interplay of a combination of viral and cellular transcription factors [50-53]. Darbinian and coll. have identified the protein P27[SI], which belongs to the DING protein family and inhibits the activity of the

Table 1 CC50 and IC50 values in peripheral blood lymphocytes and in primary macrophages

	Peripheral Blood Lymphocytes	Primary Macrophages
CC50	200 nM	140 nM
IC50	5 nM	5 nM

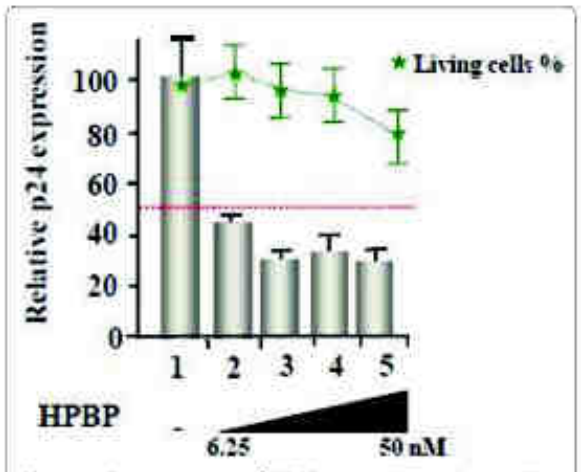

Figure 3 Dose response of HPBP on an AZT-resistant HIV-1 strain. PBL were infected with AZT resistant strain of HIV-1. Different concentrations of HPBP were added 24 h post infection and the HIV-1 replication was monitored 3 days post infection by quantification of the viral p24 protein. HIV-1 inhibition (grey columns) and percentage of living cell (green line) are shown relative to the mock treated conditions as 100%. The 100% level of replication corresponds to an average 1.1 ng of p24 protein/ml. Values correspond to an average of at least three independent experiments performed in duplicate.

HIV-1 promoter by interfering with NF-IL6, RNA pol II and Tat [45,46]. Targeting cellular factors, i.e. NT-IL6 has the advantage that resistance to these new drugs should evolve with a lower probability. More importantly, interfering with Tat will ensure a strong and selective repression of HIV-1 replication.

Since its introduction in 1996, antiretroviral therapy has changed the clinical course of HIV and AIDS. However drug resistance has occurred with all of the antiretroviral agents. It is now a major public health concern and it is crucial to design new antiretroviral drugs [54,55]. These new drugs should inhibit HIV replication by targeting new steps within the viral life cycle. Of great interest HPBP, which targets transcription, is as effective against drug resistant HIV strains as to wild type strains, highlighting the potential therapeutic advantage of HPBP. The molecular mechanism of action is unknown but currently under investigation. In the future, pharmacophores ("part of a molecule that is necessary to ensure the optimal interactions with a specific biological target and to trigger (or block) its biological response") can be inferred from the characterization of these biochemical studies.

In conclusion, this work indicates that HPBP has a potent anti HIV activity through the inhibition of transcription a not yet targeted phase of the virus cycle. However additional experiments regarding the HPBP impact on HIV replication and gene transcription have to be performed on other viral strains including several other mutant strains (NRTIs, NNRTIs and protease inhibitors). We believe that this protein or its derivatives are potentially interesting molecules and deserve further studies. As suggested for X-DING-CD4 [26], this work could also uncover a new function for proteins belonging to the DING protein family, that is a role in the innate response to infection including HIV-1. New investigations will be needed in order to precise the importance of the DING proteins. It has been previously shown that HPBP is tightly associated with HPON1 [56]. The search of a correlation between the HPBP abundance, its biologic availability, the HPBP/HPON ratio and the non progression in the disease AIDS in the "elite non progressors cohort" will be of great interest. Finally DING proteins may constitute a marker for AIDS progression since it has been shown that both HPON activity and its concentration have been altered in the presence of HIV-1 [57,58].

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Authors' contributions

TC, VE, Alicia J carried out dose response and cytotoxicity assays in lymphocytes and macrophages. Andrea T participated in dose response and cytotoxicity assays in macrophages. VLD and HH carried experiments with bisphosphorylated HPBP. CS carried out experiments in Jurkat cell line. SG and PW purified HPBP. Simarro C and OH participated in the design of the study. TC, CR and CS conceived of the study, participated in its design and coordination and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Publication 5

Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage.

Le Douce V, Herbein G, Rohr O, Schwartz C.
Retrovirology 2010

L'introduction des thérapies anti-rétrovirales hautement actives (HAART) a grandement amélioré la survie des patients. Cependant, ces traitements sont incapable de soigner définitivement les patients et ont permis de révéler la présence de réservoirs de virus quiescent, comme celles de la lignée monocyte-macrophage. La purge, ou tout au moins une réduction importante de ces réservoirs viraux durable est une condition *sine qua none* pour esperer atteindre l'éradication totale du virus. Cette revue se concentre sur les mécanismes moléculaires responsables de la persistance dans les cellules de la lignée monocyte-macrophage. La polémique entourant la latence et/ou la replication cryptique chronique sera tout particulièrement évoquée. En marge de cela s'ajoute le fait que les cellules de la lignée monocyte-macrophage infectées par le VIH-1 restent particulièrement resistantes à l'apoptose. L'incidence sur l'éradication virale inhérente à cette caractéristique de ces cellules sera également discutée. Comprendre les mécanismes de persistence du VIH-1 apparaît comme un prérequis à l'élaboration de nouvelles thérapies visant à accomplir l'éradication du virus.

J'ai réalisé l'intégralité des figures présente dans cette review et est activement participer au travail de rédaction

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REVIEW Open Access

Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage

Valentin Le Douce[1], Georges Herbein[3], Olivier Rohr*[1,2] and Christian Schwartz[1,2]

Abstract
The introduction of the highly active antiretroviral therapy (HAART) has greatly improved survival. However, these treatments fail to definitively cure the patients and unveil the presence of quiescent HIV-1 reservoirs like cells from monocyte-macrophage lineage. A purge, or at least a significant reduction of these long lived HIV-1 reservoirs will be needed to raise the hope of the viral eradication. This review focuses on the molecular mechanisms responsible for viral persistence in cells of the monocyte-macrophage lineage. Controversy on latency and/or cryptic chronic replication will be specifically evoked. In addition, since HIV-1 infected monocyte-macrophage cells appear to be more resistant to apoptosis, this obstacle to the viral eradication will be discussed. Understanding the intimate mechanisms of HIV-1 persistence is a prerequisite to devise new and original therapies aiming to achieve viral eradication.

Introduction

Human immunodeficiency 1 (HIV-1), identified in 1983 [1], remains a global health threat responsible for a world-wide pandemic. Several advances have been made in curing acquired immune deficiency syndrome (AIDS) since the introduction of the highly active antiretroviral therapy (HAART) in 1996. AIDS pandemic has stabilized on a global scale in 2008 with an estimated 33 million people infected worldwide (data from UN, 2008). Even if an effective AIDS vaccine is still lacking, the introduction of HAART greatly extended survival. This therapy can reduce plasma virus levels below detection limits (≤ 50 copies/ml). It induces a biphasic decline of HIV-1 RNA with a rapid decline of infected CD4+ T cells (half life 0.5 day) followed by a decline originating from infected tissue macrophages (half life 2 weeks) [2]. However, with very sensitive methods [3,4], a residual viremia is still detected in patients on HAART. Moreover, HIV RNA returns to a measurable plasma level in less than two weeks when HAART is interrupted [5,6]. These observations suggest that even long term suppression of HIV-1 replication by HAART cannot totally eliminate HIV-1, the virus persists in cellular reservoirs because of viral latency, cryptic ongoing replication or poor drug penetration [7-9]. In fact, the persistence of infection is not so surprising since, from an evolutionary point of view, this is the best form of adaptation of viruses to the host environment. There are essentially two theories of persistent infection: latency and ongoing replication. Latency is best described as a lack of proviral gene expression. On the other hand, ongoing replication requires continuous viral expression without cytopathic effects. It is important to distinguish between the two possibilities since they call for very different therapeutic interventions. The theory of ongoing replication suggests that drug resistance to treatment may develop. In this case treatment intensification and the design of new anti HIV-1 molecules are needed in the long term. On the other hand, if viruses are released by burst from stable reservoirs, multi drug resistance does not develop, however HAART alone is ineffective. In this case new strategies are needed to purge the reservoirs, which in combination with HAART should be able to eradicate the virus in infected patients.

Resting memory CD4+ T cells are the major cellular and the best characterized reservoir in the natural host [7,10-13]. The presence of latent proviral HIV-1 DNA in this cell population has been undoubtedly proven [10]. But there are other reservoirs. Genetic studies showed that during rebound viremia (when HAART was interrupted) the virus could be detected from another reservoir than the CD4+ T cells [14-16]. It has been proposed that peripheral blood monocytes, dendritic cells and macrophages in the lymph nodes and haematopoitic stem cells in the bone marrow can be infected latently and

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therefore contribute to the viral persistence [17-22]. It is still debated whether or not viral persistence in these latter reservoirs is due to true latency or to low level ongoing replication [23,24].

In this review, we focus on the molecular mechanisms responsible for viral persistence in cells of the monocyte-macrophage lineage since they are believed to be an important source of HIV-1 [14,19]. Several features make cells from this lineage a potential HIV-1 reservoir. Contrary to CD4+ T cells, HIV-1 infection is generally not lytic for these cells [25,26]. The particles produced in macrophages are budding into intracytoplasmic compartments which may represent favored sites for HIV-1 assembly. [27,28] (see also the accompanying review from Benaroch et al). Mechanisms underlying HIV-1 budding that involved Gag and the ESCRT pathway, were recently reviewed [29]. Cells from monocyte-macrophage are also more resistant to cytopathic effects and they are able to harbor viruses for a longer period. It may arrive that infected tissue macrophages, such as microglial cells in the brain, produce viruses during their total lifespan [30]. Finally, a major obstacle for the eradication of the virus is that HIV-1 makes infected monocyte-macrophage cells more resistant to apoptosis. Understanding the intimate mechanisms underlying HIV-1 persistence in the monocyte-macrophage lineage will be needed to devise new and original therapies to achieve viral eradication.

Evidence for the constitution of an HIV-1 reservoir by cells from the monocyte-macrophage lineage

Cells of myeloid lineage including monocytes, macrophages and dendritic cells (figure 1) play an important role in the initial infection and therefore contribute to its pathogenesis throughout the course of infection. This is mainly because these cells are critical immune cells responsible for a wide range of both innate and adaptative immune functions.

Infected monocytes have been recovered from the blood of HIV-1 infected patients, even from those on HAART and with a viral load below detectable limits [19,31]. Early studies have shown that monocytes harbor latent HIV-1 proviral DNA [32]. Interestingly, a minor monocyte subset, the CD16+ is more permissive to the infection than the more abundant CD14++CD16- monocyte subsets [33]. Although HIV-1 proviral DNA is only in less than 1% of circulating monocytes (between 0.01 to 1%), these cells are important viral reservoirs and are responsible for the dissemination of HIV-1 into sanctuaries such as the brain [19,23,31,34,35]. Infected circulating monocytes are also recruited to the gastrointestinal tract. They later differentiate into macrophages and form the HIV-1 reservoir of the intestine [36,37]. Some authors suggest that these cells are not true latent cells, since monocytes remain in circulation for only up to 3 days and replication-competent viruses may be recovered from the blood of patients. They rather suggest that a recent ongoing infection of these cells or their precursors takes place [38]. In favor of this suggestion is the viral evolution within this compartment [19].

Dendritic cells are also involved in the dissemination of HIV-1 following primary infection [39]. After capturing viruses at the site of infection, mature dendritic cells migrate into lymph nodes where they participate in the transmission of HIV-1 to CD4+ T cells [40]. Mature myeloid dendritic cells located in lymph nodes can sustain a very low level virus replication and therefore have a potential role in HIV-1 latency and/or ongoing replication. The mechanism of this viral persistence is not yet known [41-43].

Macrophages harboring the CD4 receptor and CCR5 coreceptor are now recognized as early cellular targets for HIV-1 [44]. These cells are able to produce and harbor the virus for a longer period. This is partly due to the higher resistance of these cells to cytopathic effects. It is less clear whether macrophages have a role in HIV-1 latency [22,45] or not. In patients on HAART very few lymph node macrophages are infected (about 0,005%). However, the finding of *in vivo* reactivation of these infected macrophages in response to opportunistic infections is in favor of macrophages as HIV-1 reservoirs [46,47]. Finally, resident macrophages of the central nervous system (CNS) deserve attention since they are involved in the pathogenesis of HIV-1-associated dementia [48,49]. Four types of macrophages were described in the CNS, the meningeal macrophages, the macrophages of the choroid-plexus, the perivascular macrophages and the microglial cells [48]. Among these four types, the perivascular macrophages and the microglial cells are the main targets for HIV-1 in the CNS [49]. These cells have a low turnover, 2-3 months for the perivascular macrophages and several years for the microglial cells. These features make these cells potential reservoirs for HIV-1 [30,50].

Haematopoïtic cells (HPC) have also been proposed to serve as a viral reservoir, since a subpopulation of CD34+ HPCs express CD4 and CCR5 and/or CXCR4 and these cells are susceptible to HIV-1 infection [51-54]. Furthermore, HIV-1-infected CD34+ HPCs have been detected in some patients [55,56]. Interestingly, the CD34+ CD4+ HPC subset has an impaired development and growth when HIV-1 is present. This HPC will then generate a sub population of monocytes permissive to HIV-1 infection with a low level of CD14 receptor and an increase of CD16 receptor (CD14+ CD16++). This population of monocyte may differentiate in dendritic cells in tissues such as lymph nodes [57-59]. It is not yet well understood whether the abnormalities leading to the generation of this permissive cell population are due to a direct or an

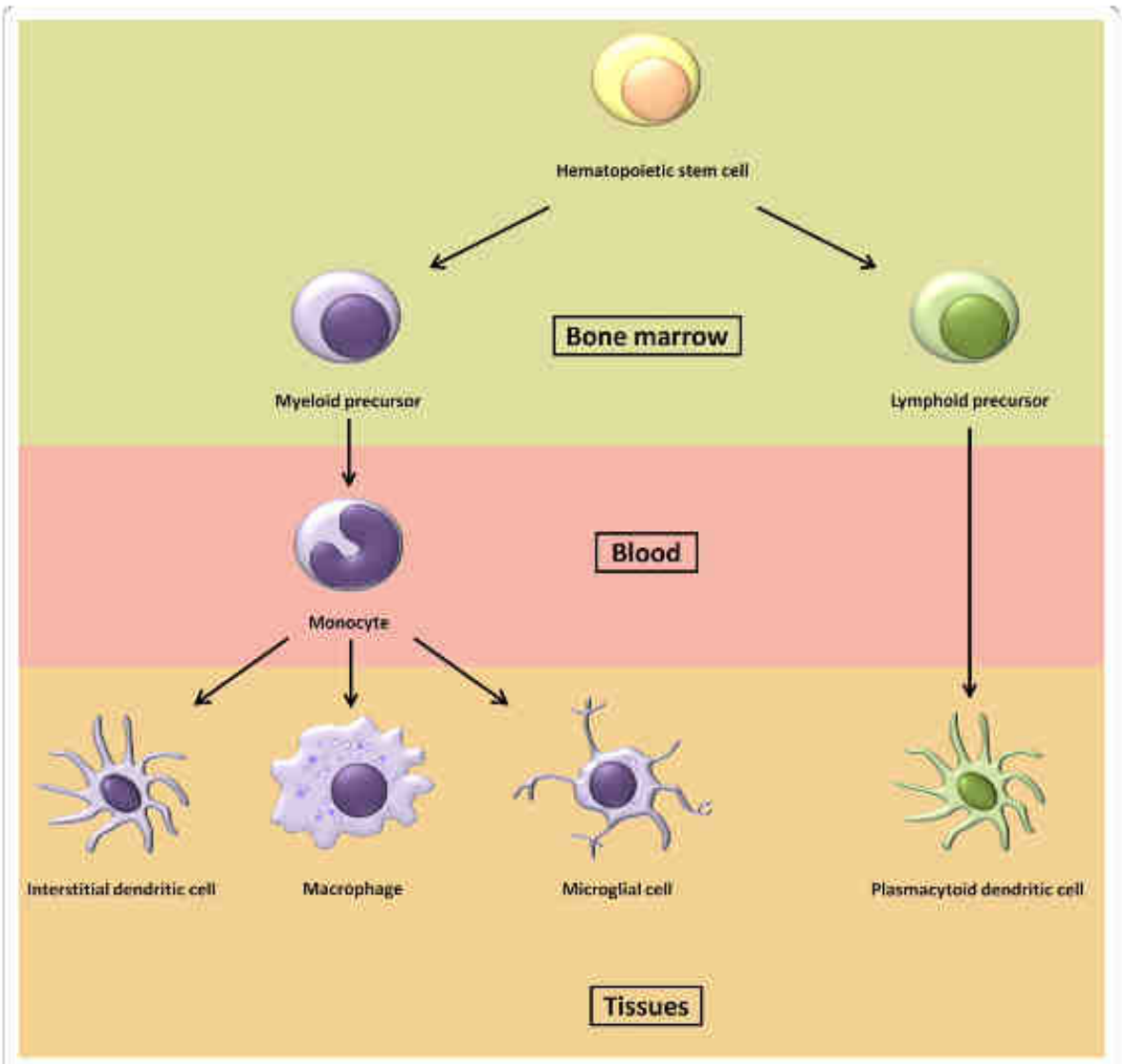

Figure 1 monocyte-macrophage lineage A. cells from the monocyte-macrophage lineage appear to derive from a same progenitor multipotent cell, the hematopoietic stem cell (HSC). The HSC, located in the bone marrow, may differentiate either into a myeloid or a lymphoid precursor, setting up the divergence between the myeloid (blue) and plasmacytoid (green) lineage. The myeloid precursor is then able to migrate into the blood stream and to differentiate into a monocyte. Monocytes migration to specific tissues and their differentiation occur upon a stimulation of a different cytokines, inter-kukins and/or other factors cocktail. Depending to the location, the monocytes become either interstitial dendritic cells, macrophages or microglial cells. Lymphoic precursor runs parallel with the myeloid one, but can directly differentiate into another type of dendritic cell, the plasmacytoid dendritic cell.

indirect interaction with HIV-1. A further investigation is needed, since these HPCs generate an infected cell lineage that may spread HIV-1 to sanctuaries.

Mechanisms of HIV-1 latency in the monocyte-macrophage lineage

Following fusion-mediated entry into the host cell, the virus is uncoated, the virus genome is reverse transcribed and the pre-integration complex enters the nucleus where the proviral DNA is integrated into the host cell genome. In productive cells, the transcription of the provirus DNA is regulated by the interplay of a combination of viral and cellular transcription factors [60-63]. However, cells that lack or have a low level of HIV-1 expression are also present and contribute to viral persistence. It is still controversial whether or not true latency occurs in infected cells

from the monocyte-macrophage lineage. For this reason, but also to avoid confusion, the word latency will be used in the following sections, not *stricto sensu* as previously defined, but in a larger sense which includes true latency and low ongoing replication. Contrary to the CD4+ T cells, in which the mechanisms of the establishment and the maintenance of true latency have been well described [64], our knowledge of the molecular mechanisms underlying latency in the monocyte-macrophage lineage is poor. Like in CD4+ T cells, two types of latency occur in cells from the monocyte-macrophage lineage.

Pre-integration latency

Pre-integration latency is frequently observed in CD4+ T cells. This form of latency has a very limited contribution to viral persistence since the half lives of the cells is very short (1 day). On the contrary, this form of latency in the monocyte-macrophage lineage may contribute to the formation of reservoirs to a larger extent and may participate in viral dissemination. This form of latency is characterized by a poor reverse transcriptase activity and therefore it is unable to synthesize the provirus DNA. Various mechanisms are involved in this form of latency, such as hypermutation of the DNA induced by the restriction factor APOBEC3, a low level of dNTP pool and an impaired nuclear importation of the pre-integration complex associated to a low level of ATP pool [65-68]. Several reports pointed out that macrophages can harbor large quantities of unintegrated viral DNA in a circular form [69,70]. Moreover, these unintegrated DNA remain stable for up to two months in non dividing macrophages [69]. Interestingly, the accessory viral protein Vpr is important for viral replication in the monocyte-macrophage lineage, but not for non dividing CD4+ T-cells [71]. Indeed, deletion of Vpr decreases transcription from unintegrated HIV-1 DNA up to 10 times [72]. A recent report suggests that infected human macrophages can support persistent transcription from this unintegrated DNA [73]. These circular forms of episomal DNA may therefore account for persistence and expression in non dividing cells such as macrophages [74].

Post-integration latency

Post-integration latency occurs once the viral genome has been reverse transcribed and has been stably integrated into the host genome. At that moment, the level of transcription is very low with a no or a low level of virus replication. Mechanisms generating HIV latency in the CD4+ T cells are well described [75,76]. Viral genome integration into repressive heterochromatin may account for the establishment of latency in some cases [77]. Transcriptional interference may be responsible for the establishment of HIV-1 latency [78,79] when viral genome integrates into active euchromatin regions. Several mechanisms acting at a transcriptional and post transcriptional level that maintain the post-integration latency in CD4+ T cells have been described, but it is unknown whether these are also effective in cells of the monocyte-macrophage lineage. However, several mechanisms generating HIV-1 post-integration latency have been described in the monocyte-macrophage, including the lack of, or dysfunctional Tat, the lack of host transcriptional activators, presence of host transcriptional repressors, influence of chromatin environment and host antiviral processes such as the one based on microRNA (miRNA).

Mechanisms involving Tat transactivation

It has been proposed that restriction of the integrated HIV-1 genome transcription is due to the lack of Tat transactivation. The recruitment of the positive transcription elongation factor (pTEFb), which is composed of two proteins, cyclin T1 (CycT1) and cyclin dependant kinase 9 (Cdk9) [80-82] makes this transactivation effective. A lack of transactivation could be due to a low level of Cyclin T1 expression since its expression is limiting for p-TEFb function. Indeed, CycT1 is undetectable in undifferentiated monocytes but activated in monocytes-differentiated macrophages [83]. However, CycT1 is not the only limiting factor involved in the transcriptional inhibition of HIV-1. The phosphorylation status of CDK9 is also important as it increases during the differentiation process of monocytes into macrophages [84].

Mechanisms involving host transcriptional factors

The lack of host transcriptional activators or the presence of host transcriptional repressors may also explain latency in these cells. It has been reported that distal LTR binding sites upstream of the NF-KB binding site are essential for the efficient transcription in monocytes and macrophages. In addition to NF-KB and Sp1 binding, NF-IL6 and/or USF protein binding to the LTR modulatory region are essential for HIV-1 transcription [85-87]. In contrast, in microglial cells the core region and the NF-KB sites are sufficient for transcription [63]. Particularly, Sp1 protein plays an essential role by anchoring directly or indirectly several cellular transcription factors to the promoter, such as NF-IL6, CREB and COUP-TF [88].

The inhibiting form of C/EBPβ/NF-IL6 (LIP), a 16 kDa inhibitory isoform that is structuraly close to C/EBPγ, is expressed in macrophages during differentiation. LIP expression is linked to the suppression of HIV-1 replication [89]. Although C/EBPβ/NF-IL6 acts as an activator of HIV-1 transcription, LIP and/or C/EBPγ act as a dominant-negative inhibitor of NF-IL6 mediated transactivation [88]. Interestingly, this latter mechanism has been proposed to explain the establishment of transcriptional HIV latency in microglial cells of a macaque model, providing the first mechanism of HIV latency in the brain [90]. The TRAF signaling pathway can activate NF-IL6

via the P38-MAPK pathway and is involved in the reactivation of latently infected macrophages [91].

The zinc-finger protein OKT18, which is produced during HIV-1 infection of macrophages, suppresses HIV-1 transcription through the viral LTR [92,93]. This protein exerts its role through the suppression of Tat-mediated HIV-1 LTR activity [94] and through two DNA binding domains which have been recently identified in the LTR: The negative-regulatory element (NRE) and the Ets binding site [93]. It appears that this regulation is cell type specific since it has been reported that OKT18 expression is only detected in brain perivascular macrophages but not in microglial cells [95]. This absence of OKT18 expression in human microglial cells is due to the down regulation of YY1 and upregulation of FoxD3 following HIV-1 infection, which leads to a repression of the OKT18 promoter activity [96]. These results point to zinc-finger proteins as important modulators of HIV-1 transcription and make them attractive for devising new drugs to control AIDS [97,98]

HIV-1 transcription is also modulated by proteins of the Sp1 family which differ in the nature of the Sp protein bound to the LTR and of the cell type. Indeed, Sp1 and Sp3 are both expressed in microglial cells, unlike CD4+ T-cells, which express only Sp1. In microglial cells, although Sp1 acts as an activator of HIV-1 transcription, the Sp3 protein represses the HIV-1 promoter activity [99]. Some factors, like IL-6, or hydroxyurea could synergistically reactivate HIV-1 replication in latently promonocytic cells by increasing the ratio of Sp1/Sp3 [100]. Serpin B2, a serine protease inhibitor induced in activated monocytes and macrophages during inflammation is also able to increase the Sp1/Sp3 ratio by inhibiting Rb-degradation, and thus may reactivate latently infected cells [101].

Importance of the chromatin environment

It is now well established that viral promoter activity depends on the chromatin environment [102]. Nucleosomes are precisely positioned at the HIV-1 promoter [103,104]. Nuc-1, a nucleosome located immediately downstream the transcription initiation site, impedes LTR activity. Epigenetic modifications and disruption of Nuc-1 are a prerequisite to activation of LTR-driven transcription and viral expression [102]. Transcriptional repressors, like Myc bind the HIV-1 promoter and recruit histone deacetylases (HDAC) together with Sp1 and induce thereby proviral latency [105]. Recently it was shown that recruitment of deacetylases and methylases on the LTR was associated with epigenetic modifications (deacetylation of H3K9 followed by H3K9 trimethylation and recruitment of HP1 proteins) in CD4+ T lymphocytes [106]. Some studies suggest that the cellular signaling pathway which involves the receptor tyrosine kinase RON could trigger the establishment and maintenance of HIV-1 latency in monocytic cell lines. A correlation was found between RON expression and inhibition of HIV-1 transcription. Transcription was affected at different levels, i.e. chromatin organization, initiation and elongation [107-109]. The retinoid signaling pathway may also be involved in the inhibition of HIV-1 reactivation. The retinoid pathway inhibits both Nuc-1 remodeling and transcription [110].

The transcription factor COUP-TF interacting protein 2 (CTIP2) has been reported [111] to play an essential role in promoting viral latency in microglial cells. This factor is a recently cloned transcriptional repressor that can associate with members of the COUP-TF family [112]. This factor is expressed in the brain and in the immune system [113]. We have previously shown that CTIP2 inhibits replication in human microglial cells [114,115]. Recently, we have shown that CTIP2 inhibits HIV-1 gene transcription through recruitment of a chromatin-modifying enzyme complex and by establishing a heterochromatic environment at the HIV-1 promoter in microglial cells [111]. Indeed, this work suggests that CTIP2 recruits histone deacetylases HDAC1 and HDAC2 to the viral promoter to promote local deacetylation of the lysine 9 from histone 3 (H3). In addition, CTIP2 has also been shown to associate to the histone methyltransferase SUV39H1, which induces trimethylation of lysine 9 from H3 therefore allowing the recruitment of heterochromatin protein 1 (HP1), heterochromatin formation and HIV-1 silencing (figure 2). Interestingly, by using a microarray analysis with a microglial cell line knocked down for CTIP2, we have shown an up regulation of the cellular cycle independent kinase inhibitor CDKN1A/p21^{waf} (unpublished data). This latter factor has been recently described as a pivotal facilitator of the HIV-1 life cycle in macrophages [116,117]. Indeed, HIV-1 infection activates p21 expression and forces a cell cycle arrest that is highly permissive for viral transcription in macrophages. We have recently reported that CTIP2 is a key transcriptional regulator of p21 gene expression [118]. CTIP2 recruited to the p21 promoter silences p21 gene transcription by inducing epigenetic modifications as described above for the HIV-1 promoter. This effect indirectly favors HIV-1 latency since activation of p21 gene stimulates viral expression in macrophages [117]. Moreover, CTIP2 counteracts HIV-1 Vpr which is required for p21 expression (see the accompanying review from Ayinde et al for more details regarding the role of Vpr in macrophage infection). We have suggested that all these factors contribute together to HIV-1 transcriptional latency in microglial cells [118]. However, p21 may have various effects along the replicative cycle of HIV-1; a very recent report from Bergamaschi et al has described p21 as an inhibitor of the HIV-1 replication [119]. Indeed, they have shown that FcγR activation can interfere with

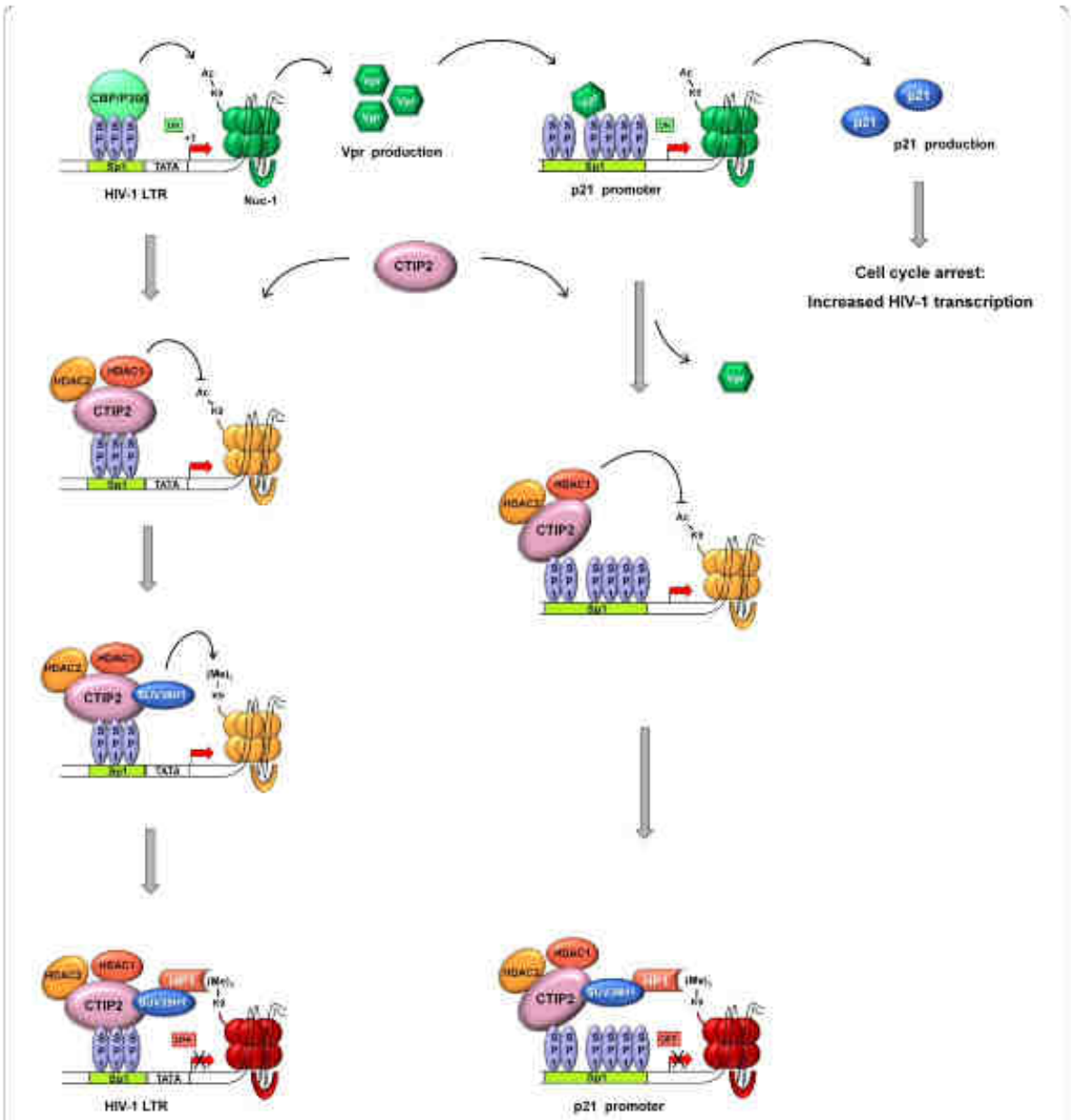

Figure 2 Functions of CTIP2 in the regulation of HIV-1 gene transcription. CTIP2 (COUP-TF interacting protein 2), a transcriptional repressor, has been pointed out as an actor of the latency establishment in the macrophage lineage. First, it has been shown that CTIP2 has a direct impact onto the HIV-1 LTR promoter by recruiting transcriptional activator such P300 (together). CTIP2 interacts with Sp1 which is anchored to the LTR switching nuc-1 from a transcriptionally active to a repressive state. Following its binding to Sp1, CTIP2 recruits sequentially histone deacetylase (HDAC) 1 and 2, which remove acetylation marks from the nuc-1 nucleosome, and then SUV39H1, which add a tri-methylation mark onto the lysine 9 of the histone protein H3. As for SUV39H1, it interacts with HP1, a protein stabilizing nuc-1 in a transcriptionally closed state. Moreover, CTIP2 is also able to indirectly repress HIV-1 gene transcription. Indeed, CTIP2 can counter the action of the viral protein Vpr. One of the roles of Vpr is to induce a cell cycle arrest through activation of the p21 gene. Sp1-mediated recruitement of Vpr to the p21 gene promoter increases the production of the p21 protein, a cell cycle regulator. Consequence of such block in cell cycle is an enhancement of the viral transcription. The binding of CTIP2 to the p21 promoter incites Vpr release, HDACs and SUV39H1 recruitment, HP1 association and p21 gene silencing.

the pre-integration step of the HIV-1 cycle and is associated to the induction of p21 expression. This role of p21 as an inhibitory factor in macrophages has also been reported for other Lentiviruses such as SIVmac and HIV-2 [119]. As discussed in this latter report and in the accompanying review (Bergamaschi and Pancino), p21 might have different effects on HIV-1 infection of macrophages depending on the targeted viral life cycle step and therefore on the time since infection.

An original post transcriptional mechanism involved in latency: the microRNA

The host antiviral processes using microRNA (miRNA) as a defense mechanism are now considered as fundamental for regulation of animal and plant gene expression [120]. Indeed miRNA, 19-25 nucleotide long non-coding RNAs, are involved in various biological processes in eukaryotic cells [121,122]. The miRNAs interact with a complementary sequence in the 3'-UTR of target mRNAs, that leads either to mRNA degradation, or more often to translational inhibition [122]. It has been shown that miRNAs are involved as well in the regulation of virus expression [123]. These processes are manipulated or quenched by HIV-1, and this favors the establishment and maintenance of latency [124]. Recently, it was shown that miRNAs regulate the expression of the histone acetyltransferase Tat cofactor PCAF, and HIV replication [125]. Moreover, an enrichment of miRNAs in clusters has been observed only in resting CD4+ T cells and not in active CD4+ T cells [126], suggesting that these miRNA clusters inhibit HIV replication and therefore contribute to HIV latency in resting primary CD4+ T cells. A similar mechanism, based on cellular miRNA, has also been described in circulating monocytes [127]. In another recent report Sung & Rice have identified a miRNA (miR-198) that is strongly down-regulated when monocytes are induced to differentiate. Moreover, they have shown that this miRNA restricts HIV-1 replication through the repression of CycT 1 expression [128]. This result confirms previous observations that translation of CycT1 mRNA is inhibited in monocytes [129]. Identification of additional miRNAs involved in the repression of host and/or viral factors that could be involved in HIV-1 restriction are needed. Altogether, these data indicate that miRNA are crucial in promoting HIV-1 latency and suggest also that a manipulation of miRNAs could be useful in therapies aiming to purge reservoirs [130].

Influence of the microenvironment in establishing latency

Finally, it has been proposed that the establishment of latently infected macrophages occurs in a suppressive microenvironment made of apoptotic cells [131]. Apoptotic cells induce an inhibition of HIV-1 transcription in the infected macrophages by a signal transduction which involves ELMO. This molecule is indeed involved in the phagocytosis of apoptotic cells [131].

Mechanisms of HIV-mediated apoptosis resistance in the monocyte-macrophage lineage

Another strategy developed by the virus in order to persist in infected cells is to render them resistant to apoptosis.

The NF-kB pathway

Several reports have pointed out that NF-kB activity prevents cells to undergo apoptosis [132,133] The pathway involving NF-kB is activated upon HIV-1 infection in monocyte cells and in primary macrophages [134] (see also the accompanying review from Herbein et al). It has been proposed that TNFα-induced NF-kB activity might be involved in the inhibition of apoptosis and the survival of monocytes and macrophages even if Tumour Necrosis Factor alpha (TNFα) is best known as a pro-inflammatory mediator capable to induce apoptosis. Persistent HIV-1 infection of macrophages results in increased levels of the transcription factor nuclear factor kappa B (NF-κB) in the nucleus secondary to increased IκBα, IκBβ, and IκBε degradation, a mechanism postulated to regulate viral persistence [135,136]. NF-κB is involved in the resistance to TNF-induced apoptosis that might result in a decreased susceptibility to apoptosis of macrophage versus T cells in the context of chronic immune activation like in HIV-1 infection. This indicates clearly that HIV-1 can manipulate the apoptotic machinery to its advantage. Moreover, HIV-1 can induce a dual regulation of the anti-apoptotic protein Bcl-2, resulting in persistent infection of monocytic cells [137]. HIV-1 infection first results in a decrease of Bcl-2 and thioredoxin, permitting an initial boost of replication. Then, as the synthesis at the transcriptional level proceeds, replication is negatively controlled by Bcl-2 to reach a balance characterized by low virus production and higher Bcl-2 and thioredoxin levels resulting in low but sustained viral production compatible with cell survival [137,138]. Recently, the absence of apoptosis in HIV-1-infected primary human macrophages has been reported to correlate with an increase in anti-apoptotic Bcl-2 and Bcl-XL proteins and a decrease of pro-apoptotic Bax and Bad proteins [139].

The role in apoptosis of viral proteins is often dual

The protein Nef is a regulating protein expressed early and abundantly throughout the course of HIV-1 infection. This protein has dual effects depending on the stage of infection. In the early stage, Nef contributes to the constitution of reservoirs with sustained virus production. It mimics the action of TNFα with subsequent activation of NF-kB and MAPK [140,141]. In latter stages, it is involved in the inhibition of apoptosis in infected cells by blocking TNF-mediated apoptosis [142-144]. The Nef protection to the HIV-1-induced apoptosis correlated with the hyper-phosphorylation and consequent inacti-

vation of the pro-apoptotic Bad protein [143]. Finally, Nef is also involved in the blockade of p53-mediated apoptosis [145]. Therefore the Nef anti-apoptotic effect could be a relevant part of the mechanism of the *in vivo* establishment of the HIV-1 macrophage reservoirs. Macrophage express 10-times lower numbers of cell surface CD4 than $CD4^+$ T cells [146], and therefore might be less susceptible to HIV-1 superinfection. Since high levels of cell surface CD4 on HIV-infected cells reportedly induce a dramatic reduction in the infectivity of released virions by sequestering the viral envelope by CD4 [147], low levels of CD4 on the cell surface of macrophage might favour the release of infectious virions from the infected cell, and thereby could optimize transmission of virions to the cells present in the vicinity. Third, the viral life cycle of HIV-1 is 6-times slower in primary macrophage than in primary T cells due to a slower reverse transcription process, suggesting that the rate of virion production might be lower in macrophage than in $CD4^+$ T cells, thereby allowing macrophage to form long-lasting virus reservoirs [148,149].

Tat and gp120 have also dual effects on apoptosis depending of the cell type. In the central nervous system, HIV-1 triggers apoptosis in neurons. This is also seen when neurons are exposed to extracellular Tat or gp120 [150]. On the other hand, microglial cells, the CNS resident macrophage, do not undergo apoptosis upon HIV-1 infection or following exposure to extracellular viral proteins such as Tat or gp120 [151-153]. Tat-mediated resistance to apoptosis in microglial cells is due to the activation of the PI-3-K/AKT cell survival signaling pathway. The protein Tat also decreases the activity of p53. The protein Tat has also been shown to mediate apoptosis resistance by up regulating Bcl-2. This anti-apoptotic factor inhibit TNFα related apoptosis-induced ligand (TRAIL mediated apoptosis) [154]. This combined action of Tat will therefore favor long term cellular survival observed in microglial cells throughout the course of HIV-1 infection [152].

Over-expression of CTIP2, described as an anti-apoptotic factor [155], in microglial cells leads to a repression of p21 expression [118]. This is partly due to the inhibition of the p53 activity on p21 transcription and also to the fact that CTIP2 counteracts Vpr. This latter protein has been shown to trigger apoptotic events in infected lymphocytes and in neurons [156-158]. Taken together, the data suggest that CTIP2 might be involved in the apoptosis resistance of microglial cells, besides its role in the establishment and maintenance of HIV-1 latency. Some investigators have shown that p21 transcription is slightly increased in monocytes recovered from chronically-infected patients and is associated with an anti-apoptosis signature [159]. The apparent discrepancy in the role of p21 in apoptosis in monocytes versus microglial cells needs to be clarified. It might arise from the dependence of the activities of p21 on the cell type, subcellular location, expression level and phosphorylation status. Moreover, p21 expression is regulated by both p53-dependent and p53-independent mechanisms. An increase in p21 expression mediated by Fcγ R activation in macrophages was not due to an induction of p53 since its silencing did not block p21 induction by Immune Complexes [119]. It should be noted that it is not clear whether p21 is an oncogene [160] (which could be involved in the inhibition of apoptosis) or an anti-oncogene [161] (which could be involved in the induction of apoptosis).

The protein gp120 produced by monocyte-macrophages inhibits TRAIL-mediated cell death by inducing the expression of macrophage-stimulating factor (M-CSF). This envelope protein also up-regulates expression of several anti-apoptotic genes such as Bfl-1 and Mcl-1 [162]. A stable signature of anti-apoptosis, comprising 38 genes including p53, MAPK and TNF signaling networks has also been identified from circulating monocytes of HIV-1 infected patients [159]. CCR5 co-receptor bound by HIV-1 can lead to apoptosis resistance in monocyte cultures. A recent report has also stressed the central role of CCR5 during HIV-1 infection [163]. This paper described a case of a HIV-1 positive patient, who received bone marrow transplantation for leukemia. In the follow-up study there was no evidence of the virus in the bloodstream even after 20 months. Myeloablation and T cells ablation were suggested to favor the elimination of the long-lived reservoirs. Indeed, transplantation was done with cells from a homozygous donor for mutation in the HIV-1 co-receptor CCR5. This mutation is well known to be associated with resistance to HIV-1 infection. Therefore development of new molecules to inhibit CCR5 coreceptor function will be a great challenge in the next years. It will be also interesting to investigate whether the interaction between CXCR4 co-receptor and HIV1 could also trigger apoptosis resistance. Apart from the critical role CCR5 plays in maintaining HIV-1 infection, this study also raises the possibility that the main target to cure the patients from AIDS are the peripherical circulating cells including the monocytes (and by the way the infected HPCs). Indeed, whole-body irradiation leading to complete remission of acute myeloid leukemia will mainly target radiosensible cells such as HPCs and peripherical circulating cells. The fact that no virus has been detected at month 20 of follow-up, might suggest that reservoirs in sanctuaries could not sustain viral replication alone. However, the importance of these reservoirs in the physiological context of infected WT-CCR5 patients should not be neglected.

Discovery of a new anti-apoptotic mechanism based on miRNA

Finally, a new mechanism has been proposed that protect cells from apoptosis and therefore extend the lifespan of infected cells. This mechanism is based on the suppression of the cell's RNAi activity by synthesis of a TAR miRNA, a small hairpin RNA. This RNA is capable to sequester the miRNA processing machinery of the cell and therefore impedes the functioning of cellular antiviral miRNAs [164]. Moreover, this TAR miRNA has also been shown to be involved in the down regulation of the expression of several proteins related to apoptosis [165].

Our knowledge of the mechanisms involved in apoptosis resistance is far from complete. The diversity of strategies used by HIV-1 to manipulate the apoptotic pathway emphasizes the capacity this virus possesses to survive in its host. We note that mechanisms involved in apoptosis resistance, at least the mechanisms involving the TNF-signaling pathway, are also involved in virus production. It seems that the nature of this reservoir is rather different from the latent reservoir. The therapeutical implications are therefore important since stimuli such as phorbol esters will not be suitable to purge the reservoir. Indeed, this treatment will reactivate the expression of HIV-1 in latent reservoir but may increase the resistance to apoptosis in viral reservoir that exhibit a sustained production of virions. The survival of viral reservoirs is of great importance since it is also an obstacle to HIV-1 eradication. The mechanisms underlying this apoptosis resistance are essential for devising new and original therapeutic strategies to purge the reservoirs, but are far from being completely known.

Implications for therapy

The introduction of HAART in 1996 has greatly improved survival, but it has been unable to eradicate the virus from latently infected reservoirs. A principle cause may be that besides the best characterized cellular reservoir of memory CD4+ T-cells there are other reservoirs, such as the monocyte-macrophage reservoir. Moreover, these cells are often found in tissue sanctuary sites, like the brain, that are protected from drug penetration [166-168]. Furthermore, several reverse transcriptase inhibitors are ineffective in chronically infected macrophages [34] and protease inhibitors have significantly lower activities in these cells compared to lymphocytes [169]. The emergence of multidrug resistant viruses has been reported in an increasing number of patients receiving HAART [110,170,171]. Finally, the nature of the reservoirs (latent reservoir with no or low virus replication versus productive reservoir which are resistant to apoptosis) has to be taken into account. These considerations (existence of several reservoirs, tissue-sanctuary sites and multidrug resistance) encourage the search for new and original anti HIV-1 treatment strategies. New methods should be developed which target each of these reservoirs. We believe that eradication of the virus could be achieved by specifically purging targeted reservoirs and concomitantly eliminating the virus by a reinforced HAART. Another way to control HIV-1 replication is to re-inforce the latency status by using transcriptional inhibitors.

Use of transcriptional inhibitors to control HIV-1 progression

At present the therapy of HIV-1-infected patients is based on a combination of HIV gp41, reverse transcriptase and protease inhibitors. We believe that new drugs should target other steps of the HIV-1 cycle. For example, they could be directed against proteins involved in the transcription of the inserted virus genome. Tat has a critical role in transcription, and constitutes a major target in therapeutic intervention in the HIV replicative cycle [172-174]. Moreover, drugs could be designed to target cellular cofactors involved in the activation of transcription. This strategy should be able to by-pass drug-resistance which arises with viral proteins. Therefore, ways to synthetize drugs which interfere with HIV-1 replication in monocyte-macrophage should be devised [175]. Several transcriptional inhibitors already characterized such as C-terminally truncated STAT5, Staf 50, Prothymosin α and thioredoxin reductase [176-179] could be used for controlling viral expression in the human macrophages. Inhibition of the NFAT5-LTR interaction by using small interfering RNA is also promising since it suppresses HIV-1 replication in primary macrophages and therefore progression of AIDS [180]. The discovery that only macrophages are able to repress HIV-1 transcription and replication in response to Il10 need further investigation since we could specifically control HIV-1 expression in these cells [129,181]. The demonstration that treatment of HIV-1 infected lymphocytes with the O-GlcNAcylation-enhancing agent glucosamine repressed viral transcription opens the way to metabolic treatment [182]. This treatment might work in the monocyte-macrophage lineage since this chemical compound affects Sp1 and therefore inhibits the activity of the LTR promoter. According to several reports, OKT18, a zinc-finger protein, can reduce HIV-1 replication in human macrophages by the suppression of Tat-induced HIV-1 LTR activity [93,183]. New approaches based on engineered transcription factors are now emerging with zinc finger protein as an attractive candidate for antiretroviral therapy since their binding to HIV-1 LTR in a sequence specific manner is associated with the repression of LTR activity [97,98]. Interestingly, zinc-finger protein can influence the chromatin compaction and nuclear organi-

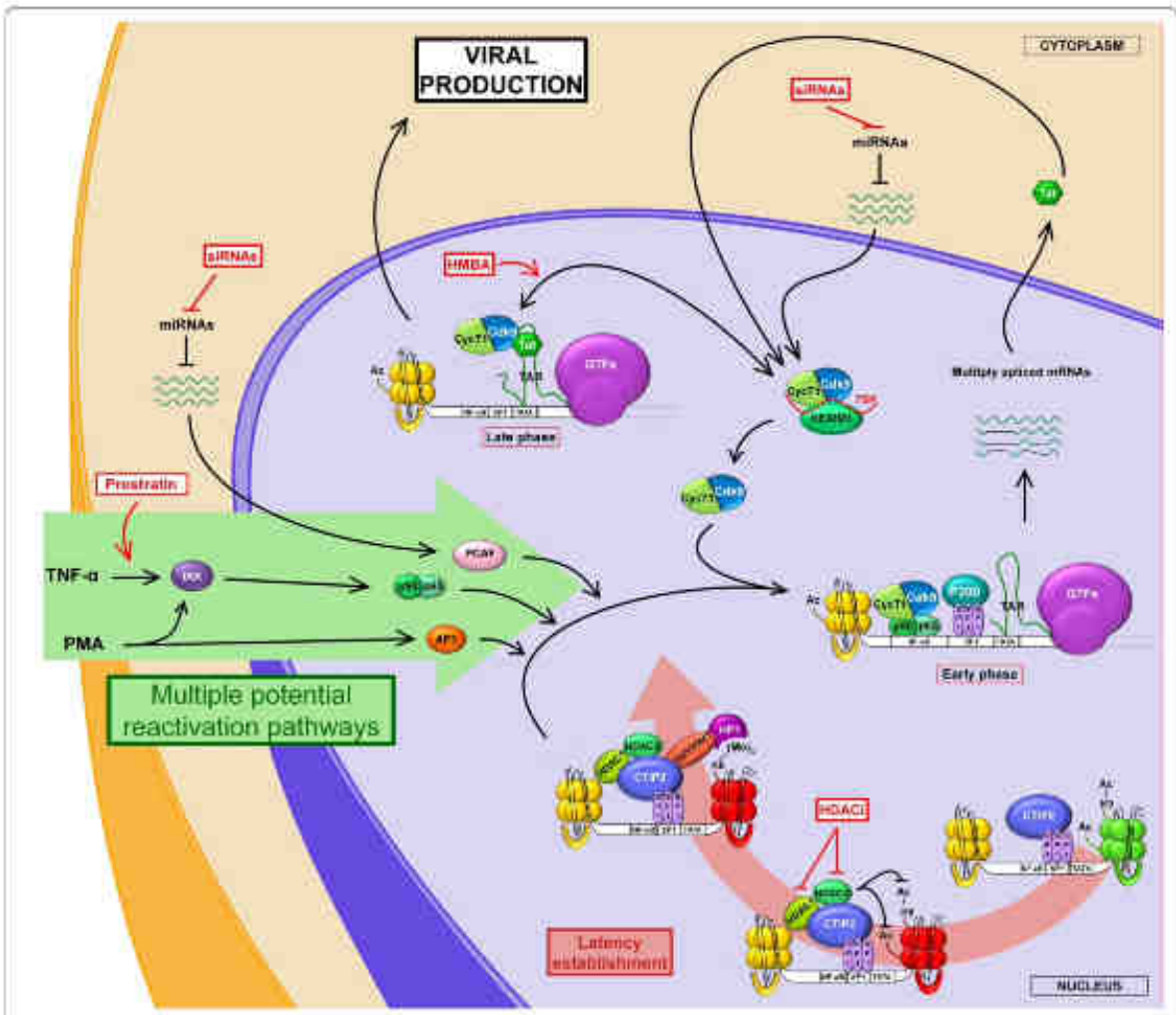

Figure 3 Pharmaceutical approaches of the potential reactivation pathways on latently integrated HIV-1 genome. Multiple ways of reactivation are possible to activate re-initiate the HIV-1 transcription, extern signals, such as TNF-α, can trigger the activation of transcriptional activator, like the heterodimer p50/p65. In the mean time, host protein balance may change leading to higher availability of transcriptional activators. For instance, miRNAs regulates the level of PCAF, a coactivator produced by the host cell (green arrow). Multiple potential reactivation pathways. There are some critical steps in this process that may be targeted to reactivate or hinder the latency establishment. Described, HDAC Inhibitors (HDACI) may prevent the formation of heterochromatin; Prostratin induces the IKK activation, which provokes the activation of transcriptions factors; HMBA increases the pTEFb release from the inactive state; it is possible to reverse the miRNAs negative impact on the mRNAs of transcriptional activators and/or Cyt T1 through specific siRNAs.

zation through regulation of proteins involved in epigenetic regulation [98].

Finally, new drugs must be designed with properties that allow them to penetrate tissue-sanctuary sites such as the brain [166].

Strategies based on virus reactivation from latent reservoirs

Recently, a new and original strategy has been proposed to eradicate the virus from infected patients. The main idea is to facilitate the reactivation of viruses from latent reservoirs, which are then destroyed by HAART (figure 3). Many factors have been involved in reactivation including physiological stimuli, chemical compounds like phorbol esters, histone deacetylase inhibitors, p-TEFb activators, and some activating antibodies (antiCD3). Many eradication protocols passed preclinical studies [2] but to date all failed in clinical trials. Some protocols failed due to the potential toxicity of treatments based on non specific cell activation such as IL2 [184]. The recent discovery that an alternatively spliced form of the cellular transcription factor Ets-1 can activate latent HIV-1 in an

NF-KB independent manner has highlighted the therapeutic potential of cellular factors for the reactivation of latent HIV-1 [185]. Future eradication protocols should use a combination of drugs targeting all the viral silencing mechanisms identified to reactivate HIV-1 from latently-infected cells. A protocol with a similar approach gave promising results. In this study, the association of a HDAC inhibitor or a DNA methylation inhibitor with prostratin (phorbol ester that stimulates protein kinase C activity) proved to have a synergistic effect on the activation of HIV-1 expression [186,187].

Strategies based on increasing apoptosis susceptibility

Increasing the susceptibility of the infected cells to apoptosis is also of great interest. Essentially, the HIV-1 proteins Tat, Nef and the envelope protein gp120 should be targeted, as these proteins have a crucial function in different steps of the virus cycle and also in the acquired resistance to apoptosis. A better comprehension of mechanisms involved in resistance to apoptosis has also allowed to devise drugs against host factors which render cells susceptible to die. For example, molecules which interfere with the chemokine receptor CCR5 are promising since these molecules are both involved in virus entry and in apoptosis resistance. Several CCR5 antagonists are already used in clinical trials [188,189]. A chemotherapeutic drug, Imatibib, restored apoptotic sensitivity of HIV-1 macrophages through the inhibition of the activity of the pro-survival cytokine macrophage colony-stimulating factor (M-CSF) [162]. Finally, several AKT inhibitors including Miltefosine are also promising molecules for targeting long-lived viral reservoirs [190].

A dramatic reduction of monocyte-macrophage reservoirs might be achieved by strategic interventions targeting both the resistance of infected cells to apoptosis and the reactivation of latently-infected cells associated with a reinforced antiretroviral therapy.

Conclusion

Cells from monocyte-macrophage lineage are an ideal reservoir for HIV-1. This is due to several features of these cells including long lifespan, absence of direct cytopathic effect, apoptosis resistance of infected cells, existence of latently infected cells (with low or no virus expression) and their localization in sanctuaries. The purging of this reservoir is therefore crucial since it constitutes one of the major obstacles to HIV-1 eradication from infected patients. A drastic reduction of this reservoir might be achieved by the development of new and original therapies which target specifically all the reservoirs, including the monocyte-macrophages. These treatments might not lead to a complete eradication of HIV-1 but there is hope that a drastic reduction could be achieved with levels of expression comparable to those in elite non progressors patients who remain asymptomatic without an antiretroviral therapy.

Competing interests
The authors declare that they have no competing interests

Authors' contributions
CS conceived and wrote the manuscript. VLD, GI and OR contributed to the writing of the manuscript. VLD created figures 1, 2 and 3 and their legends. All authors have read and approved the final manuscript

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Publication 6

HIV-1 regulation of latency in the monocyte-macrophage lineage and in CD4+ T lymphocytes.

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L'introduciton en 1996 de la HAART a donné l'espoir de l'éradication du VIH-1. Malheureusement, la découverte de réservoirs de virus latent dans les cellules T CD4+ et dans les cellules de la lignée monocyte-macrophage a ternis cet optimisme prématuré. Ces réservoirs viraux durables consitituent un obstacle majeur à l'éradiction du VIH-1. Dans cette revue, nous nous concentrons sur l'établissement et le maintien de la latence dans les deux cibles cellulaires principales du virus : les lymphocytes T CD4+ et les cellules de la lignée monocyte-macrophage. La compréhension des mécanismes d'établissement, maintien et de la réactivation de la latence virale inhérents à ces types cellulaire est cruciale à l'élaboration de thérapies efficaces. Une éradication totale du virus, le saint Graal clinique, ne pourra être atteint qu'avec des interventions stratégiques visant à la fois les cellules infectées de façon productives et latentes. Nous proposons ici que de nouvelles approches combinant différents activateurs de la transcription des provirus pourraient aider à réduire significativement la taille des réservoirs latent de virus chez les patients sous HAART.

J'ai réalisé l'intégralité des figures présente dans cette review et est activement participer au travail de rédaction

JLB

Review

HIV-1 regulation of latency in the monocyte-macrophage lineage and in CD4+ T lymphocytes

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ABSTRACT

The introduction in 1996 of the HAART raised hopes for the eradication of HIV-1. Unfortunately, the discovery of latent HIV-1 reservoirs in CD4+ T cells and in the monocyte-macrophage lineage proved the optimism to be premature. The long-lived HIV-1 reservoirs constitute a major obstacle to the eradication of HIV-1. In this review, we focus on the establishment and maintenance of HIV-1 latency in the two major targets for HIV-1: the CD4+ T cells and the monocyte-macrophage lineage. Understanding the cell-type molecular mechanisms of establishment, maintenance, and reactivation of HIV-1 latency in these reservoirs is crucial for efficient therapeutic intervention. A complete viral eradication, the holy graal for clinicians, might be achieved by strategic interventions targeting latently and productively infected cells. We suggest that new approaches, such as the combination of different kinds of proviral activators, may help to reduce dramatically the size of latent HIV-1 reservoirs in patients on HAART. *J. Leukoc. Biol.* 87: 575–588; 2010.

Introduction

The HIV-1 that causes AIDS, identified in 1983 [1], remains a global health threat with 33 million persons infected worldwide (data from United Nations, 2008). The introduction of HAART in 1996 has improved quality of life greatly and extended survival. This therapy is based on a combination of three or more drugs selected from 22 compounds that belong to three drug classes (inhibitors of RTs, proteases, and gp41) [2–4]. According to clinical trials, HAART can reduce plasma virus levels below detection limits (≤50 copies/ml plasma), raising hopes for the eradication of HIV-1. Initially, HAART induces a biphasic decline of plasma HIV-1 RNAs—a rapid decline of infected cells of the CD4+ T cells (half-life 0.5 day) is followed by a decline in plasma virus RNAs originating from infected tissue macrophages (half-life 2 weeks) [5]. Based on these studies showing biphasic decay in the level of viremia after HAART treatment, some authors predicted that the eradication might be achieved by a 3-year treatment [4]. Unfortunately, HIV-1 RNA returned to a measurable plasma level in less than 2 weeks when HAART was interrupted [6, 7]. Indeed, the long-term suppression of HIV-1 replication has unveiled the presence of latent HIV-1 reservoirs.

The first reservoir found was resting CD4+ T cells [4, 8–11]. It comprises two populations: the naïve and the memory CD4+ T cells. This latent reservoir is established very early during acute infection and therefore, limits the efficiency of HAART, even when introduced at the onset of HIV-1 infection [10, 12, 13]. Viral latency is a feature of many viruses, including other retroviruses as well. It appears that HIV-1 latency occurs at a low frequency (1 to 10^6–10^7 infected cells). As this infection is established early with X4-tropic viruses and as CD4+ T cells are CCR5-negative [11], it is difficult to understand how these cells are infected and why they are latently infected. Some investigators proposed that naïve CD4+ T cells express a low level of CCR5 and therefore, are permissive for R5-tropic viruses [15]. It has been demonstrated that interaction of naïve CD4+ T cells with follicular DCs also renders them permissive to HIV-1 infection [16]. However, their relatively short lifespan is not consistent with the fact that they can function as a long-term reservoir of HIV-1. On the other hand, the presence of latent proviral HIV-1 in the memory cells has

Abbreviations: C/EBP=CREB-binding protein, CDK9=cyclin-dependent kinase 9, CCLP-TF=chicken CNA upstream promoter transcription factor, CITED2=CDK/p300-interacting protein 2, Cyc T1=cyclin T1, DC=dendritic cell, EMC=encephalomyocarditis virus, GTF=general transcription factor, H3K9=histone 3 lysine 9, HAART=highly active antiretroviral therapy, HAT=histone acetylase, HDAC=histone deacetylase, LEDGIN=lexamethyleneblsacetamide-inducible 1, HP1=heterochromatin protein 1, HPC=hematopoietic cell, IκB=IκB kinase, LTR=long terminal repeat, mRNA=micro-RNA, Nuc-1=nucleosome 1, PCAF=p300/CBP-associated factor, pTEFb=positive transcription elongation factor b, RIP=receptor-interacting protein, RNAi=RNA interference, siRNA=small interfering RNA, SIRT=sirtuin 1, Sp1=specificity protein 1, SUV39H1=suppressor of variegation 3-9 homolog 1, TAF=transactivation response, T=transcriptional interference, TRADD=TNF-associated death domain, TRAF2=TNF-associated factor 2, Tm=transitional memory T cell

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been proven [8]. As for naïve cells, these memory cells are CCR5-negative. It is now assumed that HIV-1 latency occurs in activated CD4+ T cells, which are in the process of reverting to the resting state [17]. A recent report from the laboratory of R. P. Sékaly [18] showed the existence of two subpopulations of memory T cells serving as reservoirs for HIV-1. These cells are believed to be a major cellular reservoir for HIV-1 with different mechanisms involved to ensure viral persistence. The first reservoir—central memory T cells—can persist for decades. It is maintened through T cell survival and low-level-driven proliferation. By contrast, the second reservoir—Ttm—persists by homeostatic proliferation of infected cells and could be reduced by using drugs preventing memory T cells from dividing.

There is now evidence for the existence of other reservoirs. Some phylogenetic analyses suggest that a sustained production of the virus could indeed originate from another reservoir than the CD4+ T cell [19, 20]. It has been proposed that peripheral blood monocytes, DCs, and macrophages in the lymph nodes and hematopoitic stem cells in the bone marrow can be infected latently and therefore, contribute to the viral persistence [19, 21–25].

The monocytes are the precursors of the APCs, including macrophages and DCs. Initial studies have shown that monocytes harbor latent HIV-1 proviral DNA [26]. The detection of HIV-1 in circulating monocytes from patients under HAART [22, 27] confirmed this observation. It appears that compared with the more abundant CD14++, CD16− monocyte subsets [28], only a minor CD16+ monocyte subset is more permissive to the infection. Even if the HIV-1 proviral DNA is present only in <1% of circulating monocytes (between 0.01% and 1%), these cells constitute an important viral reservoir. Indeed, the trafficking of these bone marrow-derived monocytes is involved in the dissemination of HIV-1 into sanctuaries such as the brain [22, 27, 29, 30].

Mature myeloid DCs located in lymph nodes can sustain a very low-level virus replication and therefore, have a potential role in HIV-1 latency. The mechanisms involved in this latency are not yet known [31–33].

It is less clear whether macrophages have a potential role in HIV-1 latency [25, 34]. Patients on HAART have few lymph node macrophages infected (approximately five in 10^5). However, the finding of in vivo reactivation of these infected macrophages in response to opportunistic infections argues for macrophages as HIV-1 reservoirs [35, 36]. Although infected monocytes and macrophages contribute only a few percent to the total viral load, it is thought that these cells are important in the transmission and the pathogenesis of HIV-1 infections [35, 37–39].

In addition to the two main targets, i.e., CD4+ T cells and cells from the monocyte-macrophage lineage, HPCs have been proposed to serve as a viral reservoir, as HIV-1 CD34+ HPCs have been detected in some patients [40, 41]. Interestingly, a HPC subset (CD34+ CD4+) has an impaired development and growth when HIV-1 is present. This HPC will then generate a subpopulation of monocytes permissive to HIV-1 infection with a low level of CD14 receptor and an increase of CD16 receptor (CD14+ CD16++). It is not yet well understood whether the abnormalities leading to the generation of this permissive cell population are a result of a direct or an indirect interaction with HIV-1. A further investigation is needed, as these HPCs generate an infected cell lineage that may spread HIV-1 to sanctuaries. The importance of HPCs as a potential reservoir for HIV-1 has been highlighted by the recent report of G. Hütter et al. [42]. They showed that it may be possible to eradicate the reservoirs for HIV-1 by mycloablation and T cell ablation. Moreover, this patient became resistant to HIV-1 infection, as this treatment was followed by a repopulation of cells of the immune system deficient in *CCR5* [42].

In this review, we focus on the molecular mechanisms of the establishment and maintenance of HIV-1 latency in the two major targets of HIV-1: the CD4+ T cells and the monocyte-macrophage lineage. The two virus-infected targets are different. In CD4+ T cells, viral particles assemble at the plasma membrane and bud out of the cell, and in macrophages, a substantial amount of virus particles is budding into the intracytoplasmic compartments [43, 44]. In contrast to infection in CD4+ T cells, HIV-1 infection of macrophages is generally not lytic [45, 46]. These latter cells are also more resistant to cytopathic effects, and they are able to harbor viruses for a longer period. Sometimes, infected tissue macrophages produce viruses during the total lifespan. Deciphering the molecular mechanisms of HIV-1 latency is therefore essential for developing original strategies based on HIV-1 reactivation in association with an efficient antiretroviral therapy with, as an ultimate goal, the eradication of the virus from infected patients.

ESTABLISHMENT AND MAINTENANCE OF HIV-1 LATENCY

Models of HIV-1 latency studies

Several models have been created to study the molecular mechanisms of HIV-1 latency. These are mainly in vivo and in vitro models. Latency in primary cells is studied less because of the difficulties to grow and maintain them in ex vivo conditions in such a high density that allows HIV latency to occur even with a low frequency. Below, we discuss briefly only the most important models of latency that made it possible to better understand the molecular basis of HIV-1 latency.

In vitro models of latently infected cells. The first three model cell lines developed (Ach-2 T cell line, U1 promonocytic cell line, and JΔT T cell line) were characterized by a small constitutive expression of HIV-1, and in these, virus gene expression could be increased by treatments with cytokines and/or mitogens [47–50]. These cell lines contained virus genes that were mutated in Tat protein (U1 promonocytic cell line), TAR-RNA (Ach-2 T cell line), and the NF-κB-binding site (JΔT T cell line). Experiments suggested that these factors are important in establishing latency [51, 52]. Indeed, these elements are involved in the initiation (NF-κB) or the elongation of transcription (Tat via interaction on the TAR). Other cell lines, without mutations in the Tat gene, were also created, which better reproduce the physiology of infected cells. Marcello et al. [53] used a proviral construct containing an intact HIV-1 promoter driving GFP expression and a LTR-

driven Tat, which allowed the selection of several clonal cell lines (J-LAT) that showed no detectable GFP expression in basal conditions but a high level of GFP expression when treated with cytokines and/or mitogens [54]. In other models, cells were infected with HIV-1-based reporter viruses. In these cells, HIV-1 expression was shut down, but they were sensitive to cytokine-induced reactivation [55]. Various in vitro cell line models, i.e., myeloid cell lines at different degrees of differentiation, have also been created to reproduce HIV-1 macrophage latency [56].

Ex vivo models of latently infected cells. In vitro models do not necessarily reflect latency under in vivo conditions. Ex vivo models of primary T lymphocyte and primary-derived macrophage cell models have been developed to examine the relevant signaling pathways of HIV latency in more physiological conditions [57, 58]. However, these models are technically difficult to establish and to maintain, explaining why they are still confidential. Development of these ex vivo models is needed.

Animal models of HIV-1 latency. Animal models play an important role in understanding HIV pathogenesis. Indeed, these models provide an in vivo system to investigate the importance of the HIV-1 reservoirs. For example, Brooks et al. [58] have developed a SCID mouse model, which provided new insight into the establishment and maintenance of HIV-1 latency. Advances have also been made with reconstituted murine models harboring stem cells of human origin. For example, studies of humanized SCID models show that the function of hematopoietic stem cells is affected indirectly by HIV-1 [59]. This model is also a powerful in vivo system for preclinical studies directed toward the development of original strategies in HIV-1 reactivation. The SIV-macaque model was used to show the establishment of transcriptional HIV latency in macrophages resident in the CNS (the microglial cells) and provided the first mechanism of HIV latency in the brain [60]. This model has also been used in clinical trials to test suppressive antiretroviral therapy [61–65]. Finally, it has been confirmed in SIV macaques that the monocyte progenitor CD34+ CD11 is affected early during the virus infection [64, 65], as it was found previously in patients infected with HIV-1 [66].

Establishment of HIV-1 latency

Two different forms of HIV-1 latency have been described in CD4+ T cells. The first one is observed in naïve cells with an unintegrated DNA and is called preintegration latency [67]. This latency is characterized by a poor RT activity, and therefore, it is unable to synthesize the provirus DNA. Several mechanisms are involved in this form of latency, such as hypermutation of the DNA induced by the restriction factor APOBEC3, a low level of the dNTP pool, and an impaired nuclear importation of the preintegration complex associated with a low level of the ATP pool [68–71]. However, this form of latency is clinically not relevant, as cells carrying a full-length integral-competent HIV-1 have short half-lives (1 day) and therefore, cannot account for the long-term latency observed during HAART. This might not be true for macrophages [72, 73].

A latent reservoir, i.e., a stable postintegration of the virus genome, is required for the formation of a long-lived population of latently infected cells. This has been observed in resting T cells, which cannot proceed to integration after a direct infection, suggesting that latency arises in infected CD4+ T cells that have reverted from an activated to a quiescent state [9, 74, 75]. These cells have a specific set of surface markers together with an integrated form of the HIV-1 genome. Moreover, in these cells, cytokine and/or mitogen activation are able to induce HIV-1 virus production [76].

It has been proposed soon after the discovery of HIV-1 latency that proviral DNAs integrated into heterochromatin regions are responsible for the phenomenon of proviral silencing (**Fig. 1**) [54, 77]. Heterochromatin regions are known to be nonpermissive to proviral transcription. Different forms of proviral silencing mechanisms have been suggested. One hypothesis is that the virus takes a latent form right after DNA integration, without being transcribed. Another one suggests that some delayed forms of silencing occur only after a period of transcription of the provirus. The discovery that virus DNAs preferentially integrate into actively transcribed genes [78, 79] is in favor of this second hypothesis. This has been demonstrated in a T cell line infected in vitro and confirmed by in vivo analysis in resting CD4+ T cells [80]. Moreover, it was shown by reverse PCR that 93% of the 74 integration sites found in CD4+ memory T cell genomes were in introns of transcriptionally active genes. As many delayed forms of latency occur at transcriptionally active sites, this raises the question of why and how latency is acquired. A possible explanation is that viral DNA production is silenced by a mechanism involving different factors that interfere with viral transcription. TI is a Cis effect of one transcriptional process on a second transcriptional process [81]. It can be grouped into three different types of molecular mechanisms (Fig. 1) [82, 83]. The mechanism of promoter occlusion suggests that the polymerase complex from the upstream host promoter reads through the integrated downstream promoter of the virus. This mechanism has been confirmed recently in a CD4+ T cell line (J-LAT), which is a model for postintegration latency [84]. Another explanation for TI-promoted gene silencing is that the virus and the host genes are competing for transcription factors required for the promoter activity. TI is also possible when two transcription units—the host gene and the HIV-1 promoter—are oriented in a convergent manner. Such an orientation-dependent regulation of integrated HIV-1 transcription by a host gene transcriptional readthrough was described recently [83]. TI, especially promoter occlusion, may be an important virus-silencing mechanism, as it disables the cryptic promoter to function as a transcriptional starting-point of the virus gene [82].

A recent paper by Duverger et al. [85] suggests that below a NF-κB threshold level, the virus will integrate in a transcriptionally silent state. This means that infection leading to the establishment of latency could occur in low-level, activated CD4+ T cells or even in resting CD4+ T cells, as suggested already [86, 87]. In the view of these authors [82], once latency is established, the central mechanism maintaining latency is based on TI, as was already suggested by Han et al. [81]. This original and new view of how latency is established

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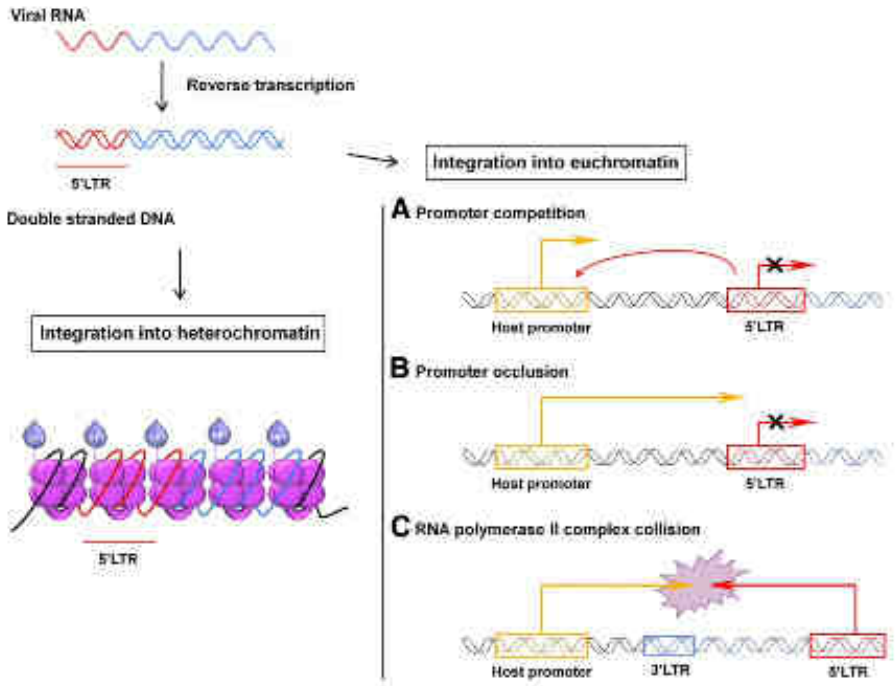

Figure 1. Establishment of postintegrational latency. After RT, viral RNA is converted into double-stranded DNA, which is then transferred into the host cell nucleus. Once the HIV-1 DNA reaches the nucleus, it is integrated into the host genome, and depending on the integration spot, postintegrational latency may occur. Different causes are distinguished to explain this phenomenon. First, the provirus ends in heterochromatin or euchromatin. In the first case (left panel), provirus lay merely in a nonreplicative part of the host genome, and so, the viral DNA is not transcribed. In the euchromatin (right panel), although the DNA is not tightly packed, the virus might enter in a latent state. Mechanisms have been described to interpret the origin of such latency set up: (A) Promoter competition: A host promoter, close to the integration site, hijacks the enhancer region of the 5′LTR, leading to transcription interferences. Enhancers interacting with the LTR preferentially induce the host gene at the expense of the provirus. (B) Promoter occlusion: Provirus is located nearby another host gene, which appears to have a stronger promoter than HIV-1. Thus, during its own transcription, the host gene prevents or displaces RNA polymerase II complexes to bind onto the proviral promoter. (C) RNA polymerase II complex collision: Promoters from the HIV-1 genome and a host gene are convergently arranged, which provokes the collision between both RNA polymerase II complexes processing the host gene and provirus.

and maintained needs further investigation, as if true, the therapeutic strategies will have to be revisited.

As it is believed that at least in CD4+ T cells, HIV-1 latency occurs in cells switching from an activated to a quiescent state, a question remains unanswered. What triggers latency in infected cells that actively produce viruses? Oscillations in NF-κB expression or stochastic fluctuations in Tat expression have been proposed to trigger the establishment of latency in CD4+ T cells [88, 89]. Weinberger et al. [89–92] have used a nice, integrated computational-experimental approach to understand the establishment of latency. Notably, they have shown that stochastic fluctuation in Tat level is a critical event, which may switch the cell from a lytic-productive to a latent-nonproductive fate. Moreover, they showed that perturbation of the Tat feedback circuit (by overexpression of the deacetylase Sirt1, which decreases Tat) changes cell fate. The probability to switch from a lytic state to a latent state increases in these cells. The probability of such events is small, but this might explain why some of the viral-producing cells become silent [89–92]. Furthermore, HIV-1 is progressively silenced in CD4+ T cells that are transfected with lentiviral vectors expressing Tat in Cis. The silencing was even greater if Tat were mutated. However, some authors suggested that the regulatory circuit involving Tat is not enough to explain cell entry into latency, as this model did not take into account the impact of other mechanisms such as epigenetic silencing [93]. Pearson et al. [93] showed that the progressive shutdown of HIV-1 expression is a result of epigenetic modifications, which affect the compaction of chromatin and thus limit HIV-1 transcription. Down-regulation of HIV expression then decreases Tat production below the level required to sustain HIV-1 transcription [93]. In the model of Weinberger et al. [89–92], overexpression of Sirt1 targets Tat acetylation, but as Sirt1 belongs to the acetylase class III family, epigenetic modifications at the HIV-1 promoter may not be excluded. We believe that any mechanism able to weaken the feedback loop of Tat can be responsible for the entry of cells into latency. In the future, mechanisms weakening the Tat circuit under physiological conditions should be explored. The epigenetic modifications observed in noninfected cells or in newly infected cells may be important in this phenomenon. Further investigations are needed in this direction.

The mechanisms proposed above for the establishment of latency in CD4+ T cells might be effective in cells from the monocyte-macrophage lineage, but this requires further investigation. The mechanism of HIV-1 latency in monocytes is not fully understood. It has been proposed that once integrated into the genome, HIV-1 transcription restriction is a result of the lack of Tat transactivation by the pTEFb, which is composed of two proteins: CycT1 and CDK9 [94–96]. Indeed, CycT1 was undetectable in undifferentiated monocytes and was induced in monocyte-differentiated macrophages [97]. However, CycT1 is not the only limiting factor involved in the transcription restriction of HIV-1: the phosphorylation status of CDK9 is also important, as it increases during monocyte differentiation into macrophage [98]. Some studies suggest

that the cellular signaling pathway, which involves the receptor tyrosine kinase RON, could trigger the establishment and maintenance of HIV-1 latency in monocytic cell lines. A correlation was found between RON expression and inhibition of HIV-1 transcription, which was affected at different levels, i.e., chromatin organization, initiation, and elongation [99–101]. The retinoid signaling pathway may also be involved in the inhibition of HIV-1 reactivation. The retinoid pathway inhibits Nuc-1 remodeling and transcription [102].

Apoptotic cells are part of a suppressive microenvironment that may help to establish reservoirs of latent macrophage populations [102]. It appears that the ability to suppress transcription is independent of phosphatidylserine and that a key signaling molecule, ELMO which participates in the phagocytosis of apoptotic cells, strongly inhibits HIV-1 transcription [103]. In addition, differences in the composition of NF-κB complexes between productive and latent macrophages have been proposed to explain the difference in the HIV-1 production between these cells [104, 105]. This requires further investigation, as the difference between NF-κB complexes has a potential role in the establishment of HIV-1 latency.

The strategies developed by the virus to survive longer, once the persistent infection is established in monocyte-macrophage cells or in memory T cells, include maintenance of HIV-1 latency (see the following section) and resistance of infected cells to apoptosis. HIV-1-infected cells do not undergo apoptosis following infection [106]. The mechanism inducing apoptosis resistance of persistently infected cells is not well understood yet. It has been proposed that the modulation of TNFR signaling by viral proteins such as Tat, Nef, and Vpr could explain the formation of viral reservoirs during HIV-1 infection [107]. Recently, a microarray approach has been used to investigate the gene expression signature in the circulating monocytes from HIV-1-infected patients [105]. A stable antiapoptosis signature comprising 38 genes, including p53, MAPK, and TNF signaling networks [108], has been identified. They showed that the CCR5 coreceptor bound by HIV-1 can lead to the apoptosis resistance in monocyte cultures.

As mentioned in the introduction, a HIV-1-positive patient has received bone marrow transplantation for leukemia. There was no evidence of the virus in the bloodstream after 20 months of follow-up [42]. Myeloablation and T cell ablation were suggested to favor the elimination of the long-lived reservoirs. This work therefore raised the possibility that the HIV-1 reservoir is confined to a population of radiosensitive cells, which might include mainly HPCs and circulating monocytes and lymphocytes. This study also points out the fact that reservoirs in sanctuaries could not sustain viral replication in the absence of peripherical-circulating cells. This latest remark would have profound therpeutic implications, as the main cellular target for eliminating the virus from infected patients would be the peripherical circulating cells (including the monocytes) and/or the hematopoietic stem cells. Moreover, this report together with the one discussed above [108] underscore the central role of CCR5 during HIV-1 infection. Indeed, transplantation was done with cells from a homozygous donor for mutation in the HIV-1 coreceptor CCR5. This mutation is well known to be associated with resistance to HIV-1 infection. The importance of the CCR5 coreceptor in HIV-1 infection, demonstrated by its role in apoptosis resistance in infected cells [108] and by the observations of Hütter et al. [42], is therefore essential, and efforts at targeting this CCR5 coreceptor will be a great challenge in the next years. It will also be interesting to investigate whether the interaction between the CXCR4 coreceptor and HIV-1 could also trigger apoptosis resistance. Another mechanism has been proposed based on HIV-1 TAR miRNA, which protects cells from apoptosis and therefore, extends the life of infected cells [109]. The survival of viral reservoirs is of great importance, as it is also an obstacle to HIV-1 eradication. Deciphering the mechanisms underlying this apoptosis resistance is essential for devising new and original therapeutic strategies to purge the reservoirs.

Maintenance of HIV-1 latency

Once viral DNA is integrated into the host genome, and latency is established, mechanisms maintaining the virus in its latent form take place. We will consider two theories: One suggests that the maintenance of latency depends on the chromatin environment, and the other one put forward a mechanism that is based on the prevention of reactivation.

The chromatin environment. It is well known that the local state of chromatin influences transcription. Indeed, a heterochromatin environment, which is more compact and structured than euchromatin, is repressive for transcription (**Fig. 2**).

On the other hand, euchromatin, a relaxed state of chromatin, is associated with active transcription. The compaction of chromatin and its permissivity for transcription depends on post-translational modifications of histones such as acetylation, methylation, sumoylation, phosphorylation, and ubiquitinylation (Fig. 2) [110]. It is also well established that viral promoter activity depend on the chromatin environment [111]. Nucleosomes are positioned precisely at the HIV-1 promoter (Fig. 2) [112, 113]. Nuc-1, a nucleosome located immediately downstream of the transcription initiation site, impedes LTR activity. Epigenetic modifications and disruption of Nuc-1 are a prerequisite of activation of LTR-driven transcription and viral expression [111]. Histones of the nucleosomes Nuc-0 and Nuc-1 are constitutively deacetylated in all model cell lines of HIV-1 latency. Enzymes with deacetylase activities are recruited by several factors, including the homodimer p50, YY1, LSF, or thyroid hormone receptors [114–116].

Furthermore, transcriptional repressors such as Myc bind the HIV-1 promoter and recruit, together with Sp1, HDAC, thereby inducing proviral latency [117]. It was found recently that recruitment of deacetylases and methylases on the LTR was associated with epigenetic modifications (deacetylation of H3K9 followed by H3K9 trimethylation and recruitment of HP1 proteins) in CD4+ T cells. In these experiments, the methylase SUV39H1 and the HP1γ proteins were knocked down by siRNA. The depletion of these factors increased the level of HIV-1 expression [118].

Recruitment of deacetylases and methylases in the microglial cells, the CNS-resident macrophages, has also been described. These cells are major targets for HIV-1 and constitute latently infected cellular reservoirs in the brain [60]. As these infected cells are protected by the blood-brain barrier, only a few drugs

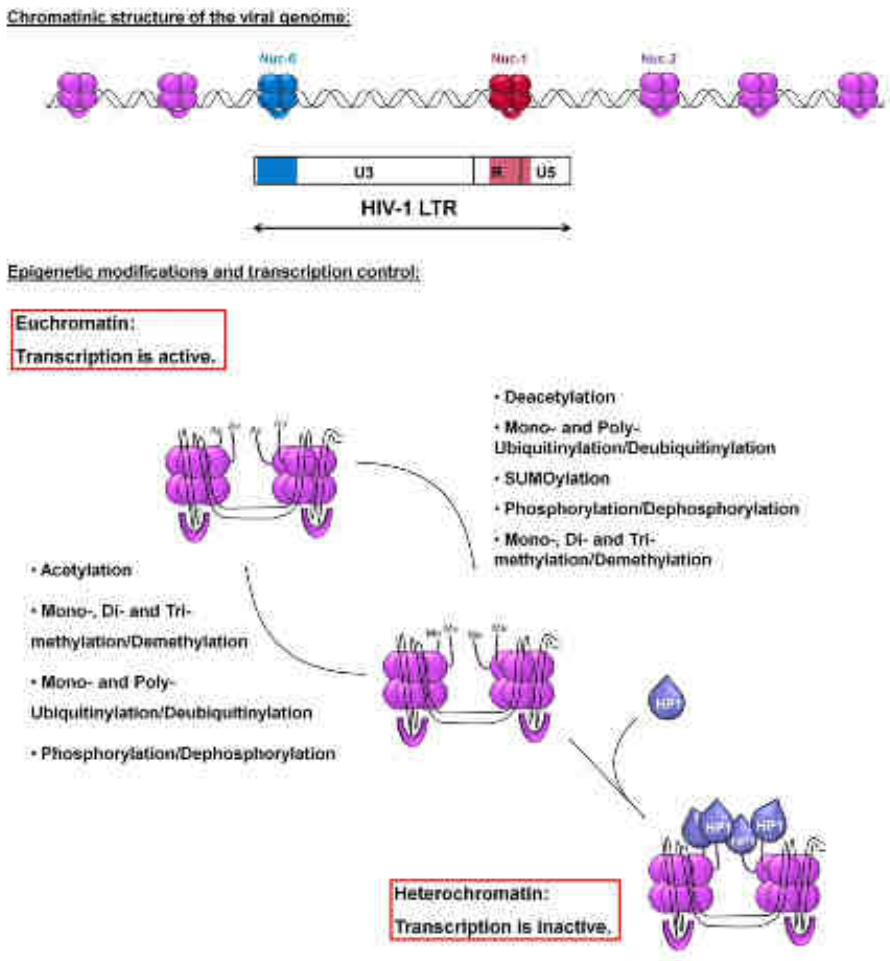

Figure 2. HIV-1 genome nucleosomal organization and epigenetic modifications of histones. 5′LTR of the HIV-1 genome presents a particular arrangement in which two nucleosomes, Nuc-0 (blue) and Nuc-1 (red), are surrounding the U3 region (upper panel). For this reason, the U3 region, which contains sites for cellular transcription factors able to modulate, enhance, and start viral replication, appears to be targetable still by chromatinic-modifying agents, even in latently infected cells. As a result of these chromatinic-modifying agents, epigenetic marks may be switched easily from repressive to activating (lower panel). Most of the epigenetic marks, carried by histone proteins, show a duality in the chromatinic state they induce. Thus, euchromatin (left) and heterochromatin (right) depend on the position rather than the presence of methylation, phosphorylation, or ubiquitinylation, and acetylation is associated with a transcriptional open state, and sumoylation is linked to a heterochromatinic closed state. Moreover, the closed state can also be stabilized through adaptor molecules such as HP1, strengthening the transcriptional repression.

are able to target them. Brain is therefore considered as a tissue sanctuary along with testes and retina. We have shown previously that CTIP2, a recently cloned transcriptional repressor that can associate with members of the COUP-TF family [119], inhibits HIV-1 replication in human microglial cells [120, 121]. CTIP2 inhibited HIV-1 gene transcription in microglial cells by recruiting a chromatin-modifying complex [122]. Indeed, our work suggests a concomitant recruitment of HDAC1, HDAC2, and methylase SUV39H1 to the viral promoter by CTIP2 (**Fig. 3**). Ordered histone modifications would allow HP1 binding, heterochromatin formation, and as a consequence, HIV silencing (Fig. 3). The heterochromatin formation at the HIV-1 promoter has been linked to postintegration latency, and this suggests that transcriptional repressors such as CTIP2 are involved in the establishment and maintenance of viral persistence and postintegration latency in the brain.

The corepressor CTIP2 has even more pleiotropic action by regulating the expression of the infected cell genes. Using a microarray analysis with a microglial cell line knocked down for CTIP2, we have seen that several genes were up- or down-regulated (unpublished results). Among these factors, the cellular CDK inhibitor CDKN1A/p21^{WAF} was up-regulated. Recruited to the p21 promoter, CTIP2 silences p21 gene transcription by inducing epigenetic modifications, as described above for the HIV-1 promoter. This effect favors HIV-1 latency indirectly, as activation of the p21 gene stimulates viral expression in macrophages [123]. Moreover, CTIP2 counteracts HIV-1 Vpr, which is required for p21 expression. We suggest that all of these factors contribute together to HIV-1 transcriptional latency in microglial cells [124].

Mechanisms preventing reactivation. Mechanisms that inhibit the reactivation of HIV-1 expression also contribute to latency. Reactivation of HIV-1 from silent, infected cells requires sequential recruitment of cellular factors to the promoter region of the virus (**Fig. 4**). The initial nucleosomal organization of the HIV-1 LTR prevents the RNA polymerase II and the GTFs to access the promoter. A nucleosome remodeling by chromatin-modifying complexes is a prerequisite for transcription. These complexes disrupt the compacted chromatin and give access to the basal transcriptional machinery complex. Two major classes of chromatin remodeling complexes can be distinguished. The first class consists of ATP-dependent remodeling complexes that alter histone-DNA

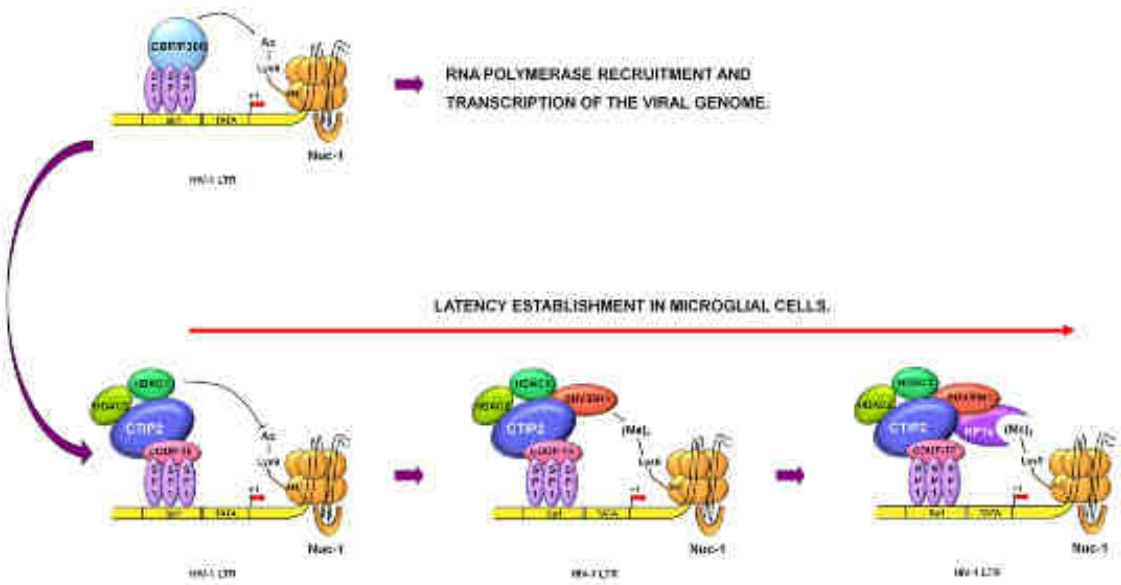

Figure 3. Postintegration latency in microglial cell. After integration in the host genome, viral DNA can be transcribed as long as the Nuc-1 is in an epigenetic open state (green). HIV-1 LTRs stay lightly packed in a euchromatinic structure, through activating epigenetic marks driven by proteins such as HAT. For instance, CBP/p300, a transcriptional coactivator, has an intrinsic HAT activity and processes Nuc-1 by interacting with Sp1, bound to HIV-1 LTR. However, it has been shown that in microglial cells, CPB/p300 can be replaced by inhibitory factors, leading to a latent HIV-1 integration. Sp1, bound to specific sites on the HIV-1 LTR, can recruit COUP-TF, which interacts with CTIP2, a known transcriptional repressor and HDAC1 and -2. Hence, histone protein 3 lysine 9 loses its acetylation in favor of a trimethylation mark induced by SUV39H1, switching Nuc-1 from a transcriptionally active to a repressive state. As a final step for latency establishment, an adaptor molecule HP1 stabilizes Nuc-1 into a heterochromatinic state (red) by targeting the newly produced trimethylation.

interactions. Enzymes of the second class regulate HAT. Cellular events, such as activation by phorbol esters (PMA, 12-*O*-tetradecanoylphorbol-13-acetate) and/or cytokines (IL-1β, IL-6, TNF-α), mitogens (PMA, prostratin), and antibodies (anti-CD3) [125], can remodel the nucleosomes (Fig. 4). HAT-regulating proteins, such as PCAF, CBP, and human GCN5, are recruited following TNF-α treatment (Fig. 4) [126]. The epigenetic modifications induced by HATs lead to better accessibility of DNA for RNA polymerase II and GTFs and allow early-phase transcription and finally, the production of Tat.

This early-phase transcription is induced by direct binding of host-cell factors to their target DNA sequences or by indirect binding via DNA-bound proteins. The ability of the viral genome to adjust its transcriptional mechanisms to specific cell types has been reviewed earlier [127]. In the early phase of transcription, multi-spliced mRNAs encoding the viral regulatory proteins (Tat, Rev, and Nef) are transcribed with low efficiency (Fig. 4). Among these viral proteins, Tat enhances gene expression by binding to TAR-RNA and associating with CycT1, which recruits Cdk9 [94–96].

The late phase of transcription is therefore under the control of Tat, which potently enhances gene expression through initiation and elongation of transcription (Fig. 4) [128]. The two steps of HIV-1 transcription occur in the two major cell types targeted by HIV-1 in a cell type-specific manner. Thus, transcription is regulated by the interplay of distinct viral and cellular transcription factors [127, 129], which bind to the LTR region of HIV-1 [130, 131]. Mechanisms preventing reactivation can act at a transcriptional and a post-transcriptional level.

Mechanisms Acting at a Transcriptional Level. One way to maintain latency of the virus is to sequestrate and/or degrade factors that would normally induce expression of the virus. MURR-1 encodes a protein involved in the cytoplasmic sequestration of NF-κB. Overexpression of this protein in memory CD4+ T cells decreases HIV-1 expression dramatically. On the other hand, knockdown of MURR-1 up-regulates HIV-1 expression. The protein Murr-1 prevents the proteasomal degradation of IκB, which interacts with NF-κB and thereby impedes reactivation of HIV expression. It is believed that this mechanism contributes to the maintenance of HIV latency [132]. Some studies point to the importance of DNA CpG methylation in the repression of transcription. These methylated sequences favor recruitment of additional factors leading to the recruitment of HDACs [133]. The use of methylase inhibitors, such as 5-aza-cytosine, induces hypomethylation and up-regulates transcription. These results strongly suggest that DNA methylation is involved in the regulation of transcription. As shown in the ACH-2 promonocytic cell lines, it may be important in the development of latency too [134, 135]. However, some reports do not support this mechanism of HIV-1 latency, as the methylase inhibitor 5-aza-cytidine induces only weak HIV-1 expression in J-LAT cells, a model cell line for latency

JLB

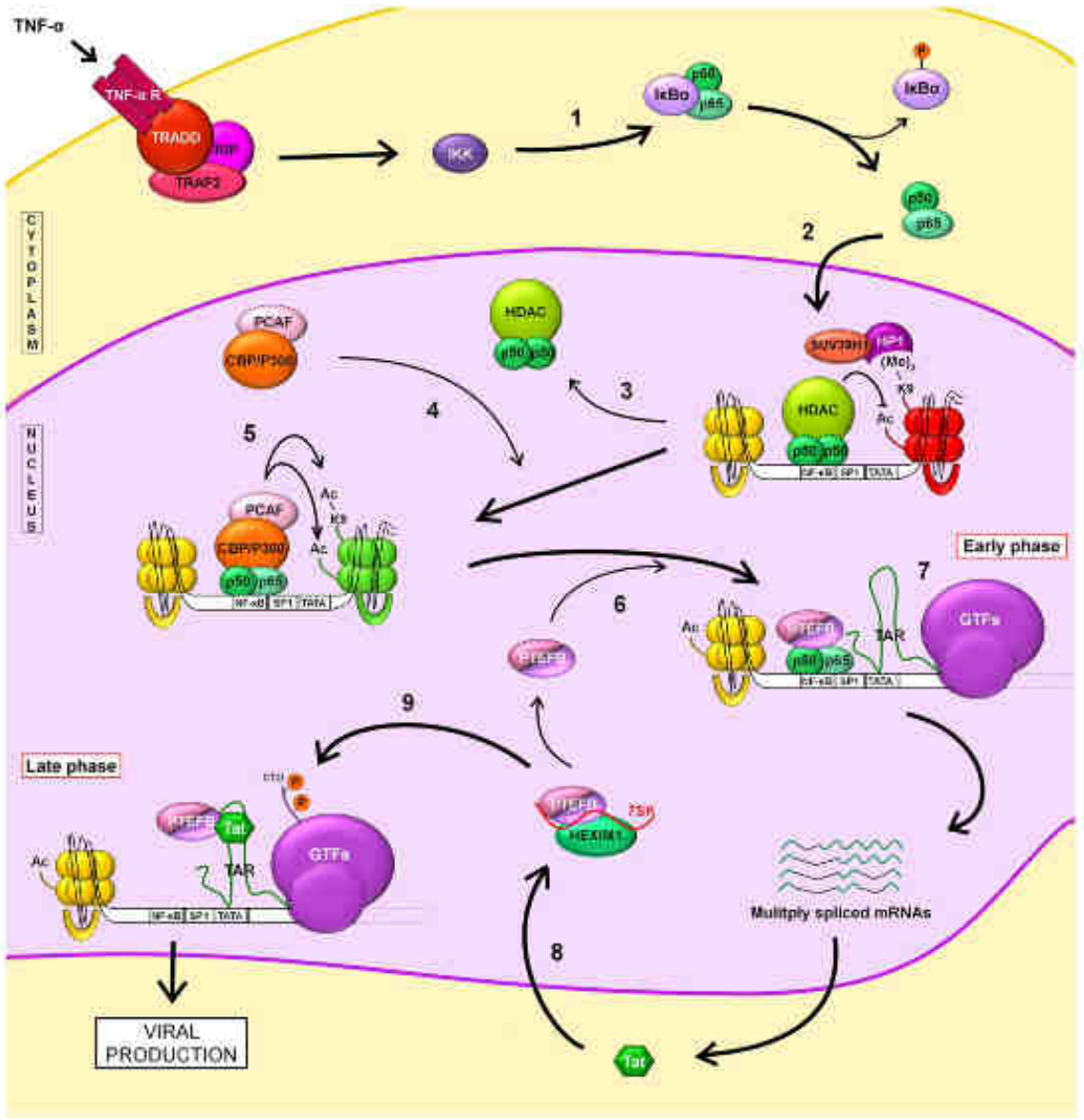

Figure 4. Mechanisms of latent HIV-1 reactivation in T lymphocyte. Reactivation of HIV-1 provirus in latently infected cells may be mediated through extracellular signals, such as the TNFα molecule. Extracellular TNFα triggers activation of IKK by direct interaction with TNFαR, located at the T lymphocyte surface. The TRADD/RIP/TRAF2 complex, associated with the TNFαR, induces a reaction cascade leading to IKK activation. Following its activation, IKK phosphorylates IκBα, protein sequestering the heterodimer p50/p65 in the cytoplasm (1). p50/p65, a transcription factor, is then released and so, migrates into the nucleus (2). Latent HIV-1 integration is a result of a tight packing of the nucleosomes on the 5′LTR, responsible for a heterochromatin structure formation (red nucleosome). The transcriptional-inactive environment is maintained through repressive epigenetic marks, for instance, histone 3 lysine 9 trimethylation or general deacetylations of the nucleosome. As described, these marks are processed, respectively, by SUV39H1 and HDACs. Moreover, trimethylation is also recruiting packing proteins, such as HP1, which enhance the chromatin-closed state. HDACs are bound onto the HIV-1 LTR by interacting with the p50/p50 homodimer, also targeting the NF-κB sites but unlike p50/p65, acts as a repressor. Thus, free p50/p65 removes p50/p50 and HDACs simultaneously (3) and recruits PCAF/CBP, a complex of coactivator and HAT, onto the HIV-1 LTR (4). This complex switches the epigenetic marks from a repressive to active form by acetylating the nucleosome, currently blocking the TATA box (5). As a result, the HIV-1 genome adopts a relaxed euchromatin form (green nucleosome). For this reason, viral DNA is accessible to other factors, such as pTEFB (6) as well as the GTFs (7), the starting point of the HIV-1 replication early-phase. Production of multiply spliced viral mRNAs following the beginning of the replication produces first, viral proteins, such as the transactivator Tat. Once synthesized, Tat recruits more pTEFB from the inactive pool of pTEFb/Hexim1/7SK RNA complexes available in the nucleus (8). The Tat/pTEFB complex, on one hand, binds to the TAR element on native viral mRNA and on the other hand, phosphorylates the RNA polymerase II, hence speeding up the process (9). The viral replication enters into the late phase, during which viruses are produced at a high rate. Viral DNA has gotten out its latent state completely.

[136]. On the other hand, some investigators conducted a cDNA screen in J-LAT cells for genes that reactivate HIV-1 and have identified a transcriptional repressor methyl-CpG-binding domain protein 2, which binds methylated DNA. Furthermore, they have shown that the association of the DNA methylation inhibitor with prostratin, a NF-κB inducer, or TNF-α has a synergistic effect on reactivation of HIV-1 expression [137]. In vivo studies are required to assess the importance of DNA CpG methylation in more physiological conditions.

Mechanisms Acting at a Post-Transcriptional Level. Several post-transcriptional mechanisms are important in maintaining HIV-1 latency. The discovery of RNAi has initiated many studies of transcriptional mechanisms. In the pioneering work of Craig Mello (University of Massachusetts Medical School) and Andrew Fire (Stanford University School of Medicine), RNAi was identified as a post-transcriptional response to exogenous double-stranded RNA in *Caenorhabditis elegans*. They also showed that RNAi is involved in the regulation of gene expression. There are two classes of RNAi. One group of RNAi has an exogenous origin and is called siRNA. These are cleavage products from enzymatic complexes such as Dicer and Drosha. RNAi of the second class is encoded by the host genome and is thought to be an endogenous regulator of gene expression. These miRNAs are short, single-stranded RNAs of 19–25 nucleotides that are involved in various biological processes in eukaryotic cells [138, 139]. The miRNAs interact with a complementary sequence in the 3′-untranslated region of target mRNAs, which lead to mRNA degradation or more often, to translational inhibition [139]. miRNAs are involved in the regulation of virus expression as well [140]. Recently, it was shown that miRNAs regulate the expression of the HAT Tat cofactor PCAF and HIV replication [141]. Moreover, in an elegant experiment using microarray technology, an enrichment of miRNAs in clusters has been observed only in resting CD4+ T cells and not in active CD4+ T cells [142]. Huang et al. [142] found that several of the miRNA clusters inhibited HIV replication. They suggested that miRNAs contribute to HIV latency in resting primary CD4+ T cells. As multispliced RNAs for Tat and Rev are present in resting T cells, even if in small amounts, it may be that degradation of these RNAs by miRNAs contributes to the maintenance of HIV latency [143]. Such a mechanism based on cellular miRNA has also been described in circulating monocytes [144]. Another post-transcriptional mechanism involves the regulation of the exportation of viral RNAs. Nuclear retention of multi-spliced RNAs has been found in memory CD4+ T cells. The mechanism underlying this nuclear retention is unclear but may involve the down-regulation of the protein poly track-binding, which interacts with RNAs. This factor is present in active CD4+ T cells, and overexpression of this protein in resting cells allowed reactivation of HIV-1 expression [145].

The molecular mechanisms of the establishment and the maintenance of latency in the two main targets for HIV-1 are multi-factorial and are involved at various stages of the viral lifecycle, as discussed above. Possibly, some forms of latency are the result of the counter-reaction of the infected cell to silence HIV-1. It is tempting to speculate that defensive mechanisms arise in cells after a long history of coevolution of mammalian cells and retroviruses. Ironically, these mechanisms contribute to the persistence of HIV-1 in infected patients.

NEW PERSPECTIVES OF THERAPEUTIC INTERVENTION IN HIV-1 INFECTION

Since the discovery of HIV-1, vast research has been done to prevent and to cure AIDS. Much research concentrated on the development of vaccines against HIV. Unfortunately, all HIV-1 vaccines have failed in the past 25 years, showing that in practice, this approach is not working. Many actors of the scientific world, including the new Nobel Prize winner in physiology and medicine, Francoise Barre-Sinoussi, voiced the need for a new direction in HIV immunology research [146–149]. The failure of the current HAART to eradicate the virus originates partly in the truly latent reservoirs [19, 150, 151]. However, phylogenetic approaches also point to an ongoing replication at a low level in certain infected cells. Moreover, these cells are often found in tissue sanctuary sites, where penetration of drugs is restricted, as in the brain [152–154]. Furthermore, several RT inhibitors are ineffective in chronically infected macrophages [29], and protease inhibitors have significantly lower activities in these cells compared with lymphocytes [155]. Finally, emergence of multidrug-resistant viruses has been reported increasingly in patients receiving HAART [102, 156, 157]. All of these considerations (existence of true latent reservoirs, tissue-sanctuary sites, and multidrug resistance) encourage the development of new and original anti-HIV-1 treatment strategies.

Today, the therapy for HIV-1-infected patients is based on a combination of HIV gp41, RT, and protease inhibitors. We believe that new drugs should target other steps of the HIV-1 cycle. For example, they could be directed against proteins involved in the transcription of the inserted virus genome. Tat has a critical role in transcription and constitutes a major target in therapeutic intervention in the HIV replicative cycle [158–160]. Moreover, drugs could be designed to target cellular cofactors involved in the activation of transcription. This strategy should be able to bypass drug resistance, which arises with viral proteins. An increasing number of studies suggest that inhibitors of cellular LTR-binding factors, such as NF-κB and Sp1, repress LTR-driven transcription [158, 160–163]. Proteins such as p27, purified from St. John's Wort, which belongs to the DING family [164], are good candidates. p27 suppresses transcription and replication of HIV-1 by interfering with the function of cellular factors (i.e., NF-IL-6) and the viral protein Tat [165, 166]. However, these drugs do not cross the blood-brain barrier easily [152], and their structures must be changed to render them accessible to the brain.

We also believe that understanding the molecular mechanisms involved in the establishment, maintenance, and reactivation is critical for designing new treatment strategies. HIV-1 transcription inhibitors could be used to prevent reactivation and keep the virus in its dormant state. However, it will not allow the complete eradication of HIV-1 and a lifelong multitherapy associated with drug side-effects and development of

drug resistance, and high treatment cost would still be required.

Recently, a new and original strategy has been proposed to eradicate the virus from infected patients. The main idea is to facilitate the reactivation of viruses from latent reservoirs, which are then destroyed by HAART. Many factors have been involved in reactivation, including physiological stimuli such as TNF-α, IL-1β, IL-2, IL-6, IFN-γ, or CD154 [49, 167–171], chemical compounds such as phorbol esters (PMA and prostratin), HDAC inhibitors (trichostatin, valproic acid, sodium butyrate, and suberoylanilide hydroxyamic acid) [112, 172–180], caspase inhibitors [181], p-TEFb activators [182], hydroxyurea [183], and some activating antibodies (anti-CD3) [11, 184]. A recent antifungal agent, amphotericine B, reactivates viruses from the THP89GFP monocytic cell line, a model macrophage cell line of HIV-1 latency [185]. This compound is specific for macrophages, but in T cells, it may induce reactivation in trans when cocultured with the amphotericine B-mediated macrophages [186]. Many eradication protocols passed preclinical studies [5], but to date, all failed in clinical trials. Valproic acid, which is a HDAC inhibitor, was promising, as it was able to reactivate virus expression in the two major reservoirs (CD4+ T cells and macrophages). Indeed, valproic acid was described as effective in the first clinical trial [179, 187], but recent clinical trials did not confirm these results [188, 189]. The p-TEFb activator hexamethylenebisacetamide is also a promising molecule currently under study. In pilot studies, it was able to reactivate latently infected cells and prevent reinfection by down-regulating CD4 receptor expression [182]. Finally, some protocols failed as a result of the potential toxicity of treatments based on nonspecific CD4+ T cell activation such as IL-2. The recent discovery that an alternatively spliced form of the cellular transcription factor Ets-1 can activate latent HIV-1 in a NF-κB-independent manner has highlighted the therapeutic potential of cellular factors for the reactivation of latent HIV-1 [190]. Future eradication protocols should combine several drugs able to reactivate HIV-1 from latently infected cells. Such an approach has been found to be promising, as the association of a HDAC inhibitor or a DNA methylation inhibitor with prostratin has a synergistic effect on the activation of HIV-1 expression [137, 191]. Viral eradication might also be achieved by strategic interventions targeting the resistance of infected cells to apoptosis and molecules involved in latency reactivation. Finally, according to recent data highlighting the existence of two subsets of memory T cells serving as a reservoir [18], a combined use of strategic intervention targeting viral replication (through reinforced HAART) and antiproliferative drugs (such as anti-cancer drugs) has been proposed. Moreover, this treatment has to be introduced very early in the course of infection (by the way, confirming the importance of early therapeutic interventions), as it reduced the constitution of the proliferative reservoir drastically (Ttm memory cells).

CONCLUSION

The latent HIV-1 reservoirs established early during infection present a major obstacle for virus eradication. Understanding the molecular mechanisms of HIV-1 latency is a prerequisite for designing new treatments that aim to eliminate the reservoirs. The mechanisms involved in the establishment and maintenance of HIV-1 latency are diverse and not understood fully. Eradication of the virus from infected patients will require a drastic reduction of the reservoirs associated to HAART, reinforced with new, potently antiretroviral drugs targeting chronically infected cells. These new drugs need to access tissue-sanctuary sites, such as the brain. As the development of a HIV-1 vaccine remains a major challenge for immunologists, we believe that future directions for a HIV-1-curative therapy will rely more on the development of original therapeutic strategies, which take into account latency, the nature of the latent reservoir, chronic replication, and accessibility to tissue sanctuary.

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Publication 7

Achieving a cure for HIV infection: do we have reasons to be optimistic?

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L'introduction en 1996 des thérapies antiretrovirales hautement actives (HAART) a transformé une maladie léthale en pathologie chronique, associé à une chute drastique de la mortalité et de la morbidité des symptôme liées au SIDA chez les patients. Cependant, la HAART n'a pas permis de soigner l'infection par le VIH du fait de l'existence de reservoirs de virus quiescents. Par ailleurs, les patients se heurtent à bien d'autres problèmes liés à la HAART (tels que des effets secondaires, une observance stricte et le coût des traitements et l'emergence de souches virale résistantes), ce qui motive d'autant plus à l'élaboration de nouvelles manières de traiter les patients. Cette revue fait le tour des dernières avancées prometteuses dans le domaine et qui pourraient être la clé d'un traitement curatif. En marge de ces nouvelles stratégies concu pour éliminer le virus, des efforts doivent également être fait pour améliorer les thérapies antirétrovirales actuelles. Nous supposons dans cette revue qu'une cure pour l'infection par le VIH-1 ne sera pas accessible à court terme et que des stratégies basées sur la purge des réservoirs se doivent d'être associées à la HAART.

J'ai réalisé l'intégralité des figures présentes dans cette review et ai activement participé au travail de rédaction

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Achieving a cure for HIV infection: do we have reasons to be optimistic?

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The introduction of highly active antiretroviral therapy (HAART) in 1996 has transformed a lethal disease to a chronic pathology with a dramatic decrease in mortality and morbidity of AIDS-related symptoms in infected patients. However, HAART has not allowed the cure of HIV infection, the main obstacle to HIV eradication being the existence of quiescent reservoirs. Several other problems have been encountered with HAART (such as side effects, adherence to medication, emergence of resistance and cost of treatment), and these motivate the search for new ways to treat these patients. Recent advances hold promise for the ultimate cure of HIV infection, which is the topic of this review. Besides these new strategies aiming to eliminate the virus, efforts must be made to improve current HAART. We believe that the cure of HIV infection will not be attained in the short term and that a strategy based on purging the reservoirs has to be associated with an aggressive HAART strategy.

Keywords: CCR5, reservoirs, latency, purge, HAART

Introduction

Human immunodeficiency virus 1 (HIV-1), identified 28 years ago,[1] remains a global health threat responsible for a worldwide pandemic with an estimated 33 million people infected.[2] More than 7000 new HIV infections occur each day, and the number of newly diagnosed infections remains far greater than the number of people (around 50%) who have access to highly active antiretroviral therapy (HAART). Advances have been made in treating AIDS since the introduction of HAART in 1996. This has transformed a lethal disease into a chronic pathology, with a dramatic decrease of mortality and morbidity of AIDS-related symptoms in infected patients.[3–6]

Why is achieving a cure important?

To date, the only way to treat patients infected with HIV relies on a combination of drugs that acts at different stages of the viral life cycle, preventing the virus from replicating. These molecules target four stages of the cycle: viral entry, reverse transcription of the viral genome, integration into the genome of the host cell and maturation of viral proteins. This therapy can reduce plasma virus levels below detection limits (≤50 copies/mL). However, with very sensitive but expensive and technically challenging methods, a residual viraemia is still detected in patients on HAART.[7–9] Moreover, HIV RNA typically returns to a measurable plasma level in less than 2 weeks when HAART is interrupted, suggesting that even long-term suppression of HIV-1 replication by HAART fails to totally eliminate HIV-1. These two latter phenomena are mainly due to the existence of HIV reservoirs.[6,9–13] The existence of integrated latent viruses or virus replicating at a very low level in different cellular reservoirs is an obstacle to the eradication of the virus, and thus the total recovery of patients, and requires strict adherence to lifelong treatment.[14–21] In addition, these cellular reservoirs are often found in tissue sanctuaries, such as the brain, where drug penetration may be several orders of magnitude lower than in other tissues.[16,18] Viral clearance from other reservoirs, such as from chronically infected macrophages, is also difficult since reverse transcriptase inhibitors are usually ineffective and protease inhibitors have significantly lower activities in these cells than in lymphocytes.[22,23] Moreover, emergence of many side effects may require the cessation of treatment.[24] Furthermore, the development of many types of resistance, related to the extreme mutability of the virus and in part to treatment interruptions, has been described in the literature.[25–28] Another major concern is related to non-AIDS events and non-AIDS mortality in patients having a residual viraemia and a normal CD4+ count, a situation also described in some HIV non-progressors. Owing to the residual viraemia, patients develop chronic inflammation that leads to several complications, for instance, cardiovascular disease, nephropathy, faster evolution of viral hepatitis and cancer.[29–33]

Last but not least, a major problem related to HAART is the cost of the treatment. Even the cost associated with the cheaper generic forms of the drugs far exceeds the abilities of many resource-limited countries in providing treatment. The cost of this treatment will be increasingly important in the future, with an overall global budget requirement to address this problem from today to 2031 being estimated at US$397–727 billion.[31] Since, to date, no effective HIV-1 vaccine is available,[32–38] it appears crucial to improve HAART and to develop new strategies to cure HIV.[39,40]

Which cure is needed: a functional or a sterilizing cure?

A sterilizing cure requires the total eradication of all HIV-infected cells, including quiescent reservoirs. On the other hand, a functional cure aims to mimic a situation encountered in some special patients called 'elite controllers' who are able to control viral replication and have less than 50 copies/mL of the virus without any treatment. Although a sterilizing cure would be the most appropriate and desirable, it may be difficult or impossible to really achieve. Only one reported case, the German case, is known in the literature that suggests a possible eradication of the virus.[41] A functional cure appears more feasible since it seems impossible to get rid of HIV from latent cells and from sanctuaries. We have to keep in mind however that the chronic inflammation described in patients under HAART has also been described in some elite controllers who have presented with residual viraemia and higher immune activation compared with healthy patients.[34–36] It is very likely that these patients will develop more non-AIDS events compared with those who are uninfected or actually cured.

How might we achieve a cure?

The best scenario would be to eradicate the virus from all infected cells. Even though this appears very difficult, we should be able to drastically decrease the HIV reservoirs by identifying and then eliminating them. Residual on-going viral replication, whatever its origin, also has to be reduced to preclude non-AIDS events.

In this article we will discuss new strategies under investigation that aim to eradicate HIV from infected patients. First we will discuss a recently described case that showed a possible eradication of HIV following transplantation of CCR5-deficient haematopoietic stem cells. This strategy may open new avenues to cure HIV-infected patients. We will also discuss novel strategies based on purging reservoirs followed by aggressive HAART. This approach has already been used in several clinical trials. Finally, we believe that HAART has to be improved and/or intensified; however, we have to keep in mind that HAART alone will not allow for a cure.

The critical role of CCR5 in maintaining HIV-1 infection

A proof of concept

A report of a German patient being transplanted with stem cells from a donor who carried the Δ32 CCR5 mutation and then controlled his HIV infection has highlighted the critical role of CCR5 in maintaining HIV infection.[42] It is well known that HIV-1 enters cells by using CD4 receptors and CCR5 or CXCR4 coreceptors and persons homozygotic for a 32 bp deletion in the gene coding for CCR5 are resistant to HIV-1 infection.[43,44] It is noteworthy that the origin of the *CCR5*-Δ32-containing ancestral haplotype is recent (estimated range of 275–1875 years) and might be related to a historic strong selective event such as an epidemic of a pathogen that, like HIV-1, utilizes *CCR5*. This hypothetical epidemic has increased the frequency of this mutation in ancestral Caucasian populations.[45] Hutter understood the significance of the CCR5 mutation and suggested that transplantation of stem cells originating from a donor homozygotic for the mutation could effectively eradicate the virus. After the relapse of leukaemia in the German patient with HIV there was no other choice but to transplant allogeneic stem cells to this person. The patient, as suggested by Hutter, received Δ32 CCR5 mutant stem cells. Following the medical intervention, the patient has stopped HAART and HIV RNA has remained below 1 copy/mL for now over 4 years. In a recent paper this group showed evidence even for a possible cure of HIV-1 infection in this patient. Indeed, they demonstrated reconstitution of both circulating and mucosal CD4+ T cells that do not express CCR5 while the patient remained free of the virus. Moreover, they also found evidence that long-lived cells such as macrophages became Δ32 CCR5. Since these cells are reservoirs for the virus along with CD4+ T memory cells, it appears that the size of the viral reservoir has decreased. This result was unexpected since the patient's CD4+ memory cells are still susceptible to productive infection by lymphotropic (CXCR4-tropic) HIV. The combination of radiotherapy and chemotherapy has allowed the eradication of long-lived reservoirs, which has prevented HIV rebound during the process of immune reconstitution following stem cell transplantation. Although this specific case is a real success, stem cell transplantation as a general strategy to cure infected patients is not yet feasible due to the high mortality of this treatment (20%–30%). This report constitutes a proof of concept and opens the development of new strategies targeting the CCR5 coreceptor.

CCR5 gene therapy

Among new treatments, CCR5 gene therapy could be a potential treatment to cure HIV (Figure 1). In preclinical trials, HIV-1 infected mice engrafted with zinc finger nuclease (ZFN)-modified CD4+ T cells had lower viral loads and higher CD4+ T cell counts than mice engrafted with wild-type CD4+ T cells, consistent with the potential to reconstitute immune function in individuals with HIV/AIDS by the maintenance of an HIV-resistant CD4+ T cell population.[46,47] Preliminary results of two Phase 1 clinical trials using this attractive approach were presented at the 2011 Conference on Retroviruses and Opportunistic Infections (CROI).[48,49] Lalezari presented data on transformed CD4+ T cells. The wild-type CD4+ T cells were obtained from six patients who had been living with HIV infection for >20 years. Participants chosen had continued low CD4+ T cell counts (ranging from 200 to 500 cells/mm^3), despite receiving antiretroviral therapy, which reduced HIV viral load to an undetectable level. Both studies showed a successful and tolerated engraftment of the transformed CD4+ T cells. At the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC),

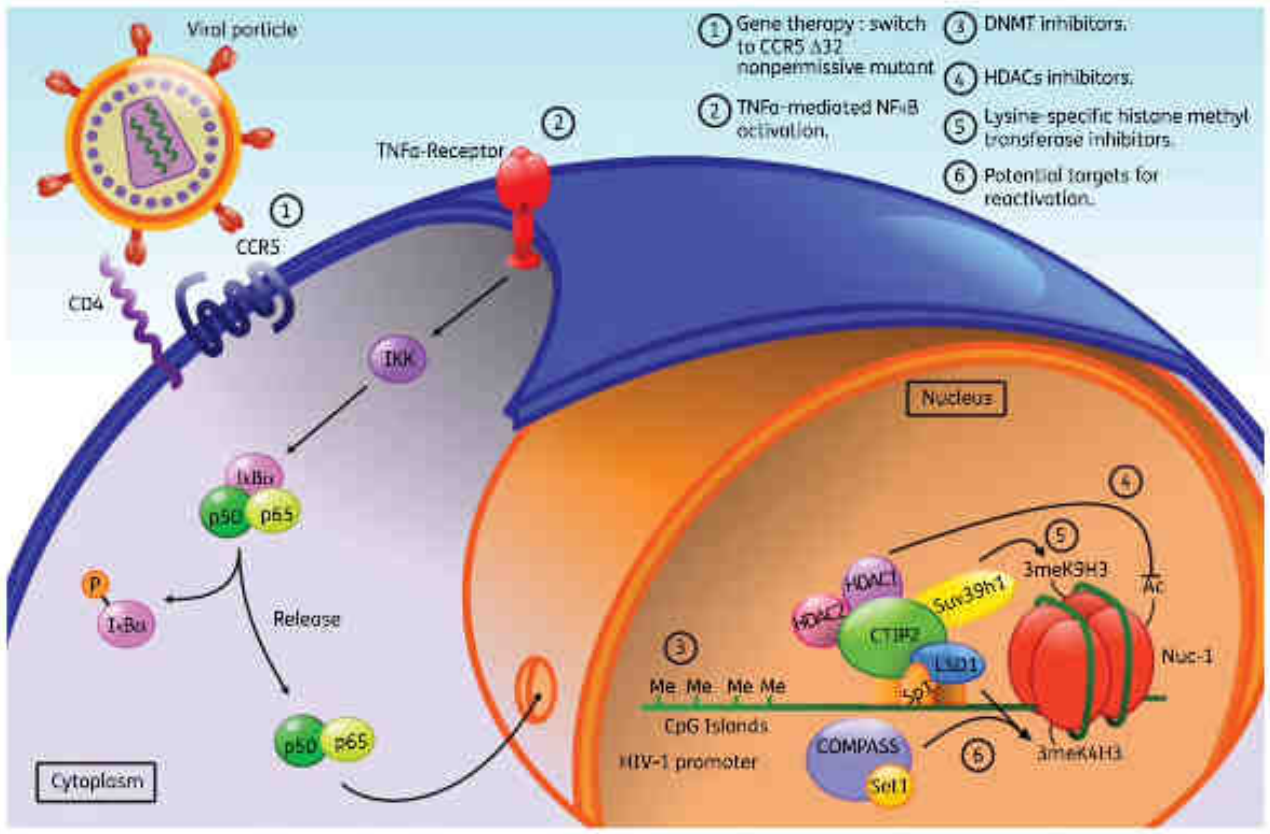

Figure 1. Promising new approaches to cure patients of HIV-1: molecular mechanisms at the macrophage level. Beside increasing the pool of new molecules and improving the currently used ones in HAART, new approaches are required to reach a full recovery from HIV-1 infection. To date, HAART can only control and prevent viral replication, but fails to achieve total viral clearance. New potential strategies include virus eradication through gene therapy and clearance of the viral reservoirs. The first strategy derived from the observation of the Δ32 CCR5 bone marrow transplanted German patient, who seems to be free of HIV-1 infection. Owing to the high risk associated with surgery and the impossibility of using this method in a large number of patients, gene therapy could be a way to disrupt the CCR5-mediated infection in order to mimic the previous results of the German patient (1). The second strategy relies on associating the current HAART with molecules activating the viral transcription and/or targeting host proteins favouring HIV-1 latency. On the one hand, the early stage of viral replication requires the transcription activator NF-κB, thus cytokines such as TNFα may allow the recovery of full viral transcription in latent reservoirs (2). On the other hand, chromatin-modifying enzymes have been associated with HIV-1 transcription extinction through fine modifications of the epigenetic code on the viral promoter. Limiting DNA methylation of the CpG islands (3), increasing activation marks, such as acetylation of histones from Nuc-1 (4), and/or avoiding marks associated with heterochromatin, such as simultaneous trimethylation of lysine 4 and lysine 9 (5,6) of histone H3 in Nuc-1 may revert the latently infected state back to productively infected macrophages. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

data from another clinical trial was also presented in which six subjects having initially >450 CD4+ T cells/mm^3 under HAART[2,3] were followed for 12 weeks after infusion of ex vivo transformed CD4+ T cells. Only one patient in this clinical trial became undetectable for the virus. However, this patient entered the clinical study with one Δ32 CCR5 mutation. Therefore a functional cure with this gene therapy was not attained. As explained during this conference, only 5% of the total CD4+ T cells were transformed, in contrast to the 100% in the German patient who benefited from stem cell transplantation. There is hope however that this small fraction of cells will rise in the body, since it is expected that the CCR5− cells infected by HIV-1 will die over time. It is possible that CCR5− mutants will be selected and will replace the normal CCR5+ cells, since the release of virus from these CCR5+ cells will not be able to infect the transfused population of CCR5− mutants. A much longer follow-up is needed to confirm these expectations.

The long-term control of HIV by the German patient who received a transplant of CCR5 deficient haematopoietic stem cells holds promise for a real cure,[5,6] but due to its toxicity, it is not a realistic one as claimed by Lewin and Rouzioux.[2,3] Further investigations in order to understand the mechanism by which HIV was eradicated have to be performed. It would also be interesting to repeat this approach in other patients, which will help us to make further conclusions.[5,6] It even raises questions such as why there is no HIV rebound from long-lived viral reservoirs

producing CXCR4-tropic viruses or what the role is of the transplantation procedure itself[54,55] in the eradication of the virus. The debate, regarding whether the treatment of the German patient represents a sterilizing cure or not, is far from over.[57] Gene therapy (including CCR5 gene therapy) is indeed a very attractive approach to cure HIV-1 infection, but it is not for immediate use, even though considerable progress in gene delivery has been made.[58,59] Moreover, the debate as to whether or not this gene therapy will lead to a sterilizing cure is still open.[60]

Purging viral reservoirs

The main drawback of HAART is that it is unable to purge the virus from quiescent reservoirs, i.e. truly latent cells,[61–63] and/or from cells with cryptic on-going HIV replication,[7,64,65] or from sanctuaries such as the brain.[66–68] Resting memory CD4+ T cells are the major cellular and the best characterized reservoirs in the natural host.[8,7,69–71] The presence of latent proviral HIV-1 DNA in this cell population has definitely been proven.[69]

Other reservoirs than resting CD4+ T cells have also been proposed.[16,18–20] Genetic studies showed that during rebound viraemia (due to HAART interruption) the virus could be detected from reservoirs other than CD4+ T cells.[52,72,73] It has been proposed that peripheral blood monocytes, dendritic cells and macrophages in the lymph nodes and haematopoietic stem cells in the bone marrow can be infected latently and therefore contribute to viral persistence.[14–17,19,51,74] It is still debated whether or not viral persistence in these latter reservoirs is due to true latency or to a low-level on-going replication.[75,76]

Deciphering the molecular mechanisms underlying HIV persistence is a prerequisite to devise novel treatments aiming to purge these reservoirs. Several recent reviews describe in more detail the mechanisms of HIV persistence with implications for the development of new therapeutic strategies.[18,20,40,77–79] Before using strategies that aim at purging the reservoir in combination with an intensified HAART, we need: (i) to identify and characterize the molecular actors involved in the persistence of latency, which relies on the chromatin environment; and (ii) to understand the mechanisms of reactivation in order to prevent it.

Persistence of latency

Once HIV-1 DNA has integrated into the host genome, and latency has been established, maintenance of HIV-1 latency depends on the chromatin environment. The chromatin organization of the HIV-1 promoter with precisely positioned nucleosomes[80,81] has been well described. Nuc-1, a nucleosome located immediately downstream of the transcription initiation site, impedes long terminal repeat (LTR) activity. Epigenetic modifications and disruption of Nuc-1 are a prerequisite of activation of LTR-driven transcription and viral expression.[82] It was recently found that recruitment of deacetylases and methylases on the LTR was associated with epigenetic modifications (deacetylation of H3K9 followed by H3K9 trimethylation and recruitment of HP1 proteins) in CD4+ T cells. In these experiments, the methylase Suv39H1 and the HP1γ proteins were knocked down by small interfering RNA (siRNA). The depletion of these factors increased the level of HIV-1 expression.[83]

Epigenetic modifications of the LTR have also been described in microglial cells, the CNS-resident macrophages. These cells are major targets for HIV-1 and constitute latently infected cellular reservoirs in the brain.[84] Previous work from our laboratory has shown that a COUP-TF interacting protein 2 (CTIP2), a recently cloned transcriptional repressor that can associate with members of the COUP-TF family,[85] inhibits HIV-1 replication in human microglial cells.[86,87] Subsequently we showed that CTIP2 inhibited HIV-1 gene transcription in these cells by recruiting a chromatin-modifying complex.[88] As demonstrated in T lymphocytes, our work suggests a concomitant recruitment of histone deacetylases HDAC1, HDAC2 and methylase SUV39H1 to the viral promoter by CTIP2. Ordered histone modifications would allow HP1 binding, heterochromatin formation and, as a consequence, HIV silencing. The heterochromatin formation at the HIV-1 promoter has been linked to post-integration latency, and this suggests that transcriptional repressors such as CTIP2 are involved in the establishment and maintenance of viral persistence and post-integration latency in the brain.

The corepressor CTIP2 has an even more pleiotropic action by regulating the expression of genes of infected cells. Recruited to the cellular cyclin-dependent kinase inhibitor CDKN1A/p21[Waf1] (p21) promoter, CTIP2 silences p21 gene transcription by inducing epigenetic modifications, as described above, for the HIV-1 promoter. This effect indirectly favours HIV-1 latency since activation of the p21 gene stimulates viral expression in macrophages.[89] Moreover, CTIP2 counteracts HIV-1 Vpr, which is required for p21 expression. We suggest that all these factors contribute together to HIV-1 transcriptional latency in microglial cells.[90] The picture regarding the importance of p21 in the replicative cycle of HIV-1 is far more complicated since p21 has been described as a restriction factor in macrophages and in resting CD4+ T cells.[91,92] The protein p21 might have different effects on HIV-1 infection of macrophages depending on the targeted viral life cycle step, and therefore on the time since infection.[93]

We have also identified a new actor involved in the maintenance of HIV-1 latency in microglial cells, the lysine-specific demethylase (LSD1).[94] We notably showed that LSD1 repressed HIV-1 transcription and viral expression in a synergistic manner with CTIP2 and reported that recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic marks. Association of both H3K4me3 and H3K9me3 epigenetic marks with LSD1 recruitment may thus constitute a new level of eukaryotic gene regulation. These observations are consistent with the discovery that H3K4 methylation at certain chromatin loci may prevent gene expression.[95] Interestingly, such a gene repression linked to H3K4me3 has been proposed to prevent the expression of cryptic promoters.[95,96] This is strengthened by the finding that HIV-1 preferentially integrates into active genes and therefore could be considered as a cryptic gene.

Surprisingly, LSD1 has been associated with activation of HIV transcription in CD4+ T cells through demethylation of K51 Tat.[97] However, in microglial cells the mechanisms underlying LSD1-mediated increase of H3K4 trimethylation is different and might rely on the ability of LSD1 to anchor other factors at the promoter rather than its own enzymatic activity. Indeed, H3K4 trimethylation was associated with the recruitment of LSD1, hSET1 and WDR5 at the Sp1 binding sites of the HIV-1 LTR. Moreover, reactivation of HIV-1 proviruses correlated with the release

of LSD1, hSET1 and WDR5 from the viral promoter and with a reduced H3K4 trimethylation. In contrast to CD4+ T cells, LSD1 is involved in the maintenance of HIV-1 latency in microglial cells by favouring a local heterochromatin structure. These two studies reporting a dual role of LSD1 through different mechanisms in two main HIV-1 targets point to the complexity of HIV latency and raise the question of how effective the use of inhibitors of LSD1 would be for full HIV-1 reactivation. Indeed, targeting LSD1 for full reactivation in microglial cells might not work in lymphocytes. Instead, in the latter cells an induction of HIV latency is expected.[96] Further investigation of the epigenetic regulation of HIV latency is therefore needed in order to design efficient drugs targeting viral reservoirs.

Another field of interest is DNA methylation, which has been involved in DNA silencing and latency.[99] It is now well established that DNA CpG methylation plays an important role in maintaining HIV-1 latency,[100,101] despite previous controversies.[102] Therefore DNA methylase inhibitors, such as 5-azacytidine, could be useful in strategies aiming to reactivate reservoirs. It is noteworthy that only a few percent of the latent viruses are methylated on their DNA, but these reservoirs of latent viruses are highly resistant to reactivation. Achieving a cure would probably require the treatment of many different types of latency simultaneously by a combination therapy approach.

Preventing reactivation

Several mechanisms acting at the transcriptional and post-transcriptional level are at work in order to preclude HIV reactivation in latent reservoirs. Affecting these mechanisms may open new ways to purge reservoirs. Sequestration of nuclear factor κB (NF-κB) in the cytoplasm of latent cells is one of these mechanisms.[103] T cell activation with tumour necrosis factor α (TNF-α) allows translocation to the nucleus of NF-κB, which then binds to the LTR and activates the early phase (Tat independent) virus transcription (Figure 1). Besides TNF-α, many other factors have been involved in HIV reactivation, including interleukins (IL) IL-1β, IL-2, IL-6, IL-7, interferon γ (IFN-γ) and CD154,[104–109] and could be used to purge the reservoirs. Among mechanisms acting at the post-transcriptional level, regulation of the exportation of viral RNAs by the poly track binding protein (PTB) seems to be important in memory CD4+ T cells.[110] Another important mechanism that acts at the post-transcriptional level involves microRNAs (miRNAs). These are single-stranded RNAs of 19–25 nucleotides involved in various biological processes in eukaryotic cells.[111,112] miRNAs interact with a complementary sequence in the 3′-untranscribed region (UTR) of target mRNAs by partial sequence matching, which leads either to mRNA degradation or, more often, to translational inhibition.[113] miRNAs are involved in the regulation of virus expression as well.[114] Recently it was shown that miRNAs regulate the expression of the histone acetyltransferase Tat cofactor PCAF and HIV replication.[115] In a recent paper, Huang et al. reported an enrichment of miRNAs in clusters, which has been observed only in resting CD4+ T cells and not in active CD4+ T cells.[116] They found that several of the miRNA clusters inhibited HIV replication, and suggested that miRNAs contribute to HIV latency in resting primary CD4+ T cells. They proposed to use specific antagomirs (anti-miRNA antisense) raised against these miRNA in order to reactivate latent CD4+ T cells.[114] However, as discussed by Sun and Rossi, the use of antagomirs to reactivate latently infected cells could be toxic for uninfected cells.[117] The feasibility of using miRNAs for HIV treatment is premature and will need far more investigation.

Implications for therapies based on purging reservoirs

Original strategies based on the combination of a purge of the reservoirs and intensifying HAART aim to eradicate the virus from infected patients. Understanding the molecular mechanisms involved in latency will allow us to devise new strategies that will facilitate the reactivation of all the reservoirs.

One strategy, known as 'Immune Activation Therapy', aims to activate T cells[118–121] (Figure 1). Many physiological stimuli that effectively activated T cells passed preclinical studies, but all failed in clinical studies.[122] IL-7 held promise since this cytokine is known to be essential for the maintenance of T cell homeostasis. Indeed, there are two subsets of memory T cells:[10] central memory T cells (Tcm), which are maintained through T cell survival and low-level driven proliferation and can persist for decades, and transitional memory T cells (Ttm), which persist, in contrast, by homeostatic proliferation of infected cells and could be reduced by using drugs preventing memory T cells from dividing. Interestingly, an IL-7-driven proliferation of Ttm cells can induce HIV expression from quiescent resting cells without the death of the infected cells. This cytokine might therefore be tested for its ability to reactivate expression of latent HIV in order to purge this quiescent HIV reservoir.[123–125] A clinical trial using IL-7 in order to reduce the size of the latent reservoir is currently running (ERAMUNE led by C. Katlama; http://www.clinicaltrials.gov). Another profound therapeutic implication, put forward by Chomont et al.,[10] is that the size of the pool of CD4− Tcm cells infected by HIV-1 should decrease with early treatment interventions.[126] Indeed, these memory Tcm cells (and the CD8+ T cells) are thought to be very important in the control of HIV infection, as shown in elite controllers.[127,128] Since IL-7 is also involved in CD8+ T cell function and T cell survival,[129–131] an early treatment that combines HAART and IL-7 will certainly help patients to control their HIV-1 infection (i.e. to get a functional cure), but might not allow the eradication of the virus (i.e. to get a sterilizing cure).

A second strategy aiming to develop rational therapeutics to flush out HIV from latency relies on the knowledge of its epigenetic regulation[114] (Figure 1). Several potential interesting candidates have emerged, such as the histone deacetylase (HDAC),[88] the histone methyltransferase,[85,88,133] DNA methyltransferases (DNMTs)[100,101] and proteins from the SWI/SNF chromatin complexes.[134,135] A switch from latent to active transcription has been described following treatment with several HDAC inhibitors such as trichostatin, trapoxin, valproic acid and sodium butyrate.[136–141] Valproic acid has been described to effectively reactivate latent HIV reservoirs in a first clinical trial,[142,143] but two other clinical trials did not confirm this.[144,145] Failure of this first clinical trial might be due to the ineffectiveness of valproic acid in inhibiting HDAC3 activity in CD4 T cells.[146] Indeed, several other HDACs, including HDAC3, contribute to the repression of HIV-1 LTR expression.[147–149] Further investigations are needed using inhibitors against newly identified epigenetic regulators of HIV latency such as chaetocine (a histone methyl transferase inhibitor) or the DNA

methyltransferase inhibitors, including well-characterized nucleotide analogue methylation inhibitors (5 azacytidine, 5-aza-2′ deoxycytidine, 5 fluoro-2′-deoxycytidine and zebularidine) and non-nucleoside DNA methylation inhibitors (procaine, procainamide, hydralazine and RG108).[78,150] Purging of latent reservoirs could also be achieved by inhibiting regulatory processes that prevent reactivation.[151] The p-TEFb activator HMBA is a promising molecule currently under study. In pilot studies it was able to reactivate latently infected cells and prevent re-infection by down-regulating CD4 receptor expression.[152]

There are several encouraging new directions in the purge of reservoirs that are based on a combination therapy approach,[153] as already used in clinical trials to treat cancer.[154–156] Such an approach has been found to be promising since the association of an HDAC inhibitor or a DNA methylation inhibitor with prostratin has a synergistic effect on the activation of HIV-1 expression.[101,157] The main benefit of this synergistic effect is that we might use drugs at suboptimal concentrations that would be sufficient to reactivate the virus but would have fewer side effects. We believe that the most promising strategy to purge the reservoirs relies on combinations of such drugs, which would be able to force viral gene expression at both the transcriptional and post-transcriptional levels.

Finally, an alternative option has been proposed, which is not based on virus reactivation, but on rendering the virus unable to replicate in latent cells without inducing cell death.[158] This original 'genome editing therapy' is based on the recognition of essential sequences within HIV-1, such as the *pol* gene by zinc finger endonuclease. Such a therapy has already been proposed to disrupt the CCR5 gene, as described previously.[159]

Improving HAART

Why is it important to improve HAART?

There are several reasons why HAART should be improved. One is the existence of a residual viraemia in patients undergoing HAART. The origin of this viraemia is still debated. There are two theories explaining this residual viraemia: (i) long-lived cells containing latent HIV provirus that can produce HIV at low levels following reactivation; and (ii) low-level cryptic on-going replication despite therapy. Latency is best described as a lack of proviral gene expression. In contrast, on-going replication requires continuous viral gene expression without cytopathic effects. Ineffective treatment in cells supporting on-going replication could result from poor drug penetration into sanctuaries such as the brain, where infected microglial cells are located,[160] or from cell-to-cell transfer of the virus.[161] It is important to distinguish between these two theories, since the therapeutic approaches they suggest are essentially different. The theory of on-going replication suggests that drug resistance to treatments might develop. In this case treatment intensification and the design of new anti-HIV-1 molecules are needed in the long term. On the other hand, if viruses are released in bursts from stable reservoirs, multidrug resistance does not develop, however, HAART alone is ineffective as well. Several studies have looked at the efficiency of such intensification of HAART on residual viraemia and only one failed to reduce it.[55,162] The second reason to improve HAART is related to the 'shock and kill' strategy discussed above. HAART by itself is not able to achieve a cure, but is still needed (to kill) in association with HIV reactivation from quiescent cells (to shock). Finally, emergence of drug resistances, toxicity and compliance with treatment are all obstacles to the current management of HIV-1 infection and therefore need improvement of HAART.[163]

How can we improve HAART?

Current management of HIV-1 treatment is based on seven classes of antiretrovirals: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry/fusion inhibitors (EIs), coreceptor inhibitors (CRIs) and integrase inhibitors (INIs).[164] The therapy of HIV-1-infected patients is based on a combination of three or more drugs from two or more classes.[165]

We believe that new drugs should target other steps of the HIV-1 cycle such as transcription, since there is no drug currently available targeting this step. An increasing number of studies suggest that inhibitors of cellular LTR-binding factors, such as NF-κB and Sp1, repress LTR-driven transcription.[166–170] Recently it has been shown that proteins of the DING family are good candidates to repress HIV-1 gene transcription.[171–173] Indeed, the inhibitory effect of the human DING protein named HPBP (human phosphate binding protein) on HIV-1 replication is very strong,[173] even compared with other canonical drugs currently used in HAART.[174] HPBP is also a potent anti-HIV-1 drug in peripheral blood lymphocytes and in primary macrophages, which is not true for several other anti-HIV-1 drugs. Very interestingly, HPBP, which targets transcription, is as effective against drug-resistant HIV strains as wild-type strains, highlighting the potential therapeutic advantage of HPBP. Moreover, such drugs could also be used to cope with chronic inflammation, which leads to non-AIDS events.[175] We believe that this protein or its derivatives are potentially interesting molecules and deserve further study. As suggested for X-DING-CD4,[172] proteins belonging to the DING protein family might have a role in the innate response to infections, including HIV-1.

Finally, the use of nanotechnology involving structures 1–100 nm in size is an exciting approach since it will make it possible to reduce toxicity and facilitate treatment adherence.[176] Indeed, these nano-delivery systems will permit: (i) modulation of drug release; (ii) protection of drugs from metabolism; and (iii) specific targeting of infected cells, even those located in sanctuaries. In corollary, this approach will allow improved bioavailability and therefore reduce toxicity.[177–179] Among new nanotechnology-based drug delivery systems are liposomes, polymeric micelles, dendrimers and nanosuspensions. Potential uses of these molecules have been reviewed.[176] This elegant approach will surely improve gene therapy, immunotherapy, vaccinology and microbicides.[180]

When to start antiretroviral therapy?

Today there is no real consensus on when HAART should be started. Until now, generally HAART was started when the CD4+ T cell count was below 350 cells/mm^3, however, several observations have pointed to a substantial benefit in reduced mortality if treatment is started at an earlier stage with no consideration of CD4+ T cell count.[181–182] This is in agreement with

the finding that starting treatment earlier reduced the size of the latently infected reservoirs, as discussed above. Another major concern with starting treatment earlier is that it should reduce the outcome of non-AIDS events and non-AIDS mortality.[183] The cost of the treatment, drug toxicities and non-adherence to the treatment by healthy patients has led some regulatory organizations in Europe not to recommend initiation of HAART in asymptomatic patients or patients having more than 350 CD4+ T cells/mm^3.

Conclusions

Are there reasons to be optimistic that a cure for HIV infection may be achieved? From our point of view the answer is 'yes', but this will not be achieved in the short term. Advances in some fields are very exciting and offer new opportunities to achieve a cure. For example, using gene therapy to confer HIV resistance (including the CCR5 gene therapy) is a valuable approach compared with chemotherapy, which has several drawbacks, including toxicities, development of resistance and cost. Several gene therapy trials are currently under way,[58] but it is premature to make definitive conclusions regarding the feasibility of these therapies. The 'holy grail' for clinicians will be to achieve a sterilizing cure with total eradication of the virus from the body, but we might only get a functional cure, with few patients who control HIV-1 infection (the elite controllers). The major concern with a functional cure will be to drastically reduce the viraemia in order to prevent non-AIDS events. The 'shock and kill' strategy has also emerged as an exciting potential way to eliminate the virus. Here, too, we might be able to achieve only a functional cure. The German case is the only case where a possible sterilizing cure has occurred, incidentally indicating a weakness of HIV. Today, however, we are limited by a lack of technology to clearly demonstrate that this patient is definitively cured. The war against this virus is far from over and will need much more work. This review has focused on current therapeutic strategies that could lead in the long term to a cure. From a military point of view, this latter strategy constitutes the first front line. However, to win a war you usually need to open a second front line, and this one is research leading to the development of an HIV vaccine. Even if in practice this approach is not yet working, efforts in this direction must be made, but might require new avenues in HIV immunology research.[36,184–186] Undoubtedly research aiming at a therapeutic cure will benefit from research aiming to develop a vaccine, and vice versa. Reasons to be optimistic come mainly from the intensive efforts made in different fields of research, i.e. a multidisciplinary approach, including immunologists, virologists, molecular biologists, clinicians, pharmacologists, chemists, physicists and mathematicians, who have already opened new ways and elaborated new concepts for therapies that are currently being tested in clinical trials.

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None to declare.

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Valentin Le Douce

Importance des facteurs cellulaires LSD1 HIC1 dans la restriction de l'expression du VIH-1 dans les cellules microgliales

Importance des facteurs cellulaires LSD1 et HIC1 dans la restriction de l'expression du VIH-1 dans les cellules microgliales.

Les multi-thérapies permettent de maintenir l'infection au VIH-1 sous contrôle, mais n'entraînent pas l'éradication du virus du fait de l'existence de réservoirs cellulaires, où le virus est intégré de façon latente. La compréhension des mécanismes impliqués dans l'extinction de la transcription virale, permettrait de contourner cet obstacle. Les cellules microgliales, cibles privilégiées du VIH-1 dans le cerveau, sont les macrophages résidents du système nerveux central et ont été décrites comme un réservoir cellulaire avec une longue durée de vie. Notre équipe à montré le rôle du facteur transcriptionnel CTIP2 dans l'établissement et le maintien de la latence dans ces cellules. Dans ces travaux, je mets en évidence le rôle répresseur des protéines LSD1 et HIC1 sur la transcription virale dans les microglies. LSD1 coopère avec CTIP2 pour promouvoir l'établissement de marques épigénétiques au niveau du promoteur viral pour induire la mise en place d'hétérochromatine, tandis que le suppresseur de tumeur HIC1, préalablement modifié post-traductionnellement par la déacétylase SIRT1, contrecarre l'activité de TAT afin d'empêcher la réactivation virale.

Mots clés : VIH-1, réservoirs, latence, microglies, CTIP2, LSD1, HIC1, SIRT1, TAT, chromatine.

Importance of cellular factors LSD1 and HIC1 on HIV-1 restriction expression in microglial cells.

Even though multitherapies maintain HIV infection under control, they unfortunately do not achieve viral eradication due to the existence of latently infected cell reservoirs. Microglial cells are resident macrophages and the main HIV-1 target in brain. They have been described as a long-lived HIV-1 cell reservoir, and so appear as a one of the main obstacle to viral clearance. Thus, understanding of the mechanisms implicated in the establishment and maintaining of viral latency in these cells is a critical step on the way towards an HIV cure. Our team already demonstrated the implication of the cellular transcription factor CTIP2 in the establishment of HIV-1 silencing. In this work, I present LSD1 and HIC1 as new HIV-1 transcriptionnal expression inhibitors in microglial cells. LSD1 cooperates with CTIP2 to promote the establishment of epigenetic marks associated to heterochromatin structure at the viral promoter, while the tumor suppressor HIC1 disrupts the TAT-mediated transactivation cycle. HIC1 is beforehand modified post-translationnaly by the deacetylase SIRT1 and then counteracts TAT activity in order to limit viral transcription reactivation.

Key words: HIV-1, reservoirs, latency, microgliale cells, CTIP2, LSD1, HIC1, SIRT1, TAT, chromatin.