Silencing of HIV-1 co-factors
Eekels, J.J.M.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Silencing of HIV-1 co-factors

Julia JM Eekels

UITNODIGING

voor het bijwonen van de openbare verdediging van het proefschrift van Julia JM Eekels

Silencing of HIV-1 co-factors

Op donderdag
24 november 2011
om 12.00

in de Agnietenkapel van de Universiteit van Amsterdam
Oudezijds Voorburgwal 231
1012 EZ Amsterdam

Receptie na afloop

Julia JM Eekels
Julianaplantsoen 301
1111XP Diemen
Julia.eekels@gmail.com

Paraminlen:
Marti Eekels
marteekels@hotmail.com

Paula Eekels
paulaeekels@hotmail.com
SILENCING OF HIV-1 CO-FACTORS

JULIA JOSEPHINA MARTINA EKELES
The research described in this thesis was performed at the Laboratory of Experimental Virology, Department of Medical Microbiology, Center of Infection and Immunity (CINIMA), Academic Medical Center, University of Amsterdam, The Netherlands. The research was financially supported by the Dutch AIDS Fund.

ISBN: 978-90-5335-467-4

The printing of this thesis was financially supported by:
Boehringer Ingelheim B.V.
Gilead Sciences Netherlands B.V.
Abbott B.V.
Clean Air Techniek B.V.
Nutricia Advanced Medical Nutrition, Danone Research, Centre for Specialised Nutrition University of Amsterdam

Cover photo: Arnaud Frich // www.arnaudfrichphoto.com
Printing: Ridderprint, Ridderkerk aan de IJssel

Copyright © 2011 by Julia Eekels. All rights reserved. No part of this publication may be reproduced, stored or transmitted in any way without prior permission from the author.
SILENCING OF HIV-1 CO-FACTORS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college van promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
op donderdag 24 november 2011, te 12:00 uur

door

Julia Josephina Martina Eekels
geboren te ’s-Hertogenbosch
Promotiecommissie:

Promotor: Prof. dr. B. Berkhout

Overige leden: Prof. dr. R.C. Hoeben
              Prof. dr. M.D. de Jong
              Prof. dr. J.M.A. Lange
              Prof. dr. H. Schuitemaker
              Prof. dr. R. Versteeg
              Dr. B. Verhasselt

Faculteit der Geneeskunde
## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General introduction</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Towards a durable treatment of HIV-1 infection using RNA interference</td>
<td>17</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Long-term inhibition of HIV-1 replication with RNA interference against cellular co-factors</td>
<td>31</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>A competitive cell growth assay for the detection of subtle effects of gene transduction on cell proliferation</td>
<td>47</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors</td>
<td>63</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>The Cyclophilins A and B have opposing roles in HIV-1 replication</td>
<td>81</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>The RNA helicase DDX3 is involved in trans-activation of the HIV-1 Long Terminal Repeat promoter</td>
<td>101</td>
</tr>
<tr>
<td>Chapter 8</td>
<td>General discussion</td>
<td>115</td>
</tr>
</tbody>
</table>

| Addendum | Samenvatting  
Summary  
Over de auteur  
Dankwoord | 123  |

| References | | 135 |
It was said that, since vast amounts of magic can seriously distort the mundane world, the Library did not obey the normal rules of space and time. It was said that it went on forever. It was said that you could wander for days among the distant shelves, that there were lost tribes of research students somewhere, that strange things lurked in forgotten alcoves and were preyed on by other things that were even stranger.*

* All this was untrue. The truth is that even big collections of ordinary books distort space, as can readily be proved by anyone who has been around a really old-fashioned secondhand bookshop, one of those that look as though they were designed by M. Escher on a bad day and has more staircases than storeys and those rows of shelves which end in little doors that are surely too small for a full-sized human to enter. The relevant equation is: Knowledge = power = energy = matter = mass; a good bookshop is just a genteel Black Hole that knows how to read."

Terry Pratchett – Guards! Guards!

"De bibliotheek was nog groter dan de vorige keer. Otto kreeg er bij het licht van de ene kaars maar stukjes en beetjes van te zien. Daardoor leken alle hoeken, gaten en nissen, alle muren en het plafond zich eindeloos voort te zetten, verborgen door de duisternis. De boekenkasten waar hij tussendoor liep leunden naar hem toe. Boven hem, in het donker, bogen ze misschien wel over hem heen. Er waren hier niet duizenden of honderdduizenden, maar vast miljoenen boeken, ontelbaar, in rijen en stapels, op de ene plank na de andere, naast elkaar, boven elkaar."

Tonke Dragt – Aan de andere kant van de deur
Leopold Amsterdam, 1992

"For almost half an hour, I wandered within the winding labyrinth, breathing in the smell of old paper and dust. I let my hand brush across the avenues of exposed spines, musing over what my choice would be. Among the titles faded by age, I could make out words in familiar languages and others I couldn't identify. I roamed through galleries filled with hundreds, thousands of volumes. After a while it occurred to me that between the covers of each of those books lay a boundless universe waiting to be discovered, while beyond those walls, in the outside world, people allowed life to pass by in afternoons of football and radio soaps, content to do little more than gaze at their navels."

Carlos Ruiz Zafon – The shadow of the wind (translated from: La Sombra del Viento)

"De bibliotheek was zijn trots. Jaar na jaar had hij de collectie langs de wanden aangevuld, net zolang tot de stroom te groot werd en de nieuwe aanwinsten over tafels een kleine verrijdbare kastjes moesten worden verdeeld. De enorme ruimte, acht meter breed, bijna 20 meter lang, bestond uit louter papier en hout. Aan weerszijden van de schouw, midden in de lange wand, was geen vierkante centimeter muur zichtbaar: van vloer tot plafond boeken. De korte muur die aan de hal grensde was bedekt met kasten die tot aan het plafond reikten en doorliepen boven de deur, die in de hoog oprijzende klif van leren en stoffen en papieren ruggen niet meer dan een donkere was, een tunnel naar een andere wereld."

Marcel Möring – In Babylon
Meulenhoff Amsterdam, 1997
Chapter 1
General introduction
HIV-1
The first cases of the Acquired Immuno Deficiency Syndrome (AIDS) were reported in 1981, and two years later the causative agent was isolated (30;125). The virus appeared to be a member of the lentiviridae, a subfamily of the Retroviridae. Most viruses from this family are known to cause chronic diseases of the central nervous system and the immune system. The virus was eventually named Human Immunodeficiency Virus type 1 or HIV-1. It is estimated that globally 33.3 million people were living with HIV-1 at the end of 2009 and 2.6 million people became newly infected that year. Most of the people infected with HIV-1 live in Sub-Saharan Africa and Southeast Asia (www.unaids.org).

HIV-1 molecular biology
The HIV-1 genome (Figure 1.1) is 9.8 kb in length and encodes nine viral genes; gag, pol, env, tat, rev, nef, vpu, vpr, and vif (284;365). The gag, pol and env genes are typically found in all retroviruses. The gag gene encodes four structural proteins; the p24 capsid (CA), the p17 matrix (MA), the p7 nucleocapsid (NC) and the p6 protein. Pol encodes for a polyprotein that is post-translationally cleaved into three enzymes; reverse transcriptase (RT), integrase (IN) and protease (PR), all three indispensable for the viral replication cycle. The env gene encodes a protein that is cleaved into two subunits; the glycoprotein 120 and glycoprotein 41, both subunits of the trimeric Envelope protein and important for recognition of receptors on the host cell. The tat and rev genes encode for the regulatory proteins Tat and Rev, which are essential for viral replication; Tat activates viral transcription and Rev is necessary for nuclear export of viral RNAs out of the nucleus (256;292). The HIV-1 genome encodes four accessory proteins: Nef, Vpu, Vpr and Vif. These proteins are not essential for viral replication in many in vitro systems, but are needed for viral replication, viral spread and pathogenesis in vivo (16;108). Nef has been implicated in several biological processes; e.g. down regulation of CD4 and Major Histocompatibility Complex molecules, enhancement of virion infectivity and stimulation of viral replication in primary T cells (119). Vpu contributes to CD4 downregulation by mediating degradation of newly synthesized CD4 molecules in the endoplasmatic reticulum. Vpu also enhances virus particle release by antagonizing the host restriction factor tetherin (99). Vpr interacts with many cellular proteins and has been implicated in several different functions, such as improving the fidelity of reverse transcription, suppression of immune activation and induction of G2 arrest and apoptosis (291). Vif protects HIV-1 from the host restriction factor APOBEC (134).

The protein-coding regions in the genome are flanked by non-coding domains; the Long-Terminal Repeats (LTRs), which are subdivided into the U3 (Unique 3’), R (Repeat) and U5 (Unique 5’) domains (127). These DNA domains encode the viral promoter and several RNA signals that are important for viral replication. For instance, the TAR (trans-activation response) RNA hairpin binds the Tat protein and is essential for Tat-mediated activation of transcription. Several transcription factors bind to the LTR, such as Sp1, NF-κB, NF-AT and USF (271;281).
HIV-1 replication cycle

Replication of HIV-1 starts when the virus encounters a host cell expressing the CD4 receptor and the CXCR4 or CCR5 co-receptor (Figure 1.2). These receptors are present on certain immune cells, especially CD4+ T lymphocytes, macrophages and dendritic cells. The Envelope protein on the outside of the virus particle binds to the CD4 receptor on the target cell, which leads to conformational changes in the Envelope protein. The second step of the virus entry process is binding to the co-receptor, in primary infections the CCR5 chemokine receptor, and subsequent fusion of the viral membrane with the target cell membrane. The contents of the virion are released into the host cell. The viral RNA genome is reverse transcribed into DNA by the viral Reverse Transcriptase and the capsid is partially disassembled. The capsid, with the viral genome is transported to the nucleus and the viral genomic DNA is subsequently integrated into the host genome by Integrase. The integrated provirus acts as a template for the production of spliced viral mRNAs and full-length RNA genomes. Viral mRNAs are translocated to the cytoplasm and translated into viral proteins. The viral proteins are, together with two copies of progeny RNA genomes, assembled in new virions at the plasma membrane, and released from the cell by budding. After maturation, the new virions are able to infect new host cells. The viral replication steps from entry to integration are referred to as “early” replication steps, while the stages from transcription to budding of new virions are referred to as “late” replication steps (121).
Treatment of HIV-1 infection and AIDS

AIDS has become a chronic disease in the Western world due to the introduction of combined anti-retroviral therapy (cART), although people living with HIV-1 still have a lower life expectancy than uninfected individuals (414). In the early days of the disease, only a single class of drugs was available and drug resistance emerged quickly due to the error-prone Reverse Transcriptase (195). cART uses a combination of drugs and was introduced in 1996, which led to the first decrease in annual AIDS deaths in the USA since the beginning of the epidemic. cART is advised to patients with a CD4 count below 350 cells/mm3, while other drugs (e.g. antibiotics) can be added to the regimen after the onset of AIDS to treat opportunistic diseases. Nowadays the following antiviral drug
classes are available: NRTIs and NNRTIs (nucleoside and non-nucleoside Reverse Transcriptase inhibitors), Protease inhibitors (PI), Integrase inhibitors and entry inhibitors (CCR5 antagonist and fusion inhibitors) (see also Figure 1.2). The most common cART drug combination consists of two NRTIs with a single PI or NNRTI. However, several drawbacks of cART have been recognized. The drugs have been associated with side-effects, from mild ones as nausea and fatigue to severe side-effects such as organ failure (10). Patients have to live with a daily drug regimen and sub-optimal drug levels in patients that do not strictly adhere to the medication can lead to the emergence of drug-resistant viral strains (315). The life-long medication is also expensive. Perhaps most importantly, none of the drugs is able to eradicate the virus from the body. As the development of a vaccine is still unsuccessful, despite intense efforts, there continues to be a real need for development of new therapeutics.

**RNA interference and its use against HIV-1**

RNA interference or RNAi is an evolutionary conserved mechanism in eukaryotes that leads to sequence-specific knockdown of gene expression (116). The hallmark molecule of this pathway is double stranded RNA (dsRNA), which can originate from different sources, such as the dsRNA intermediate in the replication of viruses, or dsRNA encoded by the genome as microRNAs (miRNAs). MiRNAs are important in cell differentiation and development and regulate gene expression by either translational repression of specific mRNAs or mRNA cleavage. For more information on the natural RNAi pathway, see Chapter 2.

![RNAi pathway diagram](image-url)

**Figure 1.3. RNAi pathway**

ShRNAs can be produced from plasmid DNA or vectors in the nucleus and are translocated into the cytoplasm via Exportin-5. Dicer cleaves the shRNA into a siRNA. Alternatively, siRNAs can be transfected into the cells. The siRNA
is loaded into the RISC complex. When the loaded RISC encounters an mRNA with sequence complementarity to the siRNA, the mRNA is cleaved and degraded, leading to gene silencing.

The natural RNAi pathway can be instructed by man-made dsRNA molecules designed to target the mRNA of interest. This can be done by transfecting synthetic small interfering (siRNAs) into cells (105), or by intracellular expression of short hairpin RNAs (shRNAs) or artificial miRNAs from DNA constructs (60;399). These artificial RNAi inducers are loaded into the RNAi pathway and can be used for therapeutic downregulation of a target gene (Figure 1.3). There are certain risks associated with the use of RNAi. Overexpression of siRNAs, either by transfection or by expression from a vector, may lead to saturation of the cellular RNAi pathway, which can lead to cell death, disturbance in cell development and even cancer (140). Another risk is the so-called “off-target” effect. As a miRNA only need 7 or 8 base pairs of sequence complementarity, there is always the possibility that not only the intended mRNA is targeted, but unrelated mRNAs as well (306). When shRNAs or siRNAs are suboptimally designed, they can also induce immune responses (58;324). However, all these problems can be circumvented or checked for, which makes the use of RNAi an appealing therapeutic possibility in the treatment of a wide variety of diseases (20;46;267;314).

In HIV-1 therapy, the RNAi treatment should be delivered cells that are susceptible to virus infection, being CD4+ T lymphocytes, macrophages and dendritic cells. Transient delivery of siRNAs has been tried and proven successful in inhibition of HIV-1 replication in a mouse model; but this approach will not be suitable for patients (188). The ultimate goal is to treat HIV-1 susceptible cells to allow the constitutive expression of antiviral shRNAs. This can be achieved by transduction of these cells with a lentiviral vector. Lentiviral vectors are derived from HIV-1 and can infect cells to integrate the therapeutic RNAi cargo into the cellular genome, but are unable to replicate (100). Lentiviral vectors can transduce many cell types, both dividing and quiescent, making these vectors ideal candidates for a gene therapy against HIV-1 (254). The target cells could be hematopoietic stem cells (HSC), which give rise to several lineages of immune cells, including HIV-1 susceptible cells such as CD4+ T lymphocytes. As HSC are long-living cells that continue to generate new immune cells, in theory a single gene therapy treatment should suffice for a life-long generation of protected cells.

In principle one would like to target early replication steps of HIV-1, and prevent proviral integration into the host genome. However, it appears that the incoming viral RNA genome is not a target for RNAi, as it is highly structured, coated with viral and cellular proteins and hidden in the viral capsid and thus inaccessible for the RNAi machinery (381). Late in infection, the newly made HIV-1 mRNAs will be targets for RNAi attack, and several shRNAs designed against conserved viral sequences were tested in vitro, of which several inhibited HIV-1 production and replication substantially. Stable knockdown cell lines expressing individual shRNAs were generated and infected with HIV-1. No replication of HIV-1 was detected for several weeks or even months, but HIV-1 eventually started replicating in these cultures (83). When the replicating virus was analyzed and sequenced, mutations in or close to the siRNA-target sequence were found. Mutations in the mRNA target abolish the perfect sequence complementarity needed for shRNA-mediated gene knockdown (83). A mutation found close to the siRNA-target site did not change the sequence complementarity, but was found to alter the secondary structure of the HIV-1
mRNA, making the target less accessible for the RNAi machinery (380). Both types of mutations made HIV-1 resistant against RNAi attack. Thus, although RNAi against HIV-1 mRNAs can be very effective, solutions are needed to prevent the development of resistant virus variants. Similar to current cART therapy more than one region in the HIV-1 genome can be targeted by the use of multiple shRNA inhibitors, and this was shown to be effective and no escape viruses could be selected (364). An alternative method to prevent viral escape would be to target the cellular proteins that assist HIV-1 to complete its replication cycle. More in-depth information of RNAi strategies against viral and cellular targets is provided in Chapter 2.

HIV-1 encodes only 15 proteins and needs many cellular proteins as co-factors to complete its replication cycle. RNAi has played a major role in the identification of these cellular co-factors. Three genome-wide RNAi screens have revealed many candidates, however, the overlap between these three studies was small and only three co-factors were identified in all three studies (53;62;181;406). This shows the importance for confirmation of candidate cellular co-factors by additional experimentation. The overlap in the pathways involved was more significant. Targeting of cellular co-factors has several advantages; it is possible to prevent proviral integration by targeting co-factors necessary for the early replication steps, e.g. viral entry. In theory one would expect that the chance of viral escape is much reduced as HIV-1 has evolved to very specific cellular co-factors, which makes it less likely that it can switch to the use of another related cellular co-factor. The main is that targeting of cellular co-factors could have adverse effects on cell growth and/or function, thus leading to side effects.

**Scope of this thesis**

In Chapter 2 a detailed overview is presented on how and why a gene therapy against HIV-1 can serve as a new therapeutic option. The focus is on the rationale of using RNAi and whether to target the virus or cellular co-factors and ongoing clinical research is reviewed. The main body of this thesis concerns the use of RNAi against cellular co-factors as antiviral approach that may prevent the selection of RNAi-resistant escape viruses. Stable knockdown cells with shRNAs against 30 different cellular co-factors were generated and challenged with HIV-1 in Chapter 3. In three cases we could inhibit HIV-1 replication up to two months and no viral escape was apparent.

Adverse effects on cell physiology due to RNAi attack on cellular co-factors are a major concern. To accurately measure even small effects on cell growth, either due to specific knockdown of the target or unknown off-target effects, we developed a new and sensitive cell proliferation assay in Chapter 4. In this competitive cell growth or CCG assay a mixture of transduced GFP-positive cells and untransduced GFP-negative cells is cultured over time. If transduced cells have a lower proliferation rate, this can be easily scored by FACS analysis as a gradual decrease in the percentage of GFP-positive cells. We show that the CCG assay is more sensitive than other well-established cell proliferation assays.

In Chapter 3 we obtained long-term inhibition of HIV-1 replication and one of the top candidates from this screen was an autophagy factor. Autophagy is a cellular pathway and its basic function is to recycle cellular components. Autophagy has also been implicated in HIV-1 replication and therefore we tested RNAi-mediated knockdown of additional autophagy factors. In Chapter 5 knockdown cells expressing shRNAs against different autophagy factors were generated and HIV-1 replication was inhibited in several of these.
When simultaneously knocking down two autophagy factors, we obtained increased virus inhibition without enhanced cytotoxicity compared to the individual knockdown cells. In Chapter 6 we study Cyclophilin A and B, cellular proteins that both have isomerase function. Cyclophilin A is known to support HIV-1 replication, but we have identified Cyclophilin B as a HIV-1 restriction factor. Thus, proteins with a similar enzymatic function can act as cellular co-factor or as restriction factor.

The DDX3 helicase supports HIV-1 replication through interaction with Rev and facilitating the nuclear export of viral transcripts. In Chapter 7 we demonstrate that DDX3 is also involved in Tat-mediated trans-activation of the HIV-1 LTR promoter, making DDX3 an interesting therapeutic candidate with a dual co-factor function. By targeting a single cellular co-factor, one could block two steps in the HIV-1 replication cycle.

The general discussion in Chapter 8 provides a short review of RNAi against cellular co-factors in the context of other pathogens, such as influenza A virus and hepatitis C virus and the intracellular bacterium *Mycobacterium tuberculosis*. 
Chapter 2
Towards a durable treatment of HIV-1 infection using RNA interference

Julia J.M. Eekels and Ben Berkhout

Published in:
Progress of Molecular Biology and Translational Science (2011).
Volume 102, Chapter 8, 141-163
Elsevier publishers
Editor: dr. Dirk Grimm

Laboratory of Experimental Virology,
Department of Medical Microbiology,
Center for Infection and Immunity Amsterdam (CINIMA),
Academic Medical Center of the University of Amsterdam
Abstract

RNA interference (RNAi) is a cellular mechanism that mediates sequence-specific gene silencing at the post-transcriptional level. RNAi can be used as an antiviral approach against human pathogens. An attractive target for RNAi therapeutics is the human immunodeficiency virus type 1 (HIV-1), and the first clinical trial using a lentiviral gene therapy was initiated in early 2008. In this chapter we will focus on some basic principles of such an RNAi-based gene therapy against HIV-1. This includes the subjects of target site selection within the viral RNA genome, the phenomenon of viral escape and therapeutic strategies to prevent viral escape. The latter anti-escape strategies include diverse combinatorial RNAi approaches that are all directed against the HIV-1 RNA genome. As an alternative strategy, we will also discuss the possibilities and restrictions of targeting cellular co-factors that are essential for virus replication, yet less important for cell physiology.

RNAi: from natural pathway to therapeutic method

The development of RNAi-based therapies against a wide variety of diseases, including cancer, neurological, autoimmune and infectious diseases was triggered by the discovery of the RNAi mechanism and RNAi-mediated gene silencing in mammalian cells (28;85;94;171;242;337;348). RNAi also holds promise for antiviral therapy against pathogenic viruses such as HIV-1. However, in order to properly evaluate RNAi therapeutic strategies - including the risks involved - it is important to describe in some detail the ins and outs of the cellular RNAi mechanism. We will first describe the natural microRNA (miRNA) pathway.

It is estimated that human cells express more than 500 miRNAs (http://microrna.sanger.ac.uk). These miRNAs are important in the process of cell differentiation and development by regulating gene expression at the post-transcriptional level (8;9;26;31;63;244). The natural miRNA pathway uses RNA polymerase II or III to produce a primary transcript or pri-miRNA that encodes one or multiple miRNAs, the latter in case of a polycistronic transcript (201). The pri-miRNA is processed by the microprocessor complex into a pre-miRNA with 5'-monophosphate and 3'-hydroxyl 2-nucleotide (nt) overhang (39). This enzyme complex contains the RNase III-like enzyme Drosha and the dsRNA-binding protein DGCR8/Pasha (91;138;147;193;200). Some miRNAs that are encoded within introns (mirtrons) are processed via a distinct route that uses the splicing machinery (68;295). Pre-miRNA processing occurs in the nucleus and the product is subsequently exported by Exportin-5 (Exp-5) to the cytoplasm (43;228;391). The cytoplasmic RNase III-like endonuclease Dicer cleaves the base-paired stem approximately 22-base pairs (bp) away from its base, generating a 2-nt overhang at the 3'-end (400). Dicer is associated with the TAR RNA binding protein (TRBP), which is required to recruit Argonaute 2 (Ago2) (73). The Ago2-RNA complex forms the minimal core of the RNA-induced silencing complex (RISC) (137;231). RISC unwinds the miRNA and keeps one RNA strand (guide strand) in the complex, while the other (passenger strand) is cleaved and subsequently degraded (146). One of the two RNA strand gets preferentially incorporated into the complex to execute the subsequent mRNA silencing step (352;353). RNAi-mediated gene silencing is thought to be elicited by translational repression of the
targeted mRNA in mammals (9). However, a recent study suggested mRNA cleavage as the favorite mode of miRNA action (141). An important determinant of RNAi action is the level of base pair complementarity between the miRNA and the targeted mRNA, leading either to mRNA cleavage with a perfect complementarity or translational repression with a near-perfect complementarity (56;95;175;192;205). The “seed” region of the miRNA (5’ terminal nucleotides 2-8) typically finds multiple target sequences in the 3’ untranslated region (3’UTR) of the mRNA. The silencing efficiency is determined by the overall arrangement of these 3’UTR targets, including the number and the inter-motif distance (303). Although most mammalian miRNAs base pair with imperfect complementarity to the mRNA to cause translational repression, at least one human case of perfect complementarity and subsequent mRNA cleavage has been reported (389). Degradation of the targeted mRNA is initiated by endonucleolytic cleavage in the 3’UTR opposite nucleotide position 10 to 11 of the miRNA.

In contrast to the action of natural miRNAs, man-made small interfering RNAs (siRNAs) that are designed to have full base pair complementarity can direct mRNA cleavage with only a single target sequence, and this site can be positioned anywhere within the mRNA molecule. Such designer double-stranded (ds) RNA molecules can be produced or synthesized by several methods. Synthetic siRNAs can be transfected directly into the cytoplasm of cells, where the dsRNA will be picked up by RISC (105). Short hairpin RNA (shRNA) transcripts (60;266) and man-made miRNA-mimics can be expressed intracellularly from a transgene construct (399). The transcripts are expressed in the nucleus and should thus be processed and transported to the cytoplasm in order to meet RISC and to induce RNAi action. The natural miRNA processing and transport pathway can be instructed with these man-made inhibitors for downregulation of a specific mRNA. This therapeutic possibility is relevant for diseases caused by overexpression of a specific mRNA. Alternatively, RNAi action can be specifically induced to target the RNA genome of invading microbes such as HIV-1 and other pathogenic viruses. Several of these types of inhibitors have been employed against HIV-1 (29;219;221;301;307).

Instruction of the cellular miRNA pathway with new siRNA specificity is associated with certain risks. A general problem is that the artificial siRNA molecules can compete with the endogenous siRNAs, and siRNA overexpression may lead to saturation of the miRNA pathway. This can have unwanted side effects because the miRNA pathway is important in the control of cellular gene expression. One could expect a disturbance in the cellular differentiation program, possibly cell death or even cancer (262). Saturation of the miRNA pathway was reported to cause the death of mice when high doses of shRNAs were delivered by an Adeno-associated virus (AAV) vector (45;65;140;241;348;361). Thus, exogenous RNAi inducers should be expressed at a balanced level. Another potential problem that is harder to avoid is the targeting of other, unintended mRNAs. Such off-target effects can occur because the miRNAs require only a seed sequence complementarity of 7 to 8 base pairs within the 3’UTR of a given mRNA (57). Off-target effects are difficult to predict and should be screened for in appropriate experimental models. Note that off-targeting can not only be elicited by the siRNA guide strand, but also by the passenger strand (114;160;161). Another problem relates to the induction of an immune response by siRNAs and shRNAs (58;324). This effect can be minimized and possibly avoided by optimal design of the si/shRNA molecule (234).
Antiviral RNAi strategies: towards a gene therapy for HIV-1

HIV-1 causes a chronic infection that ultimately leads to AIDS and death. Disease progression can be halted effectively with antiviral drugs, and in particular a combinatorial approach can avoid the evolution of drug-resistant HIV-1 variants. Problems associated with such drug regimens include serious toxicity during long-term follow-up. In the absence of any breakthrough at the anti-HIV vaccine front, one should think about the design of more durable therapeutic measures. The goal of an RNAi-based gene therapy approach against HIV-1 is to durably protect the cells of the immune system that are susceptible to HIV-1 infection. This includes the CD4+ T cells, monocytes, macrophages and dendritic cells. Such “intracellular immunization” will prevent the depletion of these immune cells during chronic HIV-1 infection. Maintenance of the immune function should prevent opportunistic infections and disease progression towards AIDS.

HIV-1 causes a persistent infection and no spontaneous viral clearance has been reported. Thus, a continuously active treatment regime is required. Repeated delivery of exogenous siRNAs as anti-HIV therapy has been described in a mouse model with a humanized immune system (188). Effective virus inhibition was observed with a concomitant prevention of the loss of human CD4+ T cells. The systemic delivery of siRNAs was recently reported in a human phase I clinical trial via targeted nanoparticles to patients with solid cancers (87). Tumour biopsies from melanoma patients obtained after treatment show the presence of intracellular nanoparticles and a reduction was found in both the targeted messenger RNA and the protein levels when compared to pre-dosing tissue.

We however seriously doubt whether such an siRNA approach would be suitable in the setting of an HIV-infected patient, where the prevention of viral escape requires the continuous presence of an effective dose of multiple siRNAs in all human cell types that can be infected, which are in fact located in many different tissues and body compartments. Instead, we would like to advocate the gene therapy concept with continuous expression of anti-HIV molecules after a single transduction of HIV-susceptible cells with an appropriate delivery vector. We think that the lentiviral vector system is particularly suited for this anti-HIV therapeutic action. The lentiviral vector is based on the genome of HIV-1 itself. The pathogenic genes were replaced by novel control and therapeutic sequences. The lentiviral vector can efficiently infect most target cell types and deposit the transgene payload in a stable manner, but it cannot replicate. A benefit of the lentiviral vector compared to other viral delivery systems is that dividing and non-dividing cell types can be transduced efficiently. Furthermore, the lentiviral vector stably integrates in the host cell genome at a random position, thus yielding permanent transduction (36;254).

Some specific problems can be encountered when using the HIV-based lentiviral vector system to target the HIV-1 RNA genome with RNAi reagents. This includes self-targeting of the vector RNA in the producer cell by the antiviral shRNAs. One can of course avoid the use of shRNAs that target HIV-1 sequences that are present in the vector genome. These problems and solutions have previously been discussed in detail by ter Brake et al (344). The introduction of miRNA cassettes also raises specific problems that can be countered by appropriate vector design (220). Alternative viral vector systems are available for delivery of a therapeutic RNAi transgene and these systems have been extensively discussed by others (88;258).
We depicted a possible gene therapy procedure for HIV-infected individuals in Figure 2.1. Hematopoietic stem cells seed the different lineages of immune cells in the blood and organs and are therefore interesting target cells for an ex vivo gene therapy, followed by autologous transplantation of these CD34+ cells back into the patient. The hematopoietic stem cells equipped with the lentiviral vector will durably supply all derived immune cells with the antiviral arsenal. In the presence of HIV-1, one expects the preferential survival of these shRNA-expressing immune cells over untreated cells because the latter are likely to become infected and will subsequently be removed by the immune system. This survival benefit should result in a gradual increase in the percentage of protected cells. It remains unclear what percentage of cells can be transduced, but the treatment should result in partial reconstitution of the immune system. When reconstitution reaches a certain level, one should be able to block HIV-1 infection from progressing towards AIDS. Ideally, a single gene therapy treatment should achieve a durable effect because the transduced stem cells will continue to generate HIV-protected immune cells in the diverse lineages. Hematopoietic stem cells transduced with a retroviral vector encoding an anti-HIV-1 ribozyme have already been evaluated in clinical trials (7;245). These trials demonstrate the feasibility and safety of the proposed stem cell approach, although little therapeutic effect was scored for the ribozyme antiviral. A recent study demonstrated the safety of the lentiviral vector in combination with ex vivo targeting of CD34+ cells (64). Another option is the treatment of the mature CD4+ T cell population, which represents the major target cell population for HIV-1. In this scenario the gene therapy should be applied repetitively because T cells have only a limited life span (98). Potent and sequence-specific HIV-1 inhibition has been reported with RNAi-inducing reagents in cell culture infections, but it soon became apparent that HIV-1 is prone to viral escape when a single shRNA inhibitor is applied (41;83;260;302;345;357;380). The ease of HIV-1 escape mimics what happens in patients treated with a single antiretroviral drug, but we know that a combinatorial drug regimen can prevent viral escape and therapy failure. Thus, the therapeutic RNAi vector to be developed for clinical testing should tackle the virus with multiple shRNA inhibitors at the same time. Such a combinatorial RNAi attack can target the virus at multiple genome positions (345), but one can also add an attack against host-encoded co-factors (139;218). One could also combine RNAi molecules with other RNA effector molecules such as decoys and ribozymes (93;209). Different RNA-based inhibitors can also be combined in a single transcript such as the conjugate of an antiviral aptamer that binds the HIV-1 Envelope protein and an antiviral siRNA (408). The aptamer not only blocks the Envelope protein on virion particles, but it also selectively ferries the siRNA to HIV-infected cells that express the Envelope protein on their surface. This conjugate demonstrated good antiviral activity in the pre-clinical model of the humanized mouse, although uncertainty remains about the efficiency of the intracellular delivery of the siRNA (255). Another elegant solution to avoid viral escape is the use of the second generation shRNAs that specifically target viral escape variants (343). The relatively high number of viral escape routes available to HIV-1 may limit the feasibility of this approach (363). In fact, we recently demonstrated the power of the second generation concept by effectively blocking favorite viral escape routes, but little therapeutic benefit was achieved because HIV-1 selected alternative escape routes (307).
Towards a durable treatment of HIV-1 infection using RNA interference

Figure 2.1. RNAi gene therapy for HIV-1
The HIV-1 infected patient that fails on regular antiretroviral therapy [1] could be offered the RNAi-based gene therapy with a lentiviral vector. The lentiviral vector is produced in 293T cells [2] transfected with the lentiviral vector (e.g. JS1) and a standard set of packaging plasmids (pRSV-Rev, pVSV-g and pSYNGP). The lentiviral vector will produce viral genomes and the packaging plasmids will produce the proteins required to assemble new viral particles. pVSV-g produces the Vesicular Stomatitis Virus glycoprotein that is used for virus pseudotyping. The patient will undergo an apheresis for the collection of hematopoietic stem cells after pretreatment with granulocyte colony stimulatory factor (GCSF) that mobilizes these cells from the bone marrow into the periphery [3]. The hematopoietic stem cells will be purified and transduced with the therapeutic lentiviral construct [4]. This “intracellular immunization” with the antiviral shRNA will protect these cells against HIV-1. Transduced cells will be infused back into the patient [5] and the HIV-resistant immune cells will hopefully prevent disease progression towards AIDS [6].

Where to target the HIV-1 RNA genome?
Several criteria can be formulated to identify the optimal target sites on the 9 kb HIV-1 RNA genome for RNAi attack. One could propose to select target sequences present in the multiply spliced HIV-1 mRNAs that are synthesized early upon infection and that encode the early viral proteins Tat, Rev and Nef. The idea is that such an early block in viral gene expression will severely impact the subsequent expression of unspliced and singly spliced mRNAs that encode the structural HIV-1 proteins. Alternatively, one could target HIV-1 genomic regions that are represented in all the spliced viral mRNAs, which is the case for small sequence stretches in the untranslated 5'-leader and 3'-trailer domains (251). Target RNA structure can effectively block an RNAi attack (379;380). Thus, targeting of “open” RNA domains is beneficial, and this selection could be helped by the recent description of the RNA secondary structure of the complete HIV-1 RNA genome (376). Another important selection criterion concerns the variability of target sequences. One should
select targets that are highly conserved among virus isolates, simply because one wants to inhibit as many virus strains as possible. Targeting of highly conserved genome regions may also restrict the evolution of viral escape mutants because well-conserved sequences will likely exhibit an important function in HIV-1 biology, such that RNAi-induced sequence variation may be expected to have an impact on the viral replication capacity and fitness (349).

An extensive shRNA screen against highly conserved sequences of the HIV-1 genome has been performed, yielding approximately 20 potent shRNAs (345). Stable shRNA-expressing T cell lines were generated that were subsequently infected with HIV-1, which yielded four durable shRNA inhibitors that restricted virus replication for more than 100 days (364). Other groups have also screened large sets of anti-HIV shRNAs (243). We and others have identified effective shRNAs and siRNAs targeting regulatory HIV-1 sequences, e.g. in the long terminal repeat (LTR) and untranslated leader RNA (163;345) and most viral genes: including gag (69;261;269;345), pol(37;69;334), vif (163), tat (76;199;334;345), rev (76;199;345), vpu (69), env (269) and nef (163). Follow-up analyses should include prolonged culturing of stably transduced T cells to score the impact on cell viability. To address safety in more detail, the off-target effects of the antiviral shRNAs on human mRNAs can be evaluated (160). Prolonged culturing in the presence of HIV-1 should be done to test the likelihood of viral escape, which is in fact a possibility of which the likelihood is difficult to predict. Care should be taken not to misinterpret the results of such escape studies. For instance, we previously reasoned that the appearance of point mutations in the viral target sequence forms proof of viral escape, and in fact it demonstrates the exquisite sequence-specificity of RNAi action. However, sometimes viral breakthrough is observed without the acquisition of escape mutations, which may be an indication of sub-optimal virus inhibition (363). Thus, detailed phenotypic and genotypic analyses are required to satisfactorily address this issue. Guidelines for the proper testing and selection of potent and safe shRNA inhibitors against HIV-1 have been formulated (364).

Combinatorial RNAi approaches

Combinatorial drug/RNAi approaches are essential to restrict HIV-1 evolution and to prevent viral escape, which will lead to therapeutic failure. A variety of strategies have been described for multiplexing of shRNA cassettes in a single therapeutic vector. As repeat sequences should be avoided in the lentiviral vector to prevent recombination-mediated deletions, the multiple shRNA cassettes generally use separate polymerase III promoters or a combination of polymerase II and III promoters (342). Multiplexed siRNAs can also be expressed from a single transcript. We and others developed extended-shRNAs that are processed into two or maximally three functional siRNAs (217;218). Another strategy uses truly long hairpin RNAs (lhRNAs) that should encode numerous siRNAs (29;182;304). A disadvantage of the lhRNA approach is that it is unknown whether the produced siRNAs will be active inhibitors (343), and it was recently demonstrated that a very low level of siRNAs are produced from such constructs (221). Polycistronic miRNA transcripts have also been developed (1). Various groups have reported toxicity of shRNAs (65;140;241;361), which can perhaps be solved by inserting the siRNA sequence into a natural miRNA backbone (241).
Conditional expression of the siRNA molecules will increase the safety of a therapeutic vector. For instance, one would like to avoid shRNA expression in transduced hematopoietic stem cells that still have to undergo hematopoiesis, a process that will be particularly sensitive to changes in the RNAi machinery. Tissue-specific miRNA expression has been described for several organs, including the liver (326). Another option is the design of constructs that are induced by HIV-1 infection (357). Selective expression in HIV-1 susceptible cells would be an elegant way to restrict putative saturation and off-target effects. Another option is the use of inducible gene expression systems such as the doxycycline-controlled Tet system (383;410). While shRNAs are generally expressed from polymerase III promoters, miRNAs are expressed from polymerase II promoters. These polymerase II systems are better equipped for tissue-specific or drug-regulated expression.

Comprehensive reviews on combinatorial RNA approaches are available (139;216). Other types of inhibitory RNA molecules can be added to the RNAi-inducing antiviral regimen and we already mentioned the anti-HIV aptamer-siRNA conjugate transcript (255;408). The currently ongoing phase I clinical trial at the City of Hope uses a lentiviral vector that encodes a TAR-decoy, CCR5-ribozyme and a shRNA targeting the HIV-1 genome in the tat-rev region (93;209). The TAR-decoy is a small nucleolar RNA molecule that absorbs the viral Tat protein, which will prevent the Tat-TAR interaction that is essential for enhanced viral promoter activity (214). The ribozyme cleaves the CCR5-encoding mRNA to cause reduced expression of this important HIV-1 receptor on the cell surface (305). Alternative antiviral RNA molecules include antisense transcripts (71;204), decoys (177), ribozymes (305) and aptamers (335). A new addition to this arsenal is an antisense molecule that can elicit transcriptional gene silencing of the viral LTR promoter (377). Another promising approach is the novel RNAu method that is based on the expression of a modified U1 small nuclear RNA that blocks polyadenylation of the targeted mRNA, which is subsequently degraded (2).

**Targeting cellular co-factors of HIV-1 replication**

An advantage of targeting host cell co-factors that are important for HIV-1 replication is the reduced change of viral escape. Silencing of several co-factors resulted in HIV-1 inhibition: nuclear factor kappa B (334), CD4 (14;261), CXCR4 (14;15;236), DDX3 (158), LEDGF/p75 (359), CCR5 (11;13;14) and stable expression of shRNAs against several co-factors could inhibition HIV-1 replication in vitro up to 2 months (103). CCR5 is a critical receptor for HIV-1 entry and a promising and well-studied target. Individuals with the delta-32 mutation in the CCR5 gene are not susceptible to HIV-1 infection. Perhaps strikingly, the gene deletion does not cause any health problems, only an increased risk for infection with the West Nile virus was reported (213). A potent shRNA targeting the mRNA for this host cell factor has been developed (11;13). The potential of down regulation of CCR5 is supported by the cure of an HIV-1 infected patient who had leukemia in addition to AIDS. This patient was treated in Berlin and received a bone marrow transplantation of a matching donor who was homozygous for the 32-bp deletion in the CCR5-gene. Surprisingly, HIV-1 has not been detected in the patient’s plasma for 600 days post transfusion (157). CCR5-tropic viruses are generally responsible for HIV-1 transmission, but the virus can also use the alternative CXCR4 receptor. Down regulation of the CCR5 receptor will potentially
set the stage for selection of CXCR4-tropic HIV-1 variants, but this evolutionary route was not observed in the Berlin patient. The same virus escape route was discussed when CCR5-blocking drugs were developed, but no such escape has been reported yet in patients treated with the CCR5-antagonist maraviroc (378).

Many cellular targets will obviously not be proper candidates for a gene therapy because they are essential for the cell and the host. For example, CXCR4 is required for homing of hematopoietic stem cells to the bone marrow and subsequent T-cell differentiation (194). Although HIV-1 is a well studied virus, many details of the viral replication cycle as well as the cellular co-factors involved remain elusive. Recently, three high-throughput RNAi gene knockdown screens were published identifying many new candidate co-factors (53;181;406). Although each of the three screens reported hundreds of new candidates, overlap between them was surprisingly small, in fact yielding only three proteins; MED7, MED8 and RELA (Figure 2.2). A number of reasons for this enormous variation in experimental results have been discussed (reviewed in (133)). Different experimental set-ups were used in the three studies, including different cell lines (293T versus HeLa cells), lab-adapted viral strains versus viral vectors and various incubation times after siRNA transfection. It was also shown in a meta-analysis of these RNAi screens that experimental variation between replicates and in the stringency of filtering thresholds contributed to the many discrepancies between the screens (62).

Thus, important candidate co-factors may have been missed in the screens, as illustrated by the fact that several well-known co-factors were not found in any of the screens, such as LEDGF/p75. A reason for this could be that as siRNAs target a single mRNA, those proteins whose function is redundant with other cellular proteins will not be found. Co-factors that need a complete knockdown before an effect on HIV-1 replication can be measured will also be missed, which is the case for the Integrase co-factor LEDGF/p75. Furthermore, many siRNAs can have toxic effects, for instance by off-target effects, and are thus excluded from further analysis, while the targeted co-factor can in fact be important for viral replication.

Although the overlap between individual co-factors identified in these screen was remarkably small, the overlap of the cellular pathways involved seems more significant. Comparing the three screens might not identify the individual co-factors that are important for HIV-1 replication, but it does give clues about the pathways that are important for viral replication. A Gene Ontology analysis of the co-factors reported in at least 2 siRNA screens highlighted the cellular processes of nuclear pore transport, GTP binding and protein complex assembly as being important for HIV-1 replication (62). Interfering with these pathways could be of therapeutic use.

The attack on cellular co-factors may have a more general advantage for the attack on other human pathogenic viruses that may use the same cellular pathways and/or cellular co-factors. Similar genome-wide screens have been performed for other human viruses, including influenza A virus, hepatitis C virus C (HCV), dengue virus and West nile virus (172;179;185;210;313). The disadvantages discussed above for the HIV-1 RNAi screens also apply to these screens. For instance, the overlap between the 2 influenza screens is remarkably restricted, which could be explained by the use of Drosophila versus a human cell line. When comparing all screens performed for the different viruses, proteins involved in cytoskeleton complexes invariably present themselves as candidate co-factors with a broad antiviral impact (179). Ten genes that were identified in at least one of the
HIV-1 screens were also picked up in the HCV screen by Li et al, and could thus be of double therapeutic value, which is important as an estimated 25-30% of HIV-infected individuals are co-infected with HCV (210).
Thus, genome-wide RNAi screens provide a powerful tool to identify novel druggable cellular co-factors, but the antiviral potential needs to be confirmed in more physiologically experimental systems. For instance, HIV-1 follow-up experiments should use T cell lines and primary T cells in combination with primary virus isolates.

Pre-clinical test systems and safety concerns
When potent antiviral shRNAs have been identified in cell culture experiments, one can move to relevant pre-clinical models to critically assess the safety and efficacy of the proposed therapy. A simple and efficient in vitro test system to measure the impact of shRNA expression on cell viability is to perform a co-culture of the GFP+ transduced cells and non-transduced cells (Eekels et al, Chapter 4). A reduction in the percentage of GFP+ cells over time forms an indication of delayed cell growth and RNAi toxicity. Outgrowth of the transduced and thereby protected cells should occur in the presence of HIV-1, which can also be screened for by using simple FACS analysis of a mixed cell culture.

![Figure 2.2. Overlap of HIV-1 co-factors identified in genome-wide RNAi screens](image)

Venn-diagram of the host co-factors for HIV-1 replication identified in three RNAi screens (53,181,406). Thirty-four genes were identified in at least two screens, and only three hits (MED7, MED8 and RELA) were scored in all three screens.

The SIV/macaque model (191) has been used extensively for vaccination studies, but can also be considered for testing of an anti-HIV-1 RNAi gene therapy. One should realize that this model has several limitations. First, anti-HIV shRNAs cannot easily be tested against SIV because of sequence dissimilarity, and the same likely holds for the genes encoding important co-factors in man versus macaque. Thus, one should either convert the anti-HIV
shRNAs into anti-SIV shRNAs, which may affect their inhibitory power or HIV-1 target sequences should be incorporated into the SIV test genome. Second, transduction of the HIV-based lentiviral vector is restricted by TRIM5α in macaque cells (330). Third, macaque experiments are rather expensive, and the number of animals that can be used is restricted. A minimally modified simian-tropic (st) HIV-1 strain has recently been developed that produces an acute viremia and persistent infection in pig-tailed macaques (150). In contrast to most infected humans, stHIV-1 infection is controlled in the macaque model after several months.

Most of these limitations do not apply to humanized immune system (HIS) mouse models (131;356). All major human myeloid and lymphoid cellular compartments develop and mature from input human stem cells in the most recent HIS mouse (203;233;322). This model provides access to in vivo and ex vivo experimentation on human T cells (202). HIS mice can be infected by intravenous injection of the virus but also via rectal and vaginal transmission routes. Infection results in viremia and the depletion of human CD4+ cells as seen in the disease course of infected patients (12;27;34;35;372;402). We used this model to test safety and efficacy of a lentiviral based gene therapy of human hematopoietic stem cells (348). These and other animal models, including their potential and limitations, have recently been reviewed (135).

An important lesson to be learned from various siRNA tests concerns the inclusion of appropriate control experiments. Several studies on the inhibition of infections and inflammation used a control siRNA that targets GFP. Results were in favor of a therapeutic effect, but it turned out that the GFP control is a rather special siRNA with very low immunogenicity compared to most other shRNAs. The therapeutic siRNAs trigger the TLR7/8 interferon pathway, but the GFP siRNA control does not (288). In other words, the observed therapeutic effect was not to be elicited by downregulation of the targeted mRNA. Another lesson comes from a study on an siRNA therapeutic designed for the treatment of age-related macular degeneration in the eye. The siRNA exhibited a therapeutic effect, but this was not likely to be elicited by the RNAi mechanism since the charged siRNA molecule cannot easily penetrate cells. Instead, the clinical effect was reported to occur through TLR-3 signaling (176). Both examples illustrate the importance of selecting the correct controls to ensure that one is looking at RNAi-specific effects. For HIV-1 therapies that target the viral genome, exclusive specificity can be demonstrated by the selection or construction of escape variants with a single point mutation in the target sequence. The sequence-specificity of a particular RNAi effector molecule must be demonstrated in vitro and in the pre-clinical animal model before one can move forward to the clinical test phase (342;348;363).

Safety issues raised in clinical trials

The first patient treated with a gene therapy in 1990 suffered from adenosine deaminase deficiency, a form of severe combined immunodeficiency (SCID) (81). A patient died in 1999 due to the administration of a gene therapy. This patient was treated for a genetic liver disease - ornithine transcarbamylase deficiency - and received an adenovirus treatment with the wild-type gene. He died four days later of a massive immune response, most likely triggered by the use of the viral vector (235). Another trial started in 2000 with SCID patients that received a gamma-retroviral gene transfer with the wild-type interleukin 2 gene. Although this procedure improved the condition of all patients – a true
success – two patients developed a leukemia-like condition of clonal lymphocyte proliferation (144). Both cases were caused by integration of the retroviral vector near the promoter of the LMO2 proto-oncogene, leading to enhanced expression of the LMO2 protein, which has a crucial role in hematopoietic development (145). More patients in this and a similar trial subsequently developed leukemia-like conditions due to insertional oncogenesis. After nearly 10 years of follow-up, gene therapy was shown to have corrected the immunodeficiency associated with SCID. Gene therapy may be an option for patients who don’t have a donor with a compatible HLA-donor for hematopoietic stem-cell transplantation, although this treatment is associated with a risk of acute leukemia (143).

By now more than 1300 clinical trials involving a gene therapy have been performed (102). From these clinical trials lessons can be learned for future improvement of gene therapies. For instance, retroviral vectors have been replaced by lentiviral vectors, which are much more safe because all transcriptional enhancer motifs have been removed (“self-inactivating” design (61)) and because these vectors tend to integrate in genes, and not near the promoter region. In addition, experiments with a lentiviral vector and hematopoietic stem cells in tumor-prone mice did not, in contrast to the retroviral vector, show signs of insertional oncogenesis (248). Other safety and regulatory issues concerning lentiviral vectors are addressed in a comprehensive review based on the expertise gained in the first lentiviral trial (232). The power of the lentiviral vector system to safely transduce CD34+ hematopoietic precursor cells was recently demonstrated in a gene therapy trial for children with adrenoleukodystrophy (64).

**Gene therapy trials for HIV-AIDS**

An overview of ongoing gene therapy trials for HIV-1 has been published (294). While positive in vitro results were obtained for these antiviral gene therapies, the clinical trials failed to demonstrate a therapeutic benefit. In studies where T cells or hematopoietic stem cells were treated with the original retroviral vectors, one of the bottlenecks was effective gene delivery to a clinically relevant number of cells (245). This problem has disappeared with the use of lentiviral vectors that have much higher transduction efficiency on a variety of cell types. In addition, many of the previously used inhibitory RNA molecules seem sub-optimal when compared to antiviral shRNAs. RNAi is therefore a promising candidate for development of a future anti-HIV-1 gene therapy.

The first clinical trial with a lentiviral vector was in fact directed against HIV-1 by expression of an extended antisense transcript against the viral RNA genome. Persistent in vivo expression of the therapeutic antisense molecule was documented by the VirXsys company (204). In addition, vector integration sites in blood cells revealed a preference for gene-rich regions, which is typical for a lentivirus, and no signs of insertional oncogenesis were observed. Another anti-HIV gene therapy trial that uses a triple RNA payload (ribozyme, decoy, shRNA) was performed at the City of Hope by the team of John Rossi. AIDS patients undergoing autologous transplantation for lymphoma were treated with gene-modified CD34+ hematopoietic progenitor cells. Persistent gene marking and sustained shRNA and ribozyme expression in blood cells for up to 24 months was documented for the first time (93). No unexpected infusion-related toxicities were reported. For safety reasons, the transplant consisted of a mixture of genetically modified and unmanipulated cells. Future optimization of the transplant procedure, in particular
the preferential infusion of transduced cells, should provide the setting for delivery of therapeutic levels of HIV-resistant cells.

Conclusion

We reviewed the current status of the development of an RNAi-based gene therapy to control HIV-1 infection and AIDS disease progression. Overall, an RNAi-based gene therapy against HIV-1 seems to be a promising candidate for a durable antiviral treatment, especially for a minority patient group for which the treatment options are exhausted due to drug toxicity or viral resistance. The potent and sequence-specific inhibition of HIV-1 with RNAi forms the corner stone for such a therapy. The superior transduction of hematopoietic stem cells with lentiviral vectors provides the means to deliver the transgene. The availability of several lentiviral production facilities is another promising development in the field. We are currently testing a candidate clinical vector that encodes multiple antiviral shRNAs to evaluate its safety and efficacy. This vector yielded very potent antiviral effects in prolonged in vitro cell cultures. The safety and efficacy are currently being addressed in a humanized mouse model and we expect to initiate a clinical trial within 2 years.
Chapter 3

Long-term inhibition of HIV-1 replication with RNA interference against cellular co-factors

Julia J.M. Eekels¹, Dirk Geerts², Rienk E. Jeeninga¹ and Ben Berkhout¹

Published in: Antiviral Research, 2011. 89(1); 43-53.

¹ Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam

² Department of Human Genetics, Academic Medical Center of the University of Amsterdam
Abstract
In this study we tested whether HIV-1 replication could be inhibited by stable RNAi-mediated knockdown of cellular co-factors. Cell lines capable of expressing shRNAs against 30 candidate co-factors implicated at different steps of the viral replication cycle were generated and analyzed for effects on cell viability and inhibition of HIV-1 replication. For half of these candidate co-factors we obtained knockdown cell lines that are less susceptible to virus replication. For three co-factors (ALIX, ATG16 and TRBP) the cell lines were resistant to HIV-1 replication for up to 2 months. With these cells we could test the hypothesis that HIV-1 is not able to escape from RNAi-mediated suppression of cellular co-factors, which was indeed not detected.

Introduction
The epidemic of human immunodeficiency virus type 1 (HIV-1) still causes millions of new infections and deaths annually. The current therapy against HIV-1, combined antiretroviral therapy or cART, targets the virus with multiple inhibitors to prevent the selection of resistant viral strains. This means that infected persons have to adhere to a strict drug regimen, frequently leading to severe side-effects. It has been proposed that a durable gene therapy based on RNA interference (RNAi) could provide an answer to these problems (5;22).

RNAi is an evolutionary conserved mechanism induced by double-stranded RNA (dsRNA) that triggers sequence-specific gene silencing at the post-transcriptional level. The dsRNA-inducer molecule consists of 19-23 nucleotides with one strand complementary to the target mRNA (116;148). RNAi has been shown to effectively inhibit the replication of different viruses, such as poliovirus, hepatitis A, B and C virus, enterovirus, coxsackievirus, rhinovirus and influenza virus (130;132;170;249;273;310;340;382). Intracellularly expressed short hairpin RNAs (shRNAs) as well as transfected small interfering RNAs (siRNAs) have been successfully used against target sequences in the HIV-1 RNA genome (76;199). In cell lines that constitutively express antiviral shRNAs, HIV-1 replication could be inhibited for several weeks, but the virus eventually escaped from the RNAi-induced pressure (345). Sequencing of the target sequences of the viral escape variants allowed the identification of several escape mechanisms. First, a point mutation in the target sequence can reduce the complementarity with the shRNA inhibitor and thereby abolish the RNAi-suppression (83). Second, the complete or part of the target region could be deleted, especially when non-essential viral genes are targeted (83). Indeed, no such deletion-based escape was observed when essential and well-conserved viral sequences were targeted (363). Third, resistance-causing mutations were infrequently observed outside the target region. These mutations elicit a structural change in the HIV-1 mRNA, thus making the target sequence inaccessible for the RNAi-machinery (380). These results demonstrate that the viral ability to escape from therapy is driven by its high mutation rate. However, HIV-1 is not able to escape when four shRNAs were used simultaneously, similar to the therapeutic success of cART (349).

Targeting of cellular co-factors, host proteins on which HIV-1 relies to complete its replication cycle, could present an alternative anti-escape approach. By targeting cellular co-factors rather than viral components two main problems concerning drug resistance...
might be solved. First, therapeutics directed against a viral RNA target have to act on all HIV-1 variants that circulate in the patient and in the epidemic, whereas a cellular mRNA target is constant. Second, it is expected that by targeting cellular co-factors the virus can only escape by evolving to use a different cellular co-factor (252;409). Depending on the targeted co-factor, such an escape route may be impossible, although this idea has not been validated experimentally. An obvious disadvantage of co-factor suppression is the possibility of adverse effects on cell metabolism and the host organism. A promising co-factor for therapeutic intervention is the CCR5 molecule, which is one of the receptors for HIV-1 entry. A proportion of the human population carries a 21-base pair deletion in the CCR5 gene and does not express this viral receptor without any physiological problems, but these individuals cannot be infected with a CCR5-using HIV-1 strain (156;215). This result suggests that other co-factors could exist that are vital for HIV-1 replication, but whose depletion would not necessarily reduce host viability.

Figure 3.1. Schematic of the HIV-1 replication cycle. Ten important steps of the replication cycle are numbered from attachment (1) to budding (10).

In recent years, much effort has been devoted to the identification of novel cellular co-factors that, directly or indirectly, contribute to the viral replication cycle. RNAi played an important role in these studies, as the effect of host protein knockdown on viral replication was assessed in large scale RNAi gene knockdown experiments. Hundreds of
novel cellular co-factors for HIV-1 replication were recently identified (53;178;181;285;390;406). Proteomics analysis of HIV-1 infected cells also revealed numerous host proteins that could participate in viral replication (287;354). However, there are some serious drawbacks to these screens, which used HEK293T or HeLa cells instead of T cells and laboratory-adapted HIV-1 variants or pseudo-typed virus. All these studies are transient in nature, based on the transfection of siRNAs rather than intracellularly expressed shRNAs. Thus, long-term effects on cell toxicity and viral replication could not be analyzed. In this study, we selected thirty candidate co-factors for stable knockdown in a human T cell line to test the impact on cell viability and HIV-1 replication. Co-factors were chosen based on their suggested importance in the viral replication cycle, although for some (like IPO7) this is not without discussion (18;412). The thirty co-factors that we selected are distributed along all steps of the HIV-1 replication cycle, as it is not clear whether targeting a specific step, e.g. early or late, has an advantage. We also tested the concept that targeting of cellular co-factors prevents viral escape. We observed durable inhibition of viral replication upon knockdown of three co-factors (ALIX, TRBP and ATG16), without detecting viral escape.

**Materials and methods**

**shRNA constructs**

pLKO.1 constructs expressing shRNA candidates and with the puromycin-resistance marker were from the MISSION TM TRC-Hs 1.0 library (293). Constructs, including the negative control constructs SHC001 and SHC002 (hereafter named SHC1 and SHC2), were obtained from Sigma-Aldrich (St. Louis, MO) as bacterial clones. Plasmid DNA was extracted using the Nucleobond Midiprep columns according to the manufacturer’s instructions (Macherey-Nagel, Düren, Germany). For every target gene, 4 to 5 shRNAs were included. Target sequences for every gene can be found on the website of Sigma-Aldrich [http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html].

**Cell culture**

Human embryonic kidney 293T (HEK293T) adherent cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human SupT1 suspension T cells were grown in Rosewell Park Memorial Institute medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell lines were cultured in a humidified chamber at 37°C and 5% CO2.

**Generation of stable knockdown cell lines**

The shRNA-expressing lentiviral vectors were produced as previously described (345). In short, HEK293T cells were co-transfected with the shRNA-construct and the packaging plasmids (pRSV-Rev, pMDLg/pRRE and pVSV-G) using Lipofectamine 2000 (Invitrogen). One day after transfection the medium was refreshed and the following day the supernatant was harvested. The virus containing supernatant was centrifuged, filtered (0.45 μm), and aliquots were stored at –80°C. A sample was taken for CA-p24 enzyme-
linked immunosorbent assay (ELISA) to monitor lentiviral particle production. SupT1 cells were seeded in a 24-wells plate (1x10^5 cells per well) and lentiviral vector, corresponding to 100 ng CA-p24, was added. After overnight incubation cells were washed with 1 ml PBS and cultured in complete medium supplemented with 1 µg/ml puromycin. After one week the puromycin selection was stopped and cell growth was monitored by counting of the cell cultures every other day using Fluorescent Activated Cell Sorting (FACS) over a period of 8 days. Gating based on side and forward scatter identified live cells. The measurements were used to calculate the doubling time of each shRNA-expressing cell line.

**RT-qPCR**

For selected cell lines the knockdown efficiency of the target mRNA was measured by RT-qPCR. RNA was isolated from 1x10^6 cells with the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer’s instructions, including the optional DNaseI on column treatment. Samples for RT-qPCR were taken over a period of 3 weeks. 250 ng RNA was reverse transcribed (Thermoscript kit, Invitrogen) using Oligo-dT primers. The cDNA synthesis reaction was incubated for 1 hour at 50°C. The resulting cDNA was serially diluted and used as a template in a SYBR Green based RT-qPCR with the SYBR Green FAST PCR kit (Qiagen) and an ABI Prism 7000 sequence detection system. Specific primers for the selected targets were used and β-actin primers served as an internal control (Quantitect primer assays, Qiagen). The level of target mRNA expression was measured using Ct (threshold cycle) in triplicate for every sample, and the ΔΔCt method was used for relative quantitation of target mRNA expression levels. The ΔCt was calculated by subtracting the Ct of β-actin RNA from the Ct of the target mRNA of interest. The ΔΔCt was calculated by subtracting the ΔCt of one cell line from the ΔCt of the control SHC1 cell line. Fold change was generated using the Equation 2^{−ΔΔCt} \quad (222).

**HIV-1 infection**

HIV-1 was produced by transfection of HEK293T cells with the molecular clone HIV-1 LAI (270) and virus production was measured by CA-p24 ELISA. SupT1 cultures (2 ml cultures in 6 wells plate, 2x10^5 cells/well) were infected with HIV-1 (0.2 ng of CA-p24). Every two days virus replication was monitored by scoring syncytia formation and supernatant samples were taken for CA-p24 ELISA.

**CA-p24 ELISA**

Culture supernatant was heat-inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as the capture antibody and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as the detection antibody. Detection was done with the Lumiphos plus system (Lumigen) in a LUMIstar Galaxy (BMG Labtechnologies) luminescence reader. Recombinant CA-p24 produced in a baculovirus system was used as the reference standard.
Results

**HIV-1 co-factor screen**

We selected thirty cellular co-factors that have been implicated in HIV-1 replication for stable RNAi-mediated knockdown in the human T cell line SupT1. These co-factors facilitate virus replication steps from entry to budding (Figure 3.1). Among others, we targeted co-factors that facilitate entry into the target cell (e.g. receptors CD4 and CXCR4), integration of the proviral DNA in the host chromosome (e.g. ATM kinase and INI1), viral transcription (e.g. TRBP), virion assembly (e.g. ABCE1) and budding from the cell surface. The thirty co-factors were numbered according to their function in HIV-1 replication, from entry to budding (Table 3.1). Some of these co-factors were previously demonstrated to support HIV-1 replication in studies with transient RNAi-mediated gene knockdown or small molecule inhibitors. We obtained shRNA expression constructs from the TRC shRNA library to produce lentiviral vectors. A control vector with an empty shRNA cassette (SHC1) and a vector that encodes a scrambled shRNA (SHC2) that lacks an identifiable mRNA target were used. Four to five candidate shRNAs were used per co-factor, which provides several advantages. First, since the different shRNAs gave varying degrees of knockdown, this aided in the analysis of those cases where too efficient knockdown may lead to cytotoxicity and too little knockdown might have no effect on HIV-1 replication. Second, when more than one shRNA per co-factor demonstrated inhibition of HIV-1 replication, it is more likely that the observed effect is gene-specific, thus ruling out off-target effects on unrelated genes. Lentiviral vector production was monitored by CA-p24 ELISA. Because lentiviral vectors are based on the HIV-1 replication machinery, silencing of candidate co-factors that support HIV-1 replication could also influence lentiviral vector production. However, we did not detect any significant differences for the shRNA-vectors compared to the empty vector SHC1 control (results not shown). We used the human T cell line SupT1 because it allows rapid detection of HIV-1 replication by the formation of syncytia. A fixed amount of lentiviral vector was used to transduce SupT1, yielding a total of 142 cell lines (30 co-factors, each 4 or 5 shRNAs and controls). Transduced cells were selected with puromycin for 1 week and cell proliferation was measured by counting of the number of cells by FACS analysis.
<table>
<thead>
<tr>
<th>Co-factor</th>
<th>Full name</th>
<th>Function in HIV-1 replication</th>
<th>Replication step (a)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CD4</td>
<td>CD4 molecule</td>
<td>Receptor</td>
<td>1</td>
<td>(82)</td>
</tr>
<tr>
<td>2 CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>Co-receptor</td>
<td>1</td>
<td>(115)</td>
</tr>
<tr>
<td>3 CD81</td>
<td>CD81 molecule</td>
<td>Membrane fusion</td>
<td>2</td>
<td>(136)</td>
</tr>
<tr>
<td>4 PPIA</td>
<td>Peptidylprolyl isomerase A</td>
<td>Assists in early replication</td>
<td>3</td>
<td>(225)</td>
</tr>
<tr>
<td>5 IPO7</td>
<td>Importin 7</td>
<td>Nuclear import of reverse transcription complex</td>
<td>5</td>
<td>(18)</td>
</tr>
<tr>
<td>6 EED</td>
<td>Embryonic ectoderm development</td>
<td>Interactor of HIV-1 integrase</td>
<td>5</td>
<td>(362)</td>
</tr>
<tr>
<td>7 ATM</td>
<td>Ataxia telangiectasia mutated</td>
<td>Provirus integration</td>
<td>6</td>
<td>(17)</td>
</tr>
<tr>
<td>8 INI1</td>
<td>Integrase interactor 1 protein</td>
<td>DNA joining activity of HIV-1 integrase</td>
<td>6</td>
<td>(169)</td>
</tr>
<tr>
<td>9 WRN</td>
<td>Werner syndrome, RecQ helicase-like</td>
<td>DNA repair after provirus integration</td>
<td>6</td>
<td>(318)</td>
</tr>
<tr>
<td>10 EP300</td>
<td>E1A binding protein p300</td>
<td>Tat-mediated transcription</td>
<td>7</td>
<td>(32)</td>
</tr>
<tr>
<td>11 ATF3</td>
<td>Activating transcription factor 3</td>
<td>Transcription factor</td>
<td>7</td>
<td>(316)</td>
</tr>
<tr>
<td>12 TRBP</td>
<td>TAR (HIV-1) RNA binding protein</td>
<td>Binds TAR-hairpin and acts in synergy with Tat</td>
<td>7</td>
<td>(128)</td>
</tr>
<tr>
<td>13 PRKRA</td>
<td>Protein kinase, IFN-inducible dsRNA dependent activator</td>
<td>Binding partner of TRBP</td>
<td>7</td>
<td>(33)</td>
</tr>
<tr>
<td>14 S U P T 5 H</td>
<td>Suppressor of Ty 5 homolog (S. cerevisiae)</td>
<td>Tat-mediated transcription</td>
<td>7</td>
<td>(386)</td>
</tr>
<tr>
<td>15 PP2A</td>
<td>Protein phosphatase 2</td>
<td>Tat-mediated transcription</td>
<td>7</td>
<td>(296)</td>
</tr>
<tr>
<td>16 PP1CA</td>
<td>Protein phosphatase 1</td>
<td>Tat-mediated transcription</td>
<td>7</td>
<td>(40)</td>
</tr>
<tr>
<td>17 PCAF</td>
<td>Lysine acetyltransferase 2B</td>
<td>Tat-mediated transcription</td>
<td>7</td>
<td>(32)</td>
</tr>
<tr>
<td>18 DHX9</td>
<td>DEAH (Asp-Glu-Ala-His) box poly peptide 9</td>
<td>Nuclear export of HIV-1 RNA via RRE</td>
<td>8</td>
<td>(207)</td>
</tr>
<tr>
<td>19 DDX3</td>
<td>DEAD (Asp-Glu-Ala-Asp) box poly peptide 3, X-linked</td>
<td>Nuclear export of HIV-1 RNA via CRM1</td>
<td>8</td>
<td>(388)</td>
</tr>
<tr>
<td>20 DDX1</td>
<td>DEAD (Asp-Glu-Ala-Asp) box poly peptide 1</td>
<td>Proper cellular colocalization of Rev</td>
<td>8</td>
<td>(113)</td>
</tr>
<tr>
<td>21 D H P S</td>
<td>Deoxyhypusine synthase</td>
<td>Biosynthesis of eIF-5a, co-factor for Rev</td>
<td>8</td>
<td>(297)</td>
</tr>
<tr>
<td>22 AMD</td>
<td>Adenosylmethylionine decarboxylase 1</td>
<td>Biosynthesis of eIF-5a, co-factor for Rev</td>
<td>8</td>
<td>(297)</td>
</tr>
<tr>
<td>23 AGFG1</td>
<td>ArfGAP with FG repeats 1</td>
<td>Co-factor for Rev</td>
<td>8</td>
<td>(42;122)</td>
</tr>
<tr>
<td>24 PAC51</td>
<td>Phosphofurin acidic cluster sorting protein 1</td>
<td>Localization of furin, which cleaves gp160 in Golgi</td>
<td>9</td>
<td>(274)</td>
</tr>
<tr>
<td>25 KIF4A</td>
<td>Kinesin family member 4A</td>
<td>Transport of Gag to cell membrane</td>
<td>9</td>
<td>(237)</td>
</tr>
<tr>
<td>26 HSPD1</td>
<td>Heat shock 60kDa protein 1 (chaperonin)</td>
<td>Incorporated into virion though Gag-interaction</td>
<td>9</td>
<td>(142)</td>
</tr>
<tr>
<td>27 M6PRBP1</td>
<td>Mannose-6-phosphate receptor-binding protein 1</td>
<td>Env incorporation into virions</td>
<td>9</td>
<td>(224)</td>
</tr>
<tr>
<td>28 ABCE1</td>
<td>ATP-binding cassette, sub-family E (OABP), member 1</td>
<td>HIV-1 capsid assembly</td>
<td>9</td>
<td>(413)</td>
</tr>
<tr>
<td>29 ALIX</td>
<td>Apoptosis-linked gene 2-interacting protein X</td>
<td>Viral budding machinery</td>
<td>10</td>
<td>(329)</td>
</tr>
<tr>
<td>30 ATG16</td>
<td>ATG16 autophagy related 16-like 1 (S. cerevisiae)</td>
<td>Autophagy factor</td>
<td>?</td>
<td>(53)</td>
</tr>
</tbody>
</table>

*Table 3.1. Cellular co-factors that support HIV-1 replication*
Cell viability

We divided the shRNA inhibitors into three categories depending on their impact in cell proliferation, which was compared to the doubling time of the control cell lines SHC1 and SHC2 (Figure 3.2 and Table 3.2). We observed either no or little effect on cell growth (cell lines had <2-fold increased doubling time), an intermediate effect (between 2 and 4-fold increase) or severe effects (>4-fold increase or cell death).

Figure 3.2. Effect on cell proliferation in shRNA-expressing cell lines. ShRNA cell lines for thirty cellular co-factors were counted by FACS over a period of 8 days. Each graph shows the doubling time in days (y-axis) per co-factor. Results for the controls SHC1 and SHC2 (black bars) and shRNA cell lines (grey bars) are shown. Data shown is from three independent experiments, error bars represent the standard error of the mean.
We classified the co-factors for their impact on cell viability as follows. For 11 of the 30 co-factors (CD4, CXCR4, EP300, TRBP, PPP1CA, PCAF, DDX3, AGFG1, KIF4A, M6PRBP1 and ATG16) no serious adverse effects on cell proliferation were observed. For 8 co-factors (CYP, EED, ATM, ATF3, AMD, PACS1, HSPD1 and ALIX) an intermediate effect on cell growth was scored, indicating that only a single of the 4 or 5 shRNAs imposed delayed cell growth. In these cases, knockdown of the target protein might be lethal for the cell, or alternatively, as only a single shRNA showed the effect, it cannot formally be excluded that a toxic off-target effect on an unrelated cellular mRNA occurred. We scored severe cytotoxicity when multiple shRNAs per co-factor had an impact on cell proliferation. This was observed for 11 of the 30 co-factors (CD81, IPO7, INI1, WRN, PRKRA, SUPT5H, PP2A, DHX9, DDX1, DHPs and ABCE1). For 3 co-factors (INI1, SUPT5H and DHX9) 3 of the 5 shRNAs induced cell death (Figure 3.2.8, 3.2.14 and 3.2.18 respectively).

**Inhibition of HIV-1 replication**

We continued with the cell lines that did not exhibit any adverse effects on cell growth and the cell lines with an intermediate effect. We challenged these over 120 cell lines, including the controls SHC1 and SHC2, with the CXCR4-using HIV-1 primary isolate LAI. In three separate infections we monitored viral spread by measuring CA-p24 production in the supernatant and by scoring syncytia formation. The control cell lines SHC1 and SHC2 showed massive syncytia formation and high CA-p24 levels at 10 days post infection. The CA-p24 measurements for the three infections are summarized in Figure 3.3. The controls SHC1 and SHC2 are depicted as black bars and the grey bars represent individual shRNAs that were grouped per co-factor. The results per shRNA inhibitor are summarized in the right column of Table 3.2. The effect on viral replication per shRNA was divided into three categories: no effect (CA-p24 values in the supernatant were above 10 ng/ml), intermediate (CA-p24 values between 1 and 10 ng/ml, marked + in Table 3.2) and strong inhibition of viral replication (CA-p24 values below 1 ng/ml or undetectable, marked ++ in Table 3.2).

Virus replication showed substantial inhibition for 15 of the 30 co-factors tested (CYP, IPO7, EED, WRN, ATF3, TRBP, PCAF, DDX3, DHPs, PACS1, KIF4A, HSPD1, M6PRBP1, ALIX and ATG16). In these cases, 2 or more shRNAs per co-factor showed an intermediate or strong inhibition. As for these co-factors 2 or more shRNAs did show the effect, this strongly suggests that this effect is caused by specific knockdown of the target protein. For 8 co-factors (CXCR4, ATM, INI1, PRKRA, PPP1CA, DHX9, DDX1 and AGFG1) we observed moderate HIV-1 inhibition. In these cases, only one shRNA per co-factor inhibited viral replication. An example is the co-factor CXCR4, where shRNA5 inhibited viral replication (Figure 3.3.2) and for which FACS analysis revealed a modest knockdown of CXCR4 expression at the cell surface (data not shown). For 7 co-factors (CD4, CD81, EP300, SUPT5H, PP2A, AMD and ABCE1) we observed that none of the shRNAs inhibited virus replication. For example see AMD (Figure 3.3.22), where the CA-p24 levels in all 4 shRNA-expressing cell lines reached levels comparable to the controls SHC1 and SHC2. The shRNAs tested may not have induced sufficient target protein knockdown to block viral replication and it thus cannot formally be excluded that this class does not represent true co-factors, illustrated by the fact that CD4 is in this category. In fact, CD4 expression is very high in the SupT1 T cell line, and although a single shRNA gave a modest reduction of
CD4 expression, no impact on HIV-1 replication was measured. We focused on the cell lines that exhibit the strongest inhibition, as these co-factors represent the most promising candidates.

Table 3.2. Cytotoxicity and HIV-1 inhibition by co-factor shRNAs

<table>
<thead>
<tr>
<th>Co-factor</th>
<th>Cytotoxicity per shRNA</th>
<th>HIV-1 inhibition per shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CD4</td>
<td>1 2 3</td>
<td>1 2 3</td>
</tr>
<tr>
<td>2 CXCR4</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3 CD81</td>
<td>1 1 1</td>
<td></td>
</tr>
<tr>
<td>4 PPIA</td>
<td>I</td>
<td>+ + +</td>
</tr>
<tr>
<td>5 IPO7</td>
<td>1 S</td>
<td>++ + +</td>
</tr>
<tr>
<td>6 EED</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>7 ATM</td>
<td>I</td>
<td>++</td>
</tr>
<tr>
<td>8 INI1</td>
<td>S S S ++</td>
<td></td>
</tr>
<tr>
<td>9 WRN</td>
<td>I S</td>
<td>+ ++</td>
</tr>
<tr>
<td>10 EP300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 ATF3</td>
<td>I</td>
<td>+ + nd</td>
</tr>
<tr>
<td>12 TRBP</td>
<td></td>
<td>++ +</td>
</tr>
<tr>
<td>13 PRKRA</td>
<td>S I I</td>
<td></td>
</tr>
<tr>
<td>14 SUPTSH</td>
<td>S S S</td>
<td></td>
</tr>
<tr>
<td>15 PP2A</td>
<td>S S</td>
<td></td>
</tr>
<tr>
<td>16 PPP1CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17 PCAF</td>
<td></td>
<td>++ ++</td>
</tr>
<tr>
<td>18 DHX9</td>
<td>S S i S</td>
<td></td>
</tr>
<tr>
<td>19 DDX3</td>
<td>nd ++ ++</td>
<td></td>
</tr>
<tr>
<td>20 DDX1</td>
<td>I I ++</td>
<td></td>
</tr>
<tr>
<td>21 DHPS</td>
<td>I S ++ +</td>
<td></td>
</tr>
<tr>
<td>22 AMD</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>23 AGFG1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 PACS1</td>
<td>I ++ +</td>
<td></td>
</tr>
<tr>
<td>25 KIF4A</td>
<td></td>
<td>+ +</td>
</tr>
<tr>
<td>26 HSPD1</td>
<td>I nd ++ +</td>
<td></td>
</tr>
<tr>
<td>27 M6PRBP1</td>
<td></td>
<td>++ +</td>
</tr>
<tr>
<td>28 ABCE1</td>
<td>S S nd</td>
<td></td>
</tr>
<tr>
<td>29 ALIX</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>30 ATG16</td>
<td></td>
<td>++ +</td>
</tr>
</tbody>
</table>

Cytotoxicity: I = Intermediate, doubling time increased 2-4 fold, S = Severe effects on cell growth, > 4-fold increase in doubling time
HIV-1 Inhibition: + = Intermediate, CA-p24 < 10 ng/ml, ++ = Strong, CA-p24 < 1 ng/ml
Grey areas: shRNAs not available or excluded due to severe cytotoxicity
nd = not determined
Figure 3.3. Inhibition of HIV-1 replication in shRNA-expressing cell lines. ShRNA cell lines for thirty different cellular co-factors were tested for inhibition of HIV-1 replication. Each graph shows the CA-p24 levels (y-axis) in the culture supernatant 7 day post infection per cellular co-factor. Results for the SHC1 and SHC2 controls (black bars) and shRNA cell lines (grey bars) are shown. Data shown is from three independent experiments, error bars represent the standard error of the mean.

Long term culturing to test for viral escape

In most cell lines, HIV-1 replication was delayed for 2 or 3 days compared to the controls SHC1 and SHC2 (data not shown). While this may represent partial, but true inhibition of
HIV-1 replication, we observed a more extended period of HIV-1 inhibition for three co-factors; ALIX with shRNA1, TRBP with shRNA4 and ATG16 with shRNA4, hereafter named ALIX-1, TRBP-4, and ATG16-4, respectively. Six independent HIV-1 challenges are shown per cell line (Figure 3.4).

For these three co-factors the reduction in target mRNA expression in the uninfected cells was measured in a RT-qPCR assay. For this we sampled the cells at day 13, 21 and 32 after transduction and the level of the targeted co-factor mRNA was measured in triplicate. The knockdown efficiency was stable over time, which allowed us to pool the data. For ALIX-1 a reduction in mRNA expression of 62.7% (± 12.7%) was measured, compared to the control SHC1 cells. For TRBP a reduction in mRNA expression of 70.2% (± 10.1%) was scored and for ATG16-4 the reduction was 62.9% (± 12.9%) (Figure 3.5). No effect of the SHC2 scrambled shRNA was apparent. While it may be surprising that a relatively low level of target knockdown triggers such a strong antiviral effect, these results may indicate that efficient HIV-1 replication requires an optimal expression level of these three cellular co-factors.
Figure 3.5. mRNA expression levels for TRBP, ALIX and ATG16 in shRNA cell lines. Target mRNA expression levels were measured by RT-qPCR. Target mRNA expression was normalized based on the target and β-actin mRNA expression levels measured in the control SHC1 cell line. The mean and standard error of the mean of the three different time points are shown.

Prolonged viral suppression allowed us to test the concept that no viral resistance will occur when a critical cellular co-factor is targeted. As evolution is a chance process, it is crucial to perform such experiments in multiplicate. For that reason, we infected 6 replicate cultures of the three most restricted cell lines. Potent virus suppression was seen in all 18 cultures for at least 10 days, after which breakthrough replication was frequently observed. No viral breakthrough was seen in a few cultures, even after more than 2 months (Figure 3.4: ALIX-1 culture 6 and ATG16-4 culture 5).

It is important to test whether virus breakthrough represents true viral escape or pseudo-escape, which represents the eventual spread of HIV-1 when inhibition is not complete and high viral titers are used in the challenge. We previously demonstrated such pseudo-escape in HIV-1 evolution studies (349). We collected cell-free virus at the peak of infection for all breakthrough cultures and tested samples on susceptible SupT1 control cells and resistant shRNA-expressing cells. Two examples are presented in Figure 3.6. Wild-type HIV-1LAI was restricted on the shRNA-cell line, but rapid viral replication could be measured in the parental cells (Figure 3.6A, left). The breakthrough virus obtained in the evolution experiment was inhibited when the shRNA-expressing cell line is infected (Figure 3.6A, right).

A single breakthrough virus showed the pattern predicted for a true escape virus, the virus harvested from ATG16-4 culture 3 (Figure 3.6B). Wild-type HIV-1 was restricted on the ATG16-4 shRNA-cell line (Figure 3.6B, left), while the breakthrough virus was able to replicate on the parental SupT1 cell line and restricted ATG16-4 cells, albeit somewhat delayed in the latter case (Figure 3.6B, right). We performed full HIV-1 genome sequencing to identify possible escape-causing mutations and found a single mutation in the C1 domain of the Envelope protein (Asp62Asn). However, introduction of this genotypic mutation in the LAI molecular clone did not encode the escape phenotype (results not shown), suggesting that this mutation was not responsible for the observed phenotype. A new batch of parental SupT1 cells was transduced with the lentiviral vector expressing ATG16-4 shRNA, but we failed to reproduce the partial escape phenotype. Based on these results, we conclude that the observed HIV-1 replication on the ATG16-4 expressing cell line shows a typical pseudo-escape profile. In conclusion, no evidence for viral escape was obtained.
Long-term inhibition of HIV-1 replication with RNA interference against cellular co-factors

Figure 3.6. Testing of the breakthrough viruses for viral escape. The parental SupT1 and shRNA cell lines ALIX-1 (A) and ATG16-4 (B) were infected with HIV-1LAI (left panels) and corresponding breakthrough virus (right panels) harvested from previously infected shRNA cultures. CA-p24 levels in the culture supernatant were monitored over time.

Discussion

We tested whether we could inhibit HIV-1 replication in a human T cell line with a stable RNAi-mediated knockdown of a cellular protein that has been implicated in HIV-1 replication. Thirty cellular co-factors were chosen, distributed along the viral replication cycle. We tested 4 or 5 shRNA inhibitors per co-factor, generating over 140 stable T cell lines. The cell lines were first monitored for an impact on cell growth and cell lines with severe growth problems were excluded from the study. We observed severe cytotoxicity for 3 co-factors with multiple shRNAs (INI1, SUPT5H and DHX9). We next tested the remaining shRNA-cell lines for inhibition of HIV-1 replication, which could be detected for 15 of the 30 co-factors tested. For 7 co-factors none of the shRNAs inhibited virus replication. For several co-factors more than a single shRNA inhibited the virus, strongly suggesting that the observed effect is gene-specific. For 8 co-factors we observed moderate inhibition (CXCR4, ATM, INI1, PPP1CA, DHX9, DDX1, AGFG1, ATF3 and PRKRA). Since the function of the co-factors from these two categories are scattered along the viral replication cycle, virus inhibition can occur at multiple steps, which may not be a surprise finding.

HIV-1 could be suppressed up to two months upon silencing of the co-factors ATG16, ALIX and TRBP. The ATG16 effect was observed for three shRNA inhibitors (2, 4 and 5), of which one shRNA inhibited viral replication over a longer period. ATG16 is a protein involved in the autophagy pathway, a cellular mechanism responsible for the degradation of long-
Chapter 3

lived proteins and organelles, and was recently identified as candidate HIV-1 co-factor in one of the previous transient RNAi-screens (53). Autophagy has been implicated in the so-called bystander effect, the massive cell death of uninfected T cells due to interaction of CXCR4 with the HIV-1 Envelop protein on the infected cell (90). However, the exact role of autophagy in HIV-1 replication has not been elucidated yet, which makes ATG16 and other autophagy factors interesting candidates for future mechanistic studies. Strong HIV-1 inhibition was observed upon silencing of the co-factor ALIX with shRNA1, but modest inhibition was also observed with shRNA2. ALIX is a cellular protein with several functions from endocytosis to cell division and it plays a role in budding of the HIV-1 virion (329). The third cellular co-factor that upon silencing induced strong viral inhibition is TRBP. TRBP or TAR RNA binding protein acts in synergy with the viral protein Tat to activate HIV-1 transcription (128).

We investigated whether HIV-1 could escape from RNAi-mediated silencing of three selected co-factors; ATG16, ALIX and TRBP. As we did obtain shRNA-expressing cell lines that inhibit the virus for an extended period, we were able to perform virus evolution experiments to select for RNAi-escape variants. Only a single candidate escape virus was selected upon silencing of the ATG16 co-factor, but its phenotype was partial and could not be reproduced. In addition, we could not identify any causative genotypic variation in the HIV-1 genome. These combined results indicate that only pseudo-escape was observed. In conclusion, we could not detect viral escape from RNAi against cellular co-factors, which argues for the implementation of host co-factor targeting in antiviral strategies. The potential of such a host co-factor therapy is supported by the recent cure of an HIV-1 infected patient. This patient had leukemia in addition of AIDS and received bone marrow transplantation of a matching donor who was homozygous for the 32-bp deletion in the CCR5-gene. Surprisingly, HIV-1 has not been detected in the patient’s plasma for 600 days post transfusion (157). Another study showed the successful use of zinc-finger nucleases to disrupt the CCR5 gene in hematopoietic stem cells, a strategy where the autologous cells of the patient can be used (154). Both cases thus provide an indirect proof of principle for targeting of cellular co-factors in a new therapeutic approach against HIV-1. In this study, only some of the results of previous transient assays with siRNA-mediated inhibition could be confirmed. This suggests to us that the status of these candidate co-factors requires further experimental validation. Testing of co-factors that support HIV-1 replication in long-term experimental setting seems critical to identify the optimal cellular targets for antiviral therapy.

Acknowledgments

This research was supported by the Dutch AIDS fund (grant n° 2006006 and 2007028). We thank Stephan Heynen for performing CA-p24 ELISA experiments. We also thank the Belgian Federal Government for financial support through the Inter-University Attraction Pole grant n° P6/41.
Chapter 4

A competitive cell growth assay for the detection of subtle effects of gene transduction on cell proliferation

Julia J.M. Eekels¹, Alexander O. Pasternak¹, Anne Marieke Schut¹, Dirk Geerts², Rienk E. Jeeninga¹ and Ben Berkhout¹

Accepted for publication in Gene Therapy

¹Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam

²Department of Human Genetics, Academic Medical Center of the University of Amsterdam
Abstract

RNA interference (RNAi) is a sequence-specific gene silencing mechanism with therapeutic potential against many human pathogens. To obtain a durable therapeutic effect, stable transduction of target cells with for instance a lentiviral vector that expresses a short hairpin (shRNA) inducer of the RNAi pathway is necessary. Apart from the intended therapeutic effect, this treatment can induce negative effects on cell proliferation via off-target effects. A careful evaluation of the transduced cells is required to develop a safe gene therapy approach. Stably transduced cells are usually selected by expression of the enhanced green fluorescent protein (GFP) marker. In this study we show that the mixed transduction culture, containing both transduced GFP+ and untransduced GFP− cells, can simply be passaged to score the GFP+/GFP− ratio by longitudinal flow cytometric analysis as a measure of the negative impact of the RNAi treatment on the cellular proliferation rate. We show that this assay is sensitive, easy to use and internally controlled for assessing subtle effects on cell proliferation of lentiviral transduction and transgene expression.

Introduction

RNA interference (RNAi) is an evolutionary conserved mechanism induced by double-stranded RNA, leading to sequence-specific gene silencing at the post-transcriptional level (116). RNAi can be induced transiently by transfecting target cells with small interfering RNAs (siRNAs) or stably by intracellular expression of short hairpin RNAs (shRNAs). Both strategies can be exploited for therapeutic purposes against a wide variety of diseases or microbial pathogens such as viral infections (101;216;221;268;404). Both viral RNA and cellular transcripts that encode co-factors necessary for viral replication can be targeted (103). Especially for the attack on viruses that cause a persistent infection the preferred method is the generation of stable knockdown cells, in which constitutive expression of the antiviral shRNAs is achieved by vector transduction. In particular, lentiviral vectors have been very successful because they are able to transduce many different cell types, both actively dividing and quiescent cells, in which the viral genome is stably integrated in the host cell DNA. A marker, which usually encodes for antibiotic resistance or a fluorescent marker protein like the green fluorescent protein (GFP), can be used to select the transduced cells.

An important consideration when developing gene therapeutic strategies based on RNA interference (RNAi) is the potential impact on the physiology and viability of the transduced cells. RNAi can have adverse effects on cellular properties and affect cell growth for several reasons. First, off-target effects of the shRNA on an unspecified mRNA with partial sequence complementarity can affect cell growth in an unpredictable manner (160). Second, overexpression of shRNA molecules can saturate components of the RNAi pathway, and thus disturb normal cellular gene regulation by microRNAs (140;173). Third, shRNA overexpression can trigger innate immune responses such as the interferon cascade (155;167;323). Fourth, targeting of a cellular protein that acts as co-factor for virus replication can affect cell growth. Caution is particularly warranted in case of stable gene knockdown, as subtle differences in cell growth rates can have a major impact on biological assays in diverse in vitro settings. The use of a stably integrating retroviral or...
lentiviral vector can cause additional adverse effects due to genome integration, e.g. disruption of the regulation of local cellular genes. Obviously, in vivo RNAi and in fact any transgene application should put an emphasis on addressing such putative adverse effects on cell physiology and cell growth.

Figure 4.1. The CCG assay versus other cell proliferation assays
The CCG assay follows the percentage of transduced GFP+ cells over time and is performed on the crude transduction mixture, providing fast and internally controlled results. Other cell proliferation assays requires the selection of pure GFP+ transduced cells, e.g. by FACS sorting and subsequent expansion. In some cases one even needs to generate clonal cell lines by limiting dilution and expand the clonal cells before proliferation assays can be performed.

Gross cell growth defects can be monitored using a variety of assays. Staining with trypan blue and other dyes, like DAPI or Hoechst 33342, can distinguish between live and dead cells. In order to calculate the cell doubling time, the increase in number of cells can be measured over time, either by direct counting of the cells (manually or automatically) or indirectly by reporter luciferase assays that measure the ATP-content of metabolically active cells. However, there is a need for a simple assay to score for more subtle cell growth effects. We explored the option to maintain the mixed culture obtained after gene transduction that consists of transduced GFP+ and non-transduced GFP- cells. The GFP+/GFP- ratio can simply be evaluated by FACS analysis over time, which allows one to score minor cell growth defects as a gradual loss of the percentage GFP+ cells. This assay is based on the competitive cell growth between transduced and non-transduced cells. We compared this Competitive Cell Growth (CCG) assay to established methods of counting cells (both by FACS and with a hemacytometer), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and a commercially available ATP bioluminescence assay. We demonstrate that the CCG assay provides a sensitive, internally controlled and simple procedure.
Materials and methods

DNA constructs

Lentiviral vector pLKO.1-constructs expressing shRNA were from the MISSION TM TRC-Hs 1.0 library (293), including the negative control constructs SHC001 and SHC002 (hereafter named SHC1 and SHC2), were obtained as bacterial clones from Sigma-Aldrich (St. Louis, MO). Plasmid DNA was extracted using the Nucleobond Midiprep columns according to the manufacturer’s instructions (Macherey-Nagel, Germany).

Sequences for shRNA1, 2 and 3 are respectively:
5’-CCGGCATCAAACCATCCTTCCTGTACTCGAGTACAGAAGGAATGGTTTGATGTTTTTG-3’,
5’CCGGCCCTGCCAAACAAGCTAATATCTCGAGATATTAGCTTGTTTGGCAGGGTTTTT-3’
5’-CCGGGAAACTGTCAAGCAAGGCGTTCTCGAGAACGCCTTGCTTGACAGTTTCTTTTT-3’

The pLKO.1 constructs from the MISSION TM TRC-Hs 1.0 library contain a puromycin selection marker and this was replaced by the GFP gene that was amplified from the lentiviral vector JS1 (311) by PCR (forward primer: 5’ GAA TTC ACC GGT CGC CAC CAT 3’, reverse primer: 5’ ACT AGT GTC GAC CCC GGG CTC 3’). The GFP-PCR product was cloned into pCR2.1-TOPO TA cloning vector using the TOPO TA-cloning kit (Invitrogen), according to the suppliers’ protocol. The BamHI-KpnI fragment from this vector, encompassing the complete GFP gene, was subsequently cloned into pLKO.1 cut with the same enzymes to replace the puromycin resistance marker. GFP expression is under control of the pGK promoter that was already present in pLKO.1. The resulting plasmid was named pLKO.1-GFP. All constructs were verified by restriction enzyme analysis and GFP expression.

Cell culture

Human embryonic kidney 293T (HEK293T) adherent cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human SupT1 T cells were grown in suspension in Rosewell Park Memorial Institute (RPMI) medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell lines were cultured in a humidified chamber at 37°C and 5% CO2.

Lentiviral vector production and transduction

Lentiviral vectors were produced as previously described (345). In short, HEK293T cells were co-transfected with shRNA-expressing construct and the packaging plasmids pRSV-Rev, pMDLg/pRRE and pVSV-G using Lipofectamine 2000 (Invitrogen). One day after transfection the medium was refreshed and the following day the supernatant was harvested. The virus containing supernatant was centrifuged, filtered (0.45 μm), and aliquots were stored at -80°C. A sample was taken for CA-p24 enzyme-linked immunosorbent assay (ELISA) to monitor lentiviral particle production. SupT1 cells were seeded in a 24-wells plate (1 x 10^5 cells/well). Lentiviral vector (0.1, 1, 10 or 100 μl) was added and incubated overnight. Excess virus was washed away on the second day.
**Generation of clonal transduced cell lines**

For the generation of clonal cell lines, the lentiviral-transduced cells were serially diluted (10-fold) in conditioned medium (filter-sterilized supernatant from a 3 day culture of SupT1 cells, culture medium RPMI + 10% FCS). For every dilution a 96-wells plate was seeded and incubated for 2 weeks in a humidified chamber at 37°C and 5% CO2. When a plate showed cell growth in equal to or less than 30 of the 96 wells, the positive cultures were considered to represent clonal cell lines, based on Poisson distribution.

**Flow cytometry**

Cell samples for analysis of GFP expression were spun down at 1500 × g for 4 minutes and the cell pellet was gently resuspended in 200 µl FACS solution (phosphate buffered saline (PBS) + 2% FCS). Flow cytometry analyses were performed on a FACS Canto cytofluorometer (BD Biosciences). Live cells were discriminated from cell debris and dead cells based on physical parameters (forward and side light scatter). Fluorescence background levels were set with untransduced and unstained cells.

**Cell proliferation assays**

For the different proliferation assays, cells were FACS sorted based on GFP expression. The sorted cell lines and SupT1 cells were seeded in 96 wells plates at a density of 1 × 10^5 cells/ml in 100 µl per well. The proliferation of these cell lines was tested in four proliferation assays, which were performed in triplicate for automated and manual counting and in six-fold for the MTT and luciferase assays. In parallel the CCG assay was performed by mixing the sorted cultures with untransduced SupT1 cells and following the GFP percentage over a period of 5 days.

The CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used as follows. A 100 µl sample was taken daily for each cell line in six-fold over a period of 5 days and stored at -20°C until measurement following manufacturer’s instructions. In brief, 20 µl sample was mixed 1:1 with CellTiter-Glo reagent in a white half-well 96 wells plate for 10 minutes. The luciferase signal, which is directly proportional to the amount of metabolically active cells in the sample, was measured on a Glomax luminometer (Promega).

For automated cell counting, daily samples were taken. Cells clusters in the culture were disrupted by gently pipetting up and down and 100 µl of cell suspension was transferred to a FACS tube containing 200 µl FACS buffer. Each sample was counted for 20 seconds with identical FACS settings. The increase in number of live cells over time in the sample is a direct measure of cell proliferation. For manual counting, a 10 µl sample was taken daily and mixed 1:1 with Trypan Blue. 10 µl was loaded into a hemacytometer (Hycor) and cells were counted in 9 squares (0.1 mm^3) to determine the number of cells/ml.

The MTT assay was performed as described earlier (250). Each day for 5 days 30 µl MTT was added to the wells of a single plate for 4 hours at 37°C. The wells on the edge of the plate were excluded to prevent variation due to evaporation and wells filled only with medium served as a negative control. After incubation 100 µl was carefully removed from each well and 100 µl dissolving solution (0.1% Triton-X, 4 mM HCl in isopropanol) was added to dissolve the formazan crystals. Formazan absorbance was measured with an ELx808 microplate reader (Biotek Instruments) at 550 nm and background levels were scored at 650 nm.
Calculations and statistical analysis

To calculate the defect in cell growth rate measured in the CCG assay, with the assumption that the decrease in the ratio of GFP+ to GFP- cells over time is exponential, the following formula was used:

\[ G_d = \frac{(T_d(GFP^+)- T_d(GFP^-))}{T_d(GFP^-)*100\%} = - \frac{T_d(GFP^-)*\log_2(y)}{(x+ T_d(GFP^-))*\log_2(y)}*100\% \]

where \( G_d \) is the calculated growth defect, \( T_d(GFP^+) \) and \( T_d(GFP^-) \) are the doubling times in days of the GFP+ and GFP- cells, respectively, \( x \) is the number of days over which the decrease in GFP percentage is measured, and \( y \) is calculated as \( \%GFP^+/%GFP^- \) at the time point \( x \) divided by the \( \%GFP^+/%GFP^- \) at the time point 0.

CCG experiments were performed on at least two occasions, each in triplicate. Cell counting with FACS and a hemacytometer were also performed in triplicate and six-fold for the MTT and ATP bioluminescence assays. All data were corrected for variation between experiments using Factor Correction (298). For cell counting, MTT, and ATP bioluminescence assays, doubling times were calculated based on exponential growth curve fitting of the cell growth curves and represented as mean doubling time ± SD. To test whether doubling times were significantly different from the control culture (SupT1), 1-way ANOVA was performed with 95% confidence interval. For the CCG assay a student-T-test was performed on the percentage difference in doubling time, and compared to SHC1, again with 95% confidence interval.

Results

Design of the competitive cell growth assay

To establish an easy and sensitive assay to monitor changes in proliferation of transduced cells we developed the competitive cell growth (CCG) assay. This assay is based on the assumption that direct competition between transduced and untransduced cells will be the most sensitive and controlled way to compare growth differences. In fact, both untransduced and transduced cells will be present in the culture after transduction and this crude transduction mixture can thus be used directly in the CCG assay. This provides a direct the advantage over other proliferation assays, for which selection of the transduced cells is necessary before one can determine the impact on cell growth (Figure 4.1).

To critically test the competitive CCG assay we used several shRNA-expressing lentiviral vectors. Two control lentiviral vectors were used; SHC1 lacking a shRNA cassette (empty vector) and SHC2 expressing a scrambled shRNA molecule that has no identifiable mRNA target in human cells. Three shRNA constructs were selected that target specific cellular mRNAs and that were previously demonstrated – based on visual inspection of long-term cultures – to have either a small effect on cell proliferation (shRNA1), an intermediate effect (shRNA2) or a severe effect (shRNA3). We currently do not know whether this toxicity is due to downregulation of the specific mRNA target or due to an unspecific off-target effect. However, the impact on cell physiology was reproducible, which made them excellent candidates to validate the CCG assay.
Lentiviral vectors encoding these shRNAs were generated and used to stably transduce the SupT1 T cell line. A fixed amount of SupT1 cells was transduced with 0.1, 1 or 10 µl of the lentivirus stocks. The titer of all vectors used in this study was comparable (data not shown). These cultures were maintained as normal SupT1 cell lines (1 in 10 split twice weekly) over a period of 26 days. Two days post transduction one usually determines the percentage of transduced, GFP-positive cells, which can be FACS-sorted for further experimentation. However, the CCG assay is performed on the raw transduction mixture of transduced and untransduced cells. This cell mixture is simply maintained and periodically scored for the percentage of GFP-positive cells.

The percentage of GFP expressing cells in the control cell lines SHC1 and SHC2 was stable over time, indicating a lack of toxicity in the transduced cells (Figure 4.2A, upper panels). These results also indicate that integration of the lentiviral vector into the host cell genome (SHC1) and the induction of the RNAi pathway (SHC2) have no effect on the cell proliferation capacity in this setting. However, a steady reduction in the percentage of GFP-expressing cells was observed over time when a vector encoding a shRNA against specific targets was used. The reduction was small in the case of the least toxic shRNA1 and increased when more toxic shRNAs were expressed (Figure 4.2A, lower panels). This reduction was observed with all three lentiviral vector inputs, thus no careful titration is needed to score toxic effects.

When one wants to determine quantitative differences, the growth defect of the GFP+ cells can be calculated based on the known doubling time of SupT1 cells, the GFP+/GFP- cell ratio at the start and end of the experiment and the time period. For each cell line and for each lentiviral vector input, we calculated the percentage growth rate defect based on a doubling time of 1.1 days for SupT1 cells. This doubling time is the average of values scored in the four alternative cell proliferation assays (Figure 4.5B, lower panels). The most prominent differences were observed for shRNA3 with 10 µl lentiviral vector input. The shRNA3 cells proliferated 21 ± 6% slower than the SupT1 control cells in the same culture (Figure 4.2B). For shRNA1 and shRNA2 also significant differences were measured when compared to SupT1 cells, with 11 ± 1% and 11 ± 0.5% reduction in growth rate, respectively. With a lower lentiviral vector input, the shRNA-expressing cells again exhibited a reduced proliferation rate, but this difference is only significant for the most toxic shRNA3. No growth rate differences were observed between SupT1 and the two control cell lines SHC1 and SHC2 (Figure 4.2B).
A competitive cell growth assay for the detection of subtle effects of gene transduction on cell proliferation

Figure 4.2. Calibration of the CCG assay
A. SupT1 cells were transduced with 0.1, 1 and 10 µl of lentiviral vector. Cells were cultured and passed as normal twice weekly and samples were taken for FACS analysis. Control cell lines SHC1 (empty vector) and SHC2 (expressing a scrambled shRNA) are shown in the upper panels and show a stable percentage of GFP-expressing cells (y-axis) over time (x-axis). Lentiviral vectors expressing shRNAs against three cellular factors (lower panels) show a gradual decrease in the number of GFP+ cells. The experiment was performed twice and one representative experiment is shown. B. The percentage difference in growth rate between SupT1 and GFP+ cells was calculated based on two experiments. No significant differences were measured in the control cell lines SHC1 and SHC2 when compared to SupT1. All shRNA-expressing cell lines showed a significant slower proliferation rate with 10 µl lentivirus input. For the most toxic shRNA3 this difference was significant even at a reduced lentiviral vector input (0.1 and 1 µl).

The CCG assay is compatible with high-throughput screening. When many transduced cultures have to be analyzed in parallel, one simply maintains the transduction mixtures in a 24 or even a 96 wells format. Many flow cytometers are equipped with a plate reader, thus allowing the screening of hundreds of cultures within an hour. For instance, we analyzed 19 shRNA-expression lentiviral vectors, including the controls SHC1 and SHC2 at a low (1 µl) and high (100 µl) transduction level (Figure 4.3). The shRNAs are shown in increasing order of cytotoxicity, shRNA4 being the least cytotoxic with a 4% decrease in
proliferation rate compared to SupT1, and shRNA20 showing the biggest decline in proliferation rate; growing 23% slower than SupT1. These cell lines were analyzed once every week, with a maximal assay time of 1 hour.

A

Days post transduction

% GFP positive

shRNA4

shRNA5

Control: SHC1

Control: SHC2

shRNA6

shRNA7

shRNA8

shRNA9

shRNA10

shRNA11

shRNA12

shRNA13

shRNA14

shRNA15

shRNA16

shRNA17

shRNA18

shRNA19

shRNA20

1 µl lentiviral vector

100 µl lentiviral vector

B

Cell growth defect (%)

SHC

shRNA

1 µl Lentiviral vector

100 µl Lentiviral vector
A competitive cell growth assay for the detection of subtle effects of gene transduction on cell proliferation

Figure 4.3. High-throughput analysis of GFP-positive cell lines
A. SupT1 cells were transduced with 1 or 100 µl lentiviral vector, expressing shRNAs against different cellular mRNAs or with the control lentiviral vectors SHC1 and SHC2. The cells were passaged normally and weekly samples were taken for FACS analysis. The percentage of GFP-positive cells (y-axis) per shRNA is shown, with the 1 µl transduced samples (triangles) and the 100 µl transduced samples (circles). Cell lines were followed for 33 days post transduction (x-axis). B. The cell growth defect was calculated for each transduced cell mixture.

Decrease in percentage of GFP-expressing cells is due to outgrowth of non-transduced cells

We reasoned that the decrease in percentage of GFP-expressing cells results from outgrowth of untransduced cells, but it cannot formally be excluded that GFP-positive cells loose transgene expression over time. Such transgene silencing has been reported on many occasions (106;152). Although the polymerase III-driven shRNA cassettes seem to be rather sturdy (346), the polymerase II driven GFP unit may be affected. To distinguish between these two possibilities, a new batch of cells was transduced with shRNA3 using a high lentiviral vector input. After sorting, this culture was 99.8% GFP-positive, but also in this culture the percentage of GFP-positive cells decreased after 40 days. Clonal transduced cells were generated by limiting dilution on day 48 post transduction and the parental culture was frozen. The clonal cells and the thawed parental culture were analyzed for GFP expression over a period of 13 days, which corresponds to 68-99 days post transduction for the clonal cells and dag 61-74 for the parental culture. FACS analysis on day 13 of the experiment indicated the expected loss of GFP-positive cells in the parental culture, but no loss of GFP expression was observed in the two clonal cells (Figure 4.4). We therefore conclude that the decrease in percentage of GFP-expressing cells in the CCG assay is due to outgrowth of untransduced over transduced cells.

Figure 4.4 Decreased GFP-expression is due to outgrowth of untransduced cells
Clonal GFP+ cells were generated from the mixed cell culture expressing shRNA3. After the generation of clonal cells via limiting dilution, the percentage of GFP-expressing cells was monitored over a time period of 13 days; light grey line (day 1) and black line (day 13). A. The percentage of GFP-expressing cells decreases in the original mixture of transduced and untransduced culture. B and C. Two GFP+ clonal cell lines demonstrate a constant high level of GFP transgene expression.
Comparison of the CCG assay with other well-established methods

A variety of methods can be used to measure the impact of a gene therapy treatment on cell proliferation. Here we compared the new CCG assay with several well-established methods. Cells transduced with 100 µl lentiviral vector were FACS sorted for GFP expression and subsequently expanded. This directly reveals an advantage of the CCG assay, where these extra steps are not required (Figure 4.1). Five different cell proliferation assays, including the CCG test, were performed in parallel over a period of 5 days. The CCG assay was performed with a mix of the sorted cells in a 1:1 ratio with untransduced SupT1 cells. A sample was analyzed daily by flow cytometry to determine the decrease in GFP percentage (Figure 4.5A). The percentage of GFP+ cells remained constant in the control cultures SHC1 and SHC2, but decreased due to shRNA1, shRNA2 and shRNA3 expression. The percentage difference in growth rates were compared to SHC1 (Figure 4.5B, upper panel). The difference in growth rates were significant higher for shRNA1, shRNA2 and shRNA3, with shRNA1 being the least toxic with a 9 ± 2% slower growth rate. A 34 ± 5% slower growth rate was measured for shRNA3 when compared to SHC1 mixed culture. Of the four alternative proliferation assays, the easiest way to measure cell proliferation in culture is by counting the number of cells over time. This can be done manually, using a hemacytometer in combination with trypan blue staining of dead cells in the culture sample (“manual counting” in Figure 4.5A). Although easy to use, this is a laborious method that is not applicable for high-throughput screening. We also determined the number of live cells by flow cytometry (“automated counting” in Figure 4.5A). A fixed volume (100 µl) of cell culture was analyzed daily. This method allows one to distinguish live from dead cells and the increase in number of cells provides a measure of cell proliferation. Furthermore, the procedure is suitable for high-throughput experiments. Some proliferation assays measure the increase in metabolic activity due to an increase in cell number. The most well-known test is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay (250). The yellow dye MTT is converted to purple formazan crystals when added to the cell culture and the absorbance can be quantified with a spectrophotometer. This conversion only takes place when reductase enzymes are active, thus only when live cells are present in the culture. The more live cells in a culture, the more purple formazan is produced, resulting in higher absorbance values over time (“MTT” in Figure 4.5A). We also tested an ATP bioluminescence assay, that measures the ATP content of a sample, which is an indicator for metabolically active cells (79). A sample is taken daily and mixed with the assay reagent containing luciferase, and the light signal is measured in a luminometer. The luciferase signal is proportional to the ATP present in the sample and thus the amount of metabolically active cells (“ATP” in Figure 4.5A). A single representative curve is shown for each of the four assays (Figure 4.5A, lower panels). To compare the proliferation assays, the doubling time of each cell line was calculated based on two experiments and normalized to SupT1, of which the doubling time was set at 1 (Figure 4.5B). The doubling times of the cell lines show a clear trend where shRNA3 is causing a cell growth delay, but these differences were not significant except for the manual counting method. No significant differences in cell growth could be scored for the weak and intermediate toxic shRNA1 and shRNA2. Thus, the CCG assay is clearly most sensitive as significant cytopathic effects were reproducibly measured for all three shRNAs.
A competitive cell growth assay for the detection of subtle effects of gene transduction on cell proliferation

Figure 4.5. Comparison of the CCG assay with other cell proliferation assay A. Sorted GFP+ cells were mixed with SupT1 and the percentage GFP+ cells was measured daily over a period of 5 days. While in SHC1 and SHC2 mixed culture the percentage is constant, in the shRNA1, shRNA2 and shRNA3 mixtures the GFP percentage decreases (upper panel). Sorted GFP+ cells were used in 4 proliferation assays; manual counting, automated counting, MTT assay and an ATP-bioluminescence assay. For every cell line growth curves were obtained for each specific assay. One representative experiment for each assay is shown. B. For the CCG assay the percentage decrease in proliferation rate compared to SupT1 shown (upper panel). Statistical analysis shows significant slower proliferation rate in the case of shRNA1, shRNA2 and shRNA3 when compared to the SHC1 mixed culture. With the growth curves of two independent experiments the relative doubling times for each cell line was calculated. Doubling time of SupT1 was set at 1. When compared to SupT1 in the manual counting assay, shRNA3 has a significant increased doubling time. Results are from two independent experiments, performed in triplicate (manual and automated counting) or six-fold (MTT and ATP bioluminescence assays). Explanation of the symbols: ns: not significant, **: P < 0.01 and ***: P < 0.001.
Discussion

In this study we describe a new method to assess small changes in the proliferation rate of cells treated with a viral vector harboring a fluorescent selection marker, e.g. GFP. This method, the competitive cell growth or CCG assay, is based on scoring the differences in proliferation rates between transduced and untransduced cells in the same culture. As transduced cells express GFP in addition to a (therapeutic) transgene, the loss in percentage of GFP-positive cells over time is a direct measure of the cell proliferation defect of transduced cells compared to untransduced cells. The CCG assay has some advantages over other well-established cell proliferation assay. First of all, the raw transduction mixture can be tested directly, meaning that no extra steps of selection and expansion of transduced cells are necessary. Importantly, the CCG assay is internally controlled as it is performed with a mixture of untransduced and transduced cells. The CCG assay is user-friendly, as no precise ratio of transduced over untransduced cells is required. There is an obvious lower limit of the assay, below approximately 5% GFP+ cells, to allow a reliable measurement of an altered GFP+/GFP- ratio (data not shown). Too high transduction efficiencies may be dangerous in the sense that multiple vector integrations may occur, which will result in increased shRNA expression levels. Indeed, such increased toxicity was scored for high-level transduction of the shRNAs and even the SHC2 scrambled shRNA control exerted some toxic effects when overexpressed. We scored such effects when over 95% of the cells were transduced.

A major advantage of the CCG assay is its extreme sensitivity. We measured a significant decrease in cellular proliferation rates when toxic shRNAs are expressed, compared to the control cell lines SHC1 and SHC2. A 10% reduction in proliferation rate can be significantly detected in the CCG assay, and although all tested proliferation assays show similar shRNA-toxicity, only manual counting revealed a significant toxic effect of the most toxic shRNA3. In the CCG assay even smaller growth defects can be observed. Although some of these values may not be statistically significant, the fact that the percentage of GFP+ cells decreases over time is a strong indication of a minor proliferation defect. For some of the shRNA cassettes we have been able to correlate cell growth problems as measured in vitro with the CCG test with in vivo results in humanized mouse models (unpublished results). The CCG assay is not limited to human T cell lines or shRNA research. Other cell types can be used, both suspension and adherent cells and even primary cells, at least when a certain level of proliferation is observed in 5 days. In case of primary cells, one should keep in mind that a culture of primary cells often consist of different subpopulations, each with a different transducibility. If GFP measurement over time can be combined with FACS analysis of cell type specific markers, one could even monitor growth of the different cell types in a single assay. It remains important to include proper controls, such as an empty lentiviral vector. In this study we scored for the impact of shRNA expression on cell growth, but any transduction event can be assessed in the CCG assay, for instance cellular problems due to vector integration or transgene expression. Not only a decrease in cell proliferation can be measured, an increase in percentage of GFP+ cells over time would indicate an increased cell proliferation rate. This may reveal oncogenic changes in the transduced cells. In exceptional cases the shRNA could have an off-target impact on the GFP reporter, which could be misinterpreted as cellular toxicity. However, such an effect will cause a reduced GFP intensity, which was not observed for the clonal cells (Figure 4.4).
An increase in percentage of GFP$^+$ cells may be observed when the transgene provides a selective advantage under the culture conditions applied. One should be cautious when the transgene can have an effect on untransduced bystander cells in the mixed culture. We used lentiviral vectors to stably transduce the shRNA cassette, but other vector systems, integrating (retroviral) or non-integrating (e.g. adenovirus or adeno-associated virus) are compatible with the transient CCG test. Therefore we propose that the CCG assay is a welcome addition to the existing array of proliferation assays, especially when one is interested in scoring subtle effects on cell growth due to stable transgene expression.

**Acknowledgements**

This research was supported by the Dutch AIDS fund (grant 2006006 and 2007028). We thank Berend Hooibrink for FACS sorting, Stephan Heynen for CA-p24 ELISA experiments and Renée van der Sluis and Dave Speijer for useful discussions. We also thank the Belgian Federal Government for financial support through the Inter-University Attraction Pole grant P6/41.
Supplement: Derivation of the equation

Let us assume exponential cell growth, then:

\[
\begin{align*}
(1) \quad N_{\text{GFP}^+}(t) &= N_{0\text{ GFP}^+} \times 2^{t/T_{d^+}} \\
(2) \quad N_{\text{GFP}^-}(t) &= N_{0\text{ GFP}^-} \times 2^{t/T_{d^-}}
\end{align*}
\]

where \( N_{\text{GFP}^+}(t) \) and \( N_{\text{GFP}^-}(t) \) are absolute numbers of GFP+ and GFP- cells at the time \( t \), \( N_{0\text{ GFP}^+} \) and \( N_{0\text{ GFP}^-} \) are the initial numbers of cells, \( T_{d^+} \) and \( T_{d^-} \) are the doubling times of GFP+ and GFP- cells, respectively.

Let us also assume that \( N_{\text{GFP}^+}/N_{\text{GFP}^-} \) decays exponentially:

\[
(3) \quad N_{\text{GFP}^+}/N_{\text{GFP}^-}(t) = N_{0\text{ GFP}^+}/N_{0\text{ GFP}^-} \times 2^{-k t}
\]

where \( k \) can be calculated if we know \( y = (N_{\text{GFP}^+}/N_{\text{GFP}^-}(x)) / (N_{0\text{ GFP}^+}/N_{0\text{ GFP}^-}) = (%_{\text{GFP}^+}/%_{\text{GFP}^-}(x)) / (%_{\text{GFP}^+}/%_{\text{GFP}^-}(0)) \), at a certain time point \( x \), as follows:

\[
\begin{align*}
y &= 2^{k x} \\
(4) \quad k &= -\log_2(y)/x
\end{align*}
\]

Knowing \( T_{d^-} \), \( x \), and \( y \), we can solve the equations (1)-(4) as follows:

\[
\begin{align*}
&N_{0\text{ GFP}^+} \times 2^{t/T_{d^+}} / N_{0\text{ GFP}^-} \times 2^{t/T_{d^-}} = N_{0\text{ GFP}^+} / N_{0\text{ GFP}^-} \times 2^{\log_2(y)/x t}, \text{ or} \\
&2^{t/T_{d^+}} = 2^{t/T_{d^-}} \times 2^{\log_2(y)/x t}, \text{ or} \\
&t/T_{d^+} = t/T_{d^-} + \log_2(y)/x t, \text{ or} \\
&1/T_{d^+} = 1/T_{d^-} + \log_2(y)/x, \text{ or} \\
&T_{d^+} = 1/(1/T_{d^-} + \log_2(y)/x)
\end{align*}
\]

Then, the cell growth defect can be calculated as follows:

\[
\begin{align*}
Gd &= ((T_{d^+} - T_{d^-})/T_{d^-}) \times 100\% = \\
&= 1/(1/T_{d^-} + \log_2(y)/x) - T_{d^-})/T_{d^-}) \times 100\% = \\
&= (1/(1 + T_{d^-} \times \log_2(y)/x) - 1) \times 100\% = \\
&= (x/(x + T_{d^-} \times \log_2(y)) - 1) \times 100\% = \\
&= - T_{d^-} \times \log_2(y)/(x + T_{d^-} \times \log_2(y)) \times 100\%
\end{align*}
\]
Chapter 5
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

Julia J.M. Eekels¹, Sophie Sagnier², Dirk Geerts³, Rienk E. Jeeninga¹, Martine Biard-Piechaczyk² and Ben Berkhout¹

Submitted for publication

¹Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam

²Centre d’études d’agents Pathogènes et Biotechnologies pour la Santé, CPBS UMR5236 CNRS UMSE, France

³Department of Human Genetics, Academic Medical Center of the University of Amsterdam
Abstract

Autophagy is a cellular process leading to the degradation of cytoplasmic components such as organelles and intracellular pathogens. It has been shown that HIV-1 relies on several components of the autophagy pathway for its replication, but the virus also blocks late steps of autophagy to prevent its degradation.

We generated stable knockdown T cell lines for 12 autophagy factors and analyzed the impact on HIV-1 replication. RNAi-mediated knockdown of 5 autophagy factors resulted in inhibition of HIV-1 replication. Autophagy analysis confirmed a specific defect in the autophagy pathway for 4 of these 5 autophagy factors. We also scored the impact on cell viability, but no gross effects were observed. Upon simultaneous knockdown of 2 autophagy factors (Atg16 and Atg5), an additive inhibitory effect was scored on HIV-1 replication. Stable knockdown of several autophagy factors inhibit HIV-1 replication without cytotoxicity. We therefore propose that targeting autophagy can be a novel therapeutic approach against HIV-1.

Introduction

Autophagy is a cellular process leading to the degradation of cytoplasmic components, such as long-lived proteins and organelles (246). The process starts with the engulfment of portions of the cytoplasm within a phagophore, eventually forming a double-membrane organelle called the autophagosome (Figure 5.1). The autophagosome subsequently fuses with lysosomes and the contents are degraded. Autophagy is mostly known as a cellular recycling mechanism in the event of nutrient starvation, but the process has also been implicated in i.e. developmental control, tissue homeostasis, tumor suppression and antigen-presentation (184;197;279;280). Autophagy has several functions in immunity, as it not only eliminates cellular components, but intracellular pathogens like viruses as well. Not surprisingly, several viruses have evolved countermeasures to evade or neutralize this pathway (110;321). For example, herpes simplex virus 1 (HSV-1) blocks two steps in the autophagy pathway with a single viral protein: ICP34.5, thereby preventing degradation of newly formed virus (264;339).

On the other hand, some viruses need the autophagy mechanism to complete their replication cycle. Several positive-stranded RNA viruses such as poliovirus remodel intracellular membrane structures as scaffolds for their replication machinery (162). These membranous structures are thought to be autophagic vacuoles. For influenza A virus, two studies highlight two different aspects of the complex interaction between the invading virus and autophagy. One study reported that the intracellular concentration of autophagy marker protein LC3-II increased during influenza virus infection and pharmacological inhibition of autophagy reduced the viral titers, indicating that influenza requires autophagy (411). However, it has also been shown that influenza arrests autophagosome degradation, for which the viral M2 protein is solely responsible. This block of autophagy makes the infected cells are more susceptible to apoptosis (126).

In case of the human immunodeficiency virus type 1 (HIV-1), it is not clear to what extent autophagy influences the viral replication cycle, or whether the virus influences the autophagy pathway. It has been reported that the expression of the HIV-1 Envelope protein (Env) on the surface of infected cells induces autophagy in bystander cells through
gp41-mediated membrane fusion (90). The induction of autophagy subsequently leads to the death of these uninfected cells (90;111). This mechanism has been used to explain the so-called “bystander-effect”, which is the massive depletion of uninfected cells in HIV-1 infected individuals. Two studies also indicated that HIV-1 inhibits autophagy in the infected CD4+ T cell, shown by reduced expression of the two autophagy marker proteins LC3 and Beclin1 and analysis of infected cells by electron microscopy (112;405). Furthermore, the viral Nef protein prevents destruction of HIV-1 components in autolysosomes, thus blocking the antiviral role of autophagy in macrophages (189).

Several autophagy factors were identified in a transient genome-wide RNAi screen for cellular co-factors of HIV-1 replication (53), suggesting that HIV-1 also needs autophagy or at least some autophagy components to complete its replication cycle. Indeed, stable knockdown of one of the identified autophagic co-factors, Atg16, resulted in long-term inhibition of HIV-1 replication (103). If HIV-1 indeed requires autophagy for its replication, inhibiting the pathway could be of therapeutic use. Since the virus relatively easily gains resistance against drugs targeting viral components, it has been hypothesized that targeting of cellular co-factors would make it more difficult for the virus to gain resistance (22;78). Therefore we sought to inhibit several autophagy factors (ATGs) via RNA interference (RNAi). Stable knockdown cell lines were generated, each cell line expressing a short hairpin RNA (shRNA) against mRNA encoding an autophagy factor. Thirteen autophagy factors were included, distributed along different steps of the autophagy pathway (Figure 5.1). Atg1/unc-51-like kinase (ULK1) is required for initiation of autophagy, and autophagosome biogenesis is coordinated by a complex containing Beclin1, class III phosphatidylinositol 3-kinase (PIK3C3) and the PI3K p150 subunit (PIK3R4). WIPI1 regulates the transport of phosphatidylinositol-3-phosphate to the membranes. Two ubiquitin-like conjugation complexes are required; the first one forms a complex between Atg5 and Atg12, and Atg16 is non-covalently bound to this complex. This conjugation is catalyzed by Atg7 and Atg10. In the second conjugation system, LC3 is cleaved by Atg4 cystein proteases, essentially Atg4A and Atg4B, making it possible for Atg7 and Atg3 to generate the phosphatidylethanolamine (PE)-bound form of LC3: LC3-II.

We show that HIV-1 replication can be delayed in stable ATG knockdown cell lines. Additive effect on inhibition of HIV-1 replication was observed when 2 ATGs were knocked down simultaneously, thus stressing the therapeutic potential of this strategy. Importantly, this HIV-1 replication delay was not accompanied by RNAi-induced cytotoxicity, suggesting autophagy can be targeted in host cells without serious side effects.
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

**Figure 5.1. Autophagy factors and their function in the autophagy pathway**

Autophagy can be induced by e.g. starvation signals. Two complexes are needed to form the phagophore. One includes ULK1, the other the class III phosphatidylinositol 3-kinase (PIK3C3), PIK3R4 and Beclin 1, together with WIPI1. To form the autophagosome, two conjugation systems play a role. The Atg12-Atg5 system forms a complex with non-covalently bound Atg16. The conjugation of LC3-I (LC3 cleaved by Atg4) with PE generates LC3-II. This process requires Atg7 and Atg3. The Atg12-Atg5-Atg16 complex is detected only during the membrane formation stage; LC3-II is detected at each step of autophagosome formation. The autophagy inhibitor 3-methyladenine (3-MA) acts on the class III phosphatidylinositol 3-kinase.

**Materials and methods**

**DNA Constructs**

pLKO.1 DNA constructs expressing a specific shRNA were from the MISSION TM TRC-Hs 1.0 library (293). Constructs including the negative control constructs SHC001 and SHC002 (hereafter named SHC1 and SHC2) were obtained from Sigma-Aldrich as bacterial clones. Plasmid DNA was extracted using the Nucleobond Midiprep columns according to the manufacturer’s instructions (Macherey-Nagel). Target sequences can be found on the website of Sigma-Aldrich [http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html]. The pLKO.1 constructs from the MISSION TM TRC-Hs 1.0 library contain a puromycin selection marker, which was replaced with the gene for enhanced eGFP (eGFP) as described earlier (Eekels et al, Chapter 4).
**Chapter 5**

**Chemicals**

The T1249 peptide (WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF, Pepscan Therapeutics) was obtained as 10,000 x stock solution (Eggink et al., 2008). The autophagy inhibitor 3-MA (Sigma-Aldrich) was diluted in 70% methanol and used at a final concentration of 10 mM. Protease inhibitors pepstatin A and E64d and the anti-LC3 antibody were purchased from Sigma-Aldrich.

**Cell lines**

The human embryonic kidney cell line HEK293T was grown in DMEM, supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. The human T cell line SupT1 was cultured in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Lentiviral vector production and generation of stable knockdown cell lines

Lentiviral vectors were produced as described earlier (345). In short, HEK293T cells were co-transfected with pLKO.1-shRNA and the packaging plasmids (pVSV-G, pMDL and pRev-RRE) with Lipofectamin 2000 (Invitrogen). The medium was refreshed one day after transfection and the culture supernatant was harvested the next day. Aliquots of the culture supernatant with the lentiviral vectors were stored at -80 °C. A sample was tested in CA-p24 ELISA.

SupT1 cells were seeded in 24-wells format at 1 × 10^5 cells/well and transduced with a fixed amount of lentiviral vector. Excess virus was washed away one day after transduction and selection of stably transduced cells was started by adding puromycin to the medium at a final concentration of 1 µg/ml. In the case of GFP-expressing cell lines, transduced cells were FACS sorted.

**CA-p24 ELISA**

Culture supernatant was heat-inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as the capture antibody and the alkaline phosphatase-conjugated anti-CA-p24 monoclonal antibody EH12-AP (International Enzymes) as the detection antibody. Detection was performed with the Lumiphos plus system (Lumigen) in a LUMIstar Galaxy luminescence reader (BMG Labtechnologies). Recombinant CA-p24 produced in a baculovirus system was used as reference standard.

**HIV-1 replication and single cycle infection**

The HIV-1 molecular clone HIV-1LAI (270) was used to produce virus by transfection of HEK293T cells. HIV-1 production was measured by CA-p24 analysis in the culture supernatant. For HIV-1 replication studies, SupT1 cells were seeded in a 6-wells plate at 4 × 10^5 cells/well and infected with HIV-1 (0.2 ng CA-p24). HIV-1 replication was monitored by scoring for syncytia formation and longitudinal measurement of CA-p24 production in the culture supernatant.

For single cycle infection experiments SupT1 cells were incubated with HIV-1 for four hours. Excess virus was washed away and the cells were cultured in the presence of entry inhibitor T1249 (Pepscan) to block subsequent rounds of viral entry. Intracellular CA-p24
was analyzed by FACS and extracellular CA-p24 was measured by ELISA at 48 hours post infection.

**Competitive cell growth assay and flow cytometry**

To assess the cytotoxicity induced by knockdown of autophagy factors, we used the competitive cell growth or CCG assay as described earlier (Eekels et al, Chapter 4). In brief, SupT1 cells were transduced with 0.1 or 1 µl lentiviral vector that expresses a shRNA and the GFP selection marker. The percentage of cells expressing GFP in the transduction mixture was analyzed longitudinally by FACS analysis. Twice weekly a sample of the culture was taken, cells were collected by centrifugation (4 min at 4000 rpm, Eppendorf centrifuge) and resuspended in FACS solution (Phosphate buffered saline (PBS) + 2% FCS) and analyzed on FACSscanto (BD Biosciences). The live cell population was determined with forward and side scatter. Fluorescence was normalized using unstained SupT1 cells. Based on the known doubling time of untransduced cells, the change in GFP+/GFP- ratio over the course of the experiment can be used to calculate the cell growth defect (%) of the GFP+ transduced cells (Eekels et al, Chapter 4).

For intracellular CA-p24 staining, cells were collected by centrifugation (4 min at 4000 rpm, Eppendorf centrifuge) and fixed in 250 µl 4% formaldehyde for 5 min at room temperature. Cells were permeabilized with 500 µl BD Perm/WashTM buffer (BD Pharmingen) and stained for at least 1 hour at 4°C in 50 µl BD Perm/WashTM buffer containing 5 µl 1:100 diluted antibody against CA-p24 conjugated with PE (monoclonal mouse, clone KC57, Coulter). Excess antibody was washed away by washing twice with 500 µl FACS solution, cells were resuspended in 250 µl FACS solution and analyzed on a FACScanto. Uninfected and unstained samples were used as negative controls.

**RT-qPCR**

mRNA knockdown levels for specific ATG targets were analyzed by RT-qPCR. RNA was isolated from 0.5 × 10^6 cells with the RNeasy kit (Qiagen) according to the manufacturer’s protocol, including the DNase I treatment on the column. 1 µg RNA was used for reverse transcription (Thermoscript, Invitrogen) with Oligo-dT primers and cDNA synthesis was performed at 50°C. The cDNA was diluted 100 times and 5 µl of the diluted sample was used as template in a SYBR Green based RT-qPCR (SYBR Green FAST PCR, Qiagen) with an ABI Prism 7000 detection system (Applied Biosciences). Primers for target genes and the internal control β-actin were from the Quantitect primer assays (Qiagen). The ΔΔCt method was used to calculate relative mRNA expression as described earlier (222).

**Analysis of autophagy**

Autophagy was induced by nutrient starvation (EBSS) for 2 hours in presence or absence of the lysosome protease inhibitors E64d and pepstatin A (10 µg/ml each) to analyze the autophagy flux. To monitor the induction of autophagy, the relative amount of the PE-conjugated form (LC3-II) was determined by immunoblot analysis of whole-cell lysate using a rabbit polyclonal antibody against LC3. Cells were washed twice in PBS and lysed in buffer containing 50 mM Tris-HCl (pH 8), 1% Triton X-100, 100 mM NaCl, 1 mM MgCl2, 150 mM PMSF, and complete mini protease inhibitor cocktail (Roche Diagnostics). Cell lysates were electrophoresed in 12% SDS-PAGE and blotted to PVDF membranes. After a blocking
step with PBS and 0.5% casein for 1 hour at room temperature, blots were incubated overnight at 4°C with the anti-LC3 antibody in the blocking buffer. After 3 washes with PBS and 0.05% Tween, the blots were incubated for 1 hour at room temperature with peroxidase-coupled antiserum diluted in blocking buffer. After further washes, the immune complexes were revealed by ECL (Millipore). The image capture was taken by the G:BOX camera system (Syngene) and intensity of the signals was analyzed with GeneTools software. The LC3-II signal was compared to that of the control housekeeping protein GAPDH.

Results

**Stable knockdown of ATG proteins inhibits HIV-1 replication**

To test whether stable knockdown of individual autophagy factors has an effect on HIV-1 replication, we generated cell lines expressing a shRNA against mRNA encoding one of the 12 autophagy factors (ATGs). Per autophagy factor 4 or 5 shRNAs were tested, resulting in 61 cell lines including 2 controls. The controls were the empty lentiviral vector SHC1 and the vector encoding a scrambled shRNA without a known mRNA target (SHC2). The testing of multiple shRNAs per ATG has several advantages. First, it allows one to score a similar phenotype for different shRNAs that target the same factor, which helps to determine whether the effect is specific. Second, as different shRNAs provide different knockdown efficiencies, the chance that at least one shRNA induces a sufficient knockdown of the specific target increases. A relatively high multiplicity of infection (MOI) for the lentiviral vectors was used, to increase the chance of observing antiviral activity. No shRNAs were available against LC3, so the shRNAs against GABARAPL1, a paralogue of LC3 that has been shown to function in autophagy (67), were included in the screen. All cell lines were tested for inhibition of HIV-1 replication in three independent experiments performed in duplicate. Stable cells were challenged with HIV-1 and the accumulation of CA-p24 in the culture supernatant was followed. We measured the average CA-p24 concentration at peak infection, 10 days post infection. In 13 cell lines at least a log decrease in CA-p24 levels and thus virus replication was observed. Inhibition was measured for the shRNAs ULK1-1 and 4, WIPI1-1 and 3, Beclin1-3, PIK3R4-3, Atg3-3, Atg4A-1 and 3, Atg5-4, Atg10-3 and 5 and Atg12-4 (Figure 5.2).
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

**Figure 5.2. Screen of shRNAs against 12 autophagy factors**

Stable knockdown cell lines with shRNAs against 12 ATGs were generated and challenged with HIV-1. The concentration of viral CA-p24 protein (ng/ml) in the culture supernatant at peak infection (day 10) is plotted. Results represent the average of three independent experiments that were performed in duplo.

To confirm these results, newly transduced cells with the suppressive shRNAs were again challenged with HIV-1 virus. Replication was followed over a period of 11 days by CA-p24...
measurement in the culture supernatant. The inhibition was confirmed for the shRNAs WIP1-1 and Beclin1-3 (Figure 5.3A) and in an independent experiment for PIK3R4-3, Atg4A-1 and 3, Atg5-4, Atg10-3 and 5 and Atg16-4 (Figure 5.3B). The Atg16 results do confirm earlier results on specific inhibition of HIV-1 replication (103). Further experiments were conducted with the 9 shRNAs against 7 different ATGs that showed an inhibitory effect in multiple experiment performed with two independently transduced cell samples.

For the selected shRNAs we first determined the mRNA knockdown of the specific ATG co-factor by RT-qPCR (Figure 5.3C). The results demonstrate a good knockdown (60-80%) for PIK3R4, Atg5, Atg10-3 and Atg16. A modest reduction in mRNA level (40-60%) was scored for Beclin1, Atg4A-1 and 3 and Atg10-5. For WIP1 only a small reduction in mRNA expression (less than 20%) was measured. These results suggest that the impact on HIV-1 replication is due to specific knockdown of the autophagy factors, but it is also apparent that the knockdown efficiency required for HIV-1 inhibition may vary per co-factor.

We determined the effect of silencing of the different ATGs on cellular autophagy by analyzing the level of LC3-II (LC3 bound to autophagic membranes) in the different cell lines. The level of LC3-II was analyzed under nutrient-rich and starvation conditions in the presence or absence of anti-proteases to analyze the autophagic flux. Starvation signals lead to conversion of LC3-I to LC3-II and the presence of protease inhibitors prevents lysosomal degradation such that LC3-II accumulates in the cell. Starvation-induced autophagy is functional in cell lines expressing WIPI, Beclin1 and Atg10 shRNAs. Results are summarized in Table 1. In contrast, the autophagy flux is impaired in cells expressing PI3KR4, Atg5 or Atg16 shRNAs. The autophagy process is dramatically altered when expression of Atg4A is shut down.

To score for effects on cell proliferation, we performed the competitive cell growth or CCG assay (Eekels et al, Chapter 4). To that purpose the puromycin selection marker in the lentiviral constructs was replaced by the eGFP marker that allows the detection of live transduced cells. Upon lentiviral-mediated transduction, the mixed culture of GFP+ and untransduced GFP- cells is followed longitudinally by FACS analysis. If transduced cells exhibit a delayed cell growth, this will result in a gradual decrease in the percentage of GFP+ cells over time, as non-transduced cells will outgrow the transduced cells. Based on the known doubling time of untransduced GFP- SupT1 cells (1.1 days, Eekels et al, Chapter 4), the decrease in GFP+/GFP- ratio over time can be used to calculate the relative cell growth capacity, with the proliferation of SHC1 cells set at 100% (Figure 5.3D). Knockdown of Atg5 has no effect on cell proliferation. For 5 shRNAs (PIK3R4, both for Atg4A and both for Atg10) a small cell growth defect of less than 10% was observed. Three shRNAs (Beclin1, WIP1 and Atg16) induce a more significant cytotoxicity with a 10-20% reduced cell growth rate.
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

Figure 3. Analysis of HIV-1 replication, ATG-mRNA levels and cell growth in stable knockdown cells

Inhibition of HIV-1 replication as observed in Figure 2 was confirmed in newly generated knockdown cells for Beclin1 and WIPI1. CA-p24 concentration was followed for 10-11 days post infection. B. In a second experiment HIV-1 inhibition was confirmed for PIK3R4-1, Atg4A-1 and 3, Atg5-4, Atg10-3 and 5 and Atg16-4. C. The mRNA knockdown was measured with RT-qPCR analysis for the indicated shRNAs. The level measured in the control SHC1 cells was set at 100%. D. Transduced cell mixtures were analyzed in the competitive cell growth assay, which is based on different growth rates of untransduced GFP versus transduced GFP+ cells. Cell proliferation rates were compared to SHC1 cells, of which the proliferation rate was set at 100%.

Table 5.1 Autophagy activity upon ATG knockdown
Table 5.1 Autophagy activity upon ATG knockdown

<table>
<thead>
<tr>
<th>Targeted ATG</th>
<th>Starvation-induced lipidation</th>
<th>LC3</th>
<th>Autophagy flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIPI1</td>
<td>+(^a)(^b)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Beclin1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PIK3R4</td>
<td>+</td>
<td>-(^c)</td>
<td></td>
</tr>
<tr>
<td>Atg4A-1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Atg4A-3</td>
<td>+/-(^d)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Atg5</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Atg10-3</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Atg10-5</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Atg16</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Results are representative of two separate experiments
\(^b\) +; LC3-II increase similar to that in control SupT1 cells
\(^c\) -; no LC3-II increase
\(^d\) +/-; minor LC3-II increase

**Knockdown of autophagy factors inhibits production of viral particles**

Single cycle infection experiments were performed to determine at which stage of the replication cycle HIV-1 is blocked in the ATG knockdown cells. Knockdown and control cells were incubated with HIV-1 for 4 hours, subsequently the virus was washed away and new infections were prevented by addition of the fusion inhibitor T1249 to the culture medium. The percentage of cells positive for intracellular CA-p24 and the concentration of CA-p24 in the culture supernatant were measured at 48 hours post infection. This allows the establishment of integrated provirus that can express new viral proteins. For 3 shRNAs (WIPI1, Beclin1 and Atg4A-1) no significant differences were measured. For 6 shRNAs a clear reduction in both intracellular and extracellular CA-p24 was measured: PIK3R4, Atg4A-3, Atg5, Atg10-3, Atg10-5 and Atg16 (Figure 5.4A, left and right panel). Less CA-p24 positive cells means that a lower percentage of cells are productively infected by HIV-1, resulting in a lower CA-p24 concentration in the culture supernatant. The mean production of CA-p24 per positive cell, shown by the mean fluorescence intensity (MFI), was not affected (Figure 5.4A, middle panel). These combined results indicate an early block (from virus entry to transcription) in the HIV-1 replication cycle in these shRNA-expressing cells.

As an alternative means to inhibit the autophagy pathway, the effect of the autophagy inhibitor 3-methyladenine (3-MA) was used, which blocks the activity of the PIK3C3 and PIK3R4 kinases (Figure 5.1). To do so, 3-MA was tested in single cycle infection experiments on wild type SupT1 cells. Cells were either pre-treated with 3-MA for 4 hours before infection, treated for 48 hours after infection or a combination of both treatments and treated cells were compared to the untreated control cells (Figure 5.4B). As reported earlier, we observed increased cell death in cultures treated with 3-MA for a prolonged period, which is true for the samples that received 3-MA 48 hours post infection (189). Treating cells before infection did not cause significant changes. The concentration of CA-p24 in the supernatant was dramatically reduced when cells were treated with 3-MA after...
infection (Figure 5.4B, right panel). However, when cultures were treated with 3-MA after infection, more cells in the culture became CA-p24 positive and the mean production of CA-p24 per actively infected cell was slightly increased (Figure 5.4B, left and middle panel). This indicates that when cells are treated with 3-MA post infection, this results in an accumulation of CA-p24 in the infected cells and a lower CA-p24 concentration in the culture supernatant. Thus a late step (from transcription to budding) in the HIV-1 replication cycle is affected by treatment with 3-MA after infection.

Figure 5.4. Single cycle infection experiments
A. ATG knockdown cells were infected with HIV-1 for 4 hours, excess virus was washed away and new infections were prevented by addition of the fusion inhibitor T1249. The percentage of CA-p24 positive cells was measured at 48 hours post infection by FACS (left panel). The mean production of CA-p24 per positive cell is represented as the mean fluorescence intensity (middle panel). The concentration of CA-p24 in the culture supernatant was determined by ELISA (right panel). B. SupT1 cells were either mock treated or incubated with 3-MA either starting 4 hours before infection or for 48 hours post infection, or both. Cells were analyzed for the percentage of CA-p24 positive cells (left panel), MFI (middle panel) and CA-p24 concentration in the culture supernatant (right panel).

Knockdown of 2 ATGs enhances HIV-1 inhibition

To test whether the simultaneous knockdown of 2 ATGs is tolerated by cells and whether the level of HIV-1 inhibition can be enhanced, we generated double-knockdown cells expressing shRNAs against Atg16 and Atg5. Atg16 was chosen as its knockdown resulted in strong inhibition of HIV-1 replication, with limited cytotoxicity (103). Atg5 knockdown resulted in good inhibition of HIV-1 replication without inducing cytotoxicity. Controls were untransduced SupT1 cells and the single-knockdown cell lines. The single knockdown cell lines were actually also transduced twice; the second transduction was performed with the SHC2 scrambled shRNA control. The first transduction used lentiviral vectors with the puromycin selection marker, while the second transduction was performed with vectors carrying a GFP-selection marker. To increase the change of scoring an additive effect, we purposely transduced cells at a relatively low multiplicity of infection (MOI) of 0.2. This will yield maximally 1 copy of each shRNA construct per doubly transduced cell to avoid saturation of the RNAi mechanism.
Cell lines were challenged with virus and a clear delay of HIV-1 replication was observed in the double-knockdown cell line (Figure 5.5A). In this low MOI setting, HIV-1 replication was not greatly delayed in the single knockdown cells. Knockdown of Atg5 and Atg16 was measured by RT-qPCR (Figure 5.5B) and knockdown of both Atg5 and Atg16 was confirmed in the double-knockdown cells. All transduced cells express GFP and could thus be tested in the competitive cell growth assay. The cell growth defect of the double-knockdown cell line is comparable to the cell growth defect of Atg16 combined with the scrambled shRNA (Figure 5.5C). Thus, cell proliferation does not seem hampered more severely by knockdown of two ATGs. In the single cycle replication assay the additive effect of the double knockdown of Atg5 and Atg16 was again observed. Fewer cells became positive for CA-p24 and the concentration of CA-p24 in the culture supernatant was reduced when compared to the single-knockdown cells and SupT1 cells (Figure 5.5D). Cells were incubated for two hours in minimal medium to generate starvation signals in the absence and presence of protease inhibitors. The level of LC3-II was detected by Western blot and normalized against the housekeeping protein GAPDH. Starvation-induced autophagy and autophagic flux were reduced in the single-knockdown cells and dramatically reduced in the double-knockdown cells (Figure 5.5E).

Figure 5.5. Additive HIV-1 inhibition by simultaneous knockdown of Atg5 and Atg16
A. Single and double knockdown cell lines were challenged with HIV-1 and replication was followed over time by measuring CA-p24 in the culture supernatant. B. Relative mRNA expression levels of Atg5 and Atg16 was determined by RT-qPCR. C. The effect of RNAi-mediated knockdown on cell growth was determined by the competitive cell growth assay. The percentage cell growth reduction is plotted. D. Single and double knockdown cells were used in single cycle infections. Cells were analyzed 48 hours post infection for the percentage of CA-p24 cells (left panel), MFI (middle panel) and CA-p24 concentration in the culture supernatant (right panel). E. Autophagy function in the single and double knockdown cells was analyzed in nutrient-rich and starvation conditions (first 2 bars for each cell line) and for autophagic flux, where the degradation step of autophagy is inhibited by protease inhibitors (last 2 bars for each cell line). The intensity of the LC3-II signal was corrected for the GAPDH level on Western blot.
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

A

CA p24 (ng/ml)

Days post infection

0 5 10 15 20

SupT1 Atg16 Atg5 Atg16 + Atg5

B

Relative expression (%)

Atg16 Atg5

SupT1 Atg16 Atg5 Atg16 + Atg5

C

Cell growth defect (%)

Atg16 Atg5 Atg16 + Atg5

D

% CA p24 positive

MFI

CA p24 (ng/ml)

SupT1 Atg16 Atg5 Atg16 + Atg5

E

Fold LC3-II/GAPDH

SupT1 Atg16 Atg5 Atg16 + Atg5

Starvation

- + - + - + - + - + - +

Protease inhibitors

- - - + + - + + - - + - +

LC3-I

LC3-II

GAPDH
Discussion

In this study we show that stable RNAi-mediated knockdown of autophagy factors can inhibit HIV-1 replication. Multiple shRNAs against 13 autophagy factors (ATGs) were tested, and inhibition of virus replication was scored for 7 ATGs. After confirming the knockdown of the targeted mRNAs and additional cell proliferation and autophagy tests, we conclude that RNAi against PIK3R4, Atg4A, Atg5 and Atg16 results in HIV-1 inhibition due to a specific block of autophagy. For these 4 ATGs a specific reduction in the target mRNA expression level and a clear defect in the autophagy pathway were measured. For Atg10-1 and 3 no block in autophagy was observed, but a specific reduction in mRNA expression levels was measured and a minor effect on cell proliferation was observed. It is possible that the effect on HIV-1 replication is due to a cellular function of Atg10 other than autophagy. Cell proliferation was not altered upon Atg5 knockdown, and only a small effect on cell growth was observed for PIK3R4 and Atg4A. Cell proliferation of Atg16 knockdown cells was 15% slower compared to untransduced cells, but for this shRNA a specific reduction in Atg16 mRNA expression levels and a clear defect in autophagy were observed. For Beclin1 and WIPI1 a delay of HIV-1 replication was measured, but in these two cell lines no effect on autophagy could be measured. In fact, both shRNAs cause a 20% slower cell proliferation and the effect on HIV-1 replication could therefore be indirect. An earlier genome-wide knockdown study has already identified ATG factors necessary for HIV-1 replication (53), although this was not confirmed in a second RNAi screen (406). However, none of the ATGs for which we scored inhibition of HIV-1 replication were identified in the knockdown study by Brass et al, except for Atg16.

When expression of Atg5 and Atg16 was inhibited in the same cell, additive HIV-1 inhibition was measured. Knockdown of both ATGs did not have a greater impact on cell proliferation than in the singly transduced cells. Knockdown of Atg16 seems to be the sole determinant of cell growth delay in the double knockdown cells, which may be surprising as both Atg5 and Atg16 mediate the same step of the autophagy pathway (Figure 5.1). Such an additive effect was measured on autophagy activity, which indicates that the modest delay in cell growth measured for Atg16 knockdown is not directly related to the impact on the autophagy pathway.

When ATG knockdown cells were analyzed in single cycle infection experiments, we observed that less cells did produce intracellular CA-p24 and the concentration of CA-p24 in the culture supernatant was concomitantly reduced. This indicates that less cells are productively infected. The mean CA-p24 production per CA-p24 positive cells (mean fluorescence intensity or MFI) was not affected by ATG knockdown. Thus, the fewer cells that are productively infected do synthesize as much CA-p24 as control cells. This result indicates that ATG knockdown leads to an early block of HIV-1 replication (e.g. entry or reverse transcription).

As an alternative to blocking autophagy with RNAi, we tested the autophagy inhibitor 3-methyladenine (3-MA). Surprisingly, we observed markedly different results in single cycle infection experiments with 3-MA compared to the shRNA-expressing cell lines. First of all, we observed that a higher percentage of the cells were positive for intracellular CA-p24, where in the shRNA-expressing cell lines this value was reduced. As normally a certain percentage of HIV-1 infections become latent, this result could indicate that treatment with 3-MA results in less latently infected cells and more productively infected cells. A
second observation was that cells treated with 3-MA after infection exhibited an increased MFI, meaning that cells produced more CA-p24 per cell than untreated cells. Less CA-p24 was measured in the culture supernatant. This is similar to what was described by Kyei et al, confirming their conclusion that blocking autophagy with 3-MA leads to inhibition of virus budding into the culture supernatant. 3-MA has been used for several years as a specific inhibitor of autophagy, however, there is accumulating evidence that 3-MA can have pleiotropic effects, and the impact on autophagy should always be confirmed with more specific inhibitors, such as shRNAs (159;385).

Autophagy is a cellular mechanism important in many viral infections. Thus blocking the autophagy pathway could be of therapeutic use. In addition to viral infections, blocking autophagy could also present a new approach against cancer. Cancerous cells appear to have increased autophagy activity that provides a survival mechanism when the cell is treated with e.g. chemotherapy (19). Blocking autophagy with the drug 3-MA in combination with anti-cancer drugs has been used against several types of cancer, such as breast and colorectal cancer, and siRNAs to silence the ATGs Beclin1 and Atg5 have been tested against cervical cancer (3;206;278). Therefore RNAi-mediated knockdown of autophagy factors could be a therapeutic approach against viruses and other diseases.

In this study we show that stable RNAi-mediated knockdown of several autophagy factors inhibits HIV-1 replication without inducing cytotoxicity. Targeting autophagy factors could therefore be used in a new therapeutic approach against HIV-1 infection.

Acknowledgements

We thank Stephan Heynen for performing CA-p24 ELISA experiments and Berend Hooibrink for FACS sorting. This research is financially supported by the Dutch AIDS fund, grant 2006006 and 2007028 and by SIDACTION. We also thank the Belgian Government for financial support through the Inter University Attraction Pole program, grant P6/41.
Chapter 6
The cyclophilins A and B have opposing roles in HIV-1 replication

Julia J.M. Eekels, Marja L. van Gerven, Ben Berkhout and Rienk E. Jeeninga

Submitted for publication

Laboratory of Experimental Virology,
Department of Medical Microbiology,
Center for Infection and Immunity Amsterdam (CINIMA),
Academic Medical Center of the University of Amsterdam
Abstract

HIV-1 modifies the cellular environment to support efficient virus replication and the cell reacts to virus infection by the production of antiviral factors. This results in a change in the expression level of many cellular proteins. We previously studied these effects in a proteomics analysis of HIV-infected T cells. We validated the results by stable RNAi-mediated knockdown of 76 cellular proteins that were either up or downregulated. Knockdown of 38 host proteins resulted in inhibition of virus replication and knockdown of 3 targets caused increased virus replication. For 29 targets no significant effect on HIV-1 replication was scored relative to the control cells. Cellular proliferation was seriously affected upon knockdown of 6 targets, which prevented reliable follow-up studies. We focused on the cellular protein Cyclophilin B (PPIB) because its knockdown did significantly enhance viral replication. Whereas the well-studied family member Cyclophilin A (PPIA) is a cellular co-factor for HIV-1 replication, we show that PPIB is a candidate cellular restriction factor that blocks HIV-1 at an early replication step.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection induces changes in the physiology of the infected cells. HIV-1 reprograms cells for efficient virus production and tries to prevent detection by (innate) immune responses of the host. HIV-1 may induce the expression of certain host cell proteins, in particular those that act as cellular co-factors in the viral replication cycle. Three genome-wide RNAi screens were performed to identify cellular co-factors of HIV-1 replication (54;180;407). Other studies focused on the influence of HIV-1 replication on cellular processes, either by microarray analysis of differential mRNA expression or proteomic analyses (286;358). We previously performed a proteomics screen in the human T cell line PM1 (286). To further corroborate these results, we now studied the effect of stable RNAi-mediated knockdown of 76 cellular targets on HIV-1 replication in the SupT1 T cell line. For 6 targets no reliable data could be obtained due to cellular toxicity upon shRNA-mediated silencing of the target protein. Knockdown of 38 targets resulted in a decrease of HIV-1 replication, thus confirming their status as candidate co-factor. For 29 targets no significant effect on virus replication was observed. For 3 cellular targets we measured increased virus replication upon their knockdown, which - combined with their increased expression upon HIV-1 infection - makes them candidate restriction factors.

One of the cellular targets for which knockdown resulted in increased virus replication was Cyclophilin B (PPIB) that forms the focus of this study. This protein belongs to a large family of peptidyl-prolyl isomerases (PPIs) (see (198;370) for review). Members of this family play a role in a variety of cellular processes including the immune response, transcription, mitochondrial function, chemotaxis, chaperone functions in protein synthesis and protein folding, and cell death. All members of this family are able to catalyze the trans-cis transition of the peptide bond preceding a proline residue during protein folding. The immunosuppressive drug cyclosporine A (CsA) has an affinity for a subset of the family members that are consequently named Cyclophilins. CsA is used as an immunosuppressive drug to treat graft failure after tissue transplantation. The immunosuppressive activity of CsA results from binding to Cyclophilins and the
subsequent sequestration of calcineurin. This sequestration blocks the calcineurin phosphatase activity that is needed for activation of the transcription factor NF-AT, which is required for upregulation of T cell function. All members of the PPI family share the 109 amino acid Cyclophilin-like domain (CLD). The domains surrounding the CLD are unique for each family member and determine their subcellular localization and function. One of the best studied members of the PPI-family is Cyclophilin A (PPIA, Figure 6.1) that was identified as a protein with high affinity for CsA. This protein acquired particular interest when it was reported to be important for HIV-1 replication (Figure 6.1) (366). PPIA binds to the capsid domain (CA) of the HIV-1 Gag polyprotein and is specifically incorporated into newly assembling virion particles (48;120;226). The interaction between CA and PPIA is required for an early step of HIV-1 replication, either by promoting a viral mechanism such as virion uncoating or by blocking cellular restriction factors (49-51;211;355;393). Surprisingly, it is not the PPA in virions but the PPIA expressed in the infected cell that plays this role as viral co-factor (151;328).

![Figure 6.1. Schematic of the interactions between HIV-1 and the Cyclophilins A and B](image)

Cyclophilin A (PPIA) localizes to the cytoplasm and is incorporated into virions through binding to HIV-1 Gag (CA). PPIA expression in the infected cell is important to support early HIV-1 replication. Cyclophilin B (PPIB) resides mainly in the ER and is also capable of Gag binding, but PPIB is not incorporated into the virion. In this study we show that PPIB blocks an early HIV-1 replication step. The immunosuppressive drug cyclosporine A (CsA) inhibits both PPIA and PPIB and blocks Envelope protein incorporation into the virion in a Cyclophilin-independent manner.
The Cyclophilins A and B have opposing roles in HIV-1 replication

Similar to PPIA, PPIB is also capable of binding to the HIV-1 Gag polyprotein and the processed CA-p24 protein in vitro, but PPIB is not detected in viral particles (Figure 6.1)(48;226). The PPIA and PPIB proteins are very similar, but PPIB contains a signal sequence for localization to the endoplasmatic reticulum (ER)-Golgi, whereas PPIA resides in the cytoplasm. PPIB mainly localizes in the lumen of the ER (275;374), but a subset of PPIB can be found in the cytoplasm and ER-substructures on the cytoplasmic side (52;374). Functional studies demonstrated that PPIB when complexed with other proteins can also function in the nucleus and extracellularly (263;275;300;396;397). PPIB interacts with cytoplasmic proteins such as interferon response factor-3 (IRF3) to mediate the IRF3 response in dendritic cells (263). Both PPIA and PPIB have been implicated in the replication of other viruses. PPIA interacts with the core of vaccinia virus, is incorporated into virions and localizes at the sites of virus replication in the nucleus (66). PPIA is also incorporated in SARS-coronavirus particles and blocking of its interaction with the extracellular protein CD147 interferes with SARS virus infectivity (72). PPIB is important in hepatitis C virus (HCV) replication as it promotes the RNA binding properties of the HCV NS5B polymerase (374). Several CsA-derivatives that block this interaction are currently being tested in clinical trials against HCV infection (117). This strategy could have a double therapeutic benefit as these CsA-derivatives are also active against HIV-1, and these viruses are frequently diagnosed as co-infections.

We previously documented a significant increase in PPIB expression when human T cells are infected with HIV-1 (286). In this study, we demonstrate that stable RNAi-mediated knockdown of PPIB has a positive effect on an early step of virus replication, which may indicate a role of PPIB as restriction factor. Thus, PPIA and PPIB appear to have opposing roles in an early step of the HIV-1 replication cycle, PPIA acting as cellular co-factor and PPIB as candidate restriction factor. Interestingly, the replication defect in PPIA knockdown cells can be rescued by PPIB knockdown. This result indicates that the PPIA/PPIB balance is important and cautiously suggests that PPIB could be the proposed restriction factor that is counteracted by PPIA.

Materials and Methods

Cells and viruses

C33A cervix carcinoma cells (ATCC HTB31) (25) and human embryonic kidney 293T cells were grown as a monolayer at 37°C and 5% CO₂ in advanced Dulbecco’s minimal essential medium supplemented with 10% (v/v) fetal calf serum (FCS), 40 U/ml penicillin, 40 µg/ml streptomycin, 20 mM glucose and minimal essential medium nonessential amino acids. The human T lymphocytic cell line SupT1 (ATCC CRL-1942) (325) was cultured in advanced RPMI 1640 medium (Gibco BRL) supplemented with 1% (v/v) FCS, 40 U/ml penicillin, and 40 µg/ml streptomycin at 37°C and 5% CO₂. Virus infections were performed with C33A-produced HIV-1 stocks of the HIV-1 LAI molecular clone (270). The cell line SupT1::HIV-rtTA was obtained by limiting dilution as previously described (165). This clonal cell line has an integrated doxycycline inducible HIV-rtTA provirus with a wild-type Tat gene (165). These cell lines were maintained as the parental cell lines, but always in the presence of the fusion inhibitor T1249 to prevent new rounds of infection.
Chapter 6

TRC library
The shRNA-constructs from the MISSION™ TRC-Hs 1.0 library (247;293) were provided as bacterial clones. Plasmid DNA was extracted using the Nucleobond™ miniprep columns according to the manufacturer’s instructions (Macherey-Nagel). For every target gene 4 to 5 shRNAs are included and the targeted sequences are provided on the website of Sigma-Aldrich [http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html].

Construction of the GFP marker in the shRNA expression plasmids
The pLKO.1 constructs from the MISSION™ TRC-Hs 1.0 library contain the puromycin resistance gene, which was replaced by the GFP gene that was amplified from the lentiviral vector JS1 (312) by PCR (forward primer: 5’ GAA TTC ACC GGT CGC CAC CAT 3’, reverse primer: 5’ ACT AGT GTC GAC CCC GGG CTC 3’). The GFP-PCR product was cloned into pCR2.1-TOPO TA cloning vector using the TOPO TA-cloning kit (Invitrogen) according to the suppliers’ protocol. The BamHI-KpnI fragment from this vector, encompassing the complete GFP gene, was subsequently cloned into pLKO.1 cut with the same enzymes to replace the puromycin resistance marker. GFP expression is under control of the pGK promoter that was already present in pLKO.1. The resulting plasmid was named pLKO.1-GFP. All constructs were verified by restriction enzyme analysis and the ability to express GFP.

Lentiviral vector production and transduction
The lentiviral vectors were produced as previously described (347). In short, HEK293T cells were co-transfected with the shRNA-construct and the packaging plasmids (pRSV-Rev, pMDL and pVSV-G) using Lipofectamine 2000 (Invitrogen). The medium was refreshed one day after transfection and the supernatant was harvested the following day. The virus-containing culture supernatant was cleared from cellular debris by low speed centrifugation, filtered (0.45 µm), and aliquots were stored at –80°C. A sample was taken for CA-p24 enzyme-linked immunosorbent assay (ELISA) to monitor lentiviral particle production. SupT1 cells were seeded in a 24-wells plate (1x10^5 cells per 500 µl), the lentiviral vector (corresponding to 100 ng CA-p24) was added and incubated overnight. Cells were washed with 1 ml phosphate buffered saline (PBS) the next day and cultured in complete growth medium supplemented with 1 µg/ml puromycin.

Virus production and replication
C33A cells were transfected by the calcium phosphate method with 5 µg of pLAI plasmid DNA (270) to produce virus stocks as described previously (84). Virus stock concentrations were determined by measuring the CA-p24 concentration. HIV-1 replication on the cells was performed in 96-well format. Infections were initiated by adding HIV-1 virus (corresponding to 1 ng of CA-p24) to 4 x 10^5 cells, which were subsequently divided over two wells. Per 96-well plate two independent infections (thus 4 wells in total) with wild-type SupT1 cells were included as positive control. Starting at day 3 post infection, microscopic inspection of each culture was performed every 24h to score for syncytia formation. A 50 µl supernatant sample was taken for CA-p24 ELISA to measure virus
The Cyclophilins A and B have opposing roles in HIV-1 replication production and replaced by 50 µl fresh medium. Infection of the PPIB knockdown cells was performed in T25 culture flasks with 1 × 10^6 cells and an amount of HIV-1 corresponding to 5 ng CA-p24.

**Single round infection assays**

SupT1 cells were infected with HIV-1 as described (270). Excess virus was washed away after 3h and the cells were cultured in the presence of entry inhibitor T1249 to block subsequent rounds of viral entry. We measured extracellular CA-p24 production in the cell culture medium 48h post infection by ELISA and intracellular CA-p24 by FACS analysis.

**Competitive cell growth assay**

The competitive cell growth (CCG) assay was used as described earlier (Eekels et al). In brief, SupT1 cells were transduced with 25 or 100 µl of lentiviral vector expressing a shRNA and a GFP selection marker. The percentage of cells positive for GFP was analyzed daily by FACS over a period of 10 days, starting at 3 days post transduction. To do so, a sample was removed from the culture to collect cells by low-speed centrifugation (as above). The cells were resuspended in FACS buffer and analyzed on a FACScanto (BD biosciences) flow cytometer. Cell populations were defined based on forward/sideward scattering. Untransduced SupT1 cells were used to normalize the GFP signal. Based on the known doubling time of untransduced SupT1 cells and the change in GFP+/GFP- ratio over the course of the experiment the difference in cell proliferation rate was calculated as described (Eekels et al, Chapter 4).

**Western blot**

Cells (1×10^5) were collected by centrifugation (4 min 400 x g, Eppendorf centrifuge) and resuspended in 100 µl SDS-PAGE loading buffer, boiled for 10 minutes and 15 µl was loaded onto a 15% SDS-PAGE gel. Proteins were transferred to an Immobilon-P membrane (Millipore) via semi-dry transfer. Membranes were treated for 1h at room temperature in blocking solution (PBS + 5% milk + 0.1% Tween-20) and detection was performed with anti-PPIB (Abcam, ab74173, mouse, 1:1000), anti-β-actin (Sigma, clone AC-74, mouse, 1:5000) and HRP-labeled goat anti-mouse IgG (1:5000). Luminometric detection of proteins was performed with Western Lightning ECL (PerkinElmer Life Sciences) and membranes were analyzed on a LAS3000 imager (GE Healthcare).

**Intracellular staining and fluorescence-activated cell sorting (FACS)**

Flow cytometry was performed with RD1 or FITC-conjugated mouse monoclonal anti-CA-p24 (clone KC57, Coulter) and the mouse [k2E2] monoclonal to PPIB (ab74173, Abcam) in combination with a FITC or Cy5-conjugated goat anti mouse secondary antibody. Cells from a 1 ml culture sample were collected (4 min 400 x g, Eppendorf centrifuge) and fixated in 250 µl 4% formaldehyde for 5 min at room temperature. The cells were washed with 500 µl BD Perm/Wash™ buffer (BD Pharmingen) and stained for at least 30 min at 4°C in 20 µl of BD Perm/Wash™ buffer and 5 µl of the appropriate antibody (diluted 1 in 100 for CA-p24 and secondary antibodies and 1 in 20 for PPIB). Excess antibody was removed by washing the cells with 500 µl BD Perm/Wash™ buffer. The cells were collected and resuspended in 750 µl FACS buffer (PBS with 2% FCS). Cells were analyzed on a FACScanto.
Chapter 6

flow cytometer with FACSDiva software (BD biosciences). Cell populations were defined based on forward/sideward scattering. Isotype controls or uninfected control cells were used to set markers. The data from different experiments was corrected for between-session variation with the factor correction program (299).

CA-p24 ELISA

Culture supernatant was heat inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as the capture antibody and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as the detection antibody. Detection was done with the lumiphos plus system (Lumigen) in a LUMIstar Galaxy (BMG labtechnologies) luminescence reader. Recombinant CA-p24 produced in a baculovirus system was used as the reference standard.

Reagents

Cyclosporin A (Sigma) was dissolved in absolute ethanol at a concentration of 1 mM. Final concentrations are indicated in the experiment. In each experiment equal amounts of absolute alcohol was added to the control cultures to control for effects of the solvent. The fusion inhibitor T1249 (WQEWEQKITALEQAQIQQEKNEYELQKLDKWASLWEWF, Pepscan Therapeutics) was obtained as a 10.000x stock solution of 1 mg/ml. Doxycycline (Sigma D9891) was prepared as a 1000x stock in sterile milliQ water and used at a final concentration of 1000 ng/ml.

Results

Design of the shRNA screen and HIV-1 replication studies

We previously reported on proteomic changes that occur in the PM1 T cell line upon HIV-1 infection (286). To further investigate the role of these proteins in HIV-1 replication, we selected 76 candidates for follow-up based on availability of specific short hairpins RNAs (shRNAs) for knockdown experiments. We used a stable RNAi knockdown approach in the SupT1 T cell line to score the effects on cell viability and susceptibility to HIV-1 infection, which should broaden the initial PM1 observations. The MISSION™ TRC-Hs 1.0 library contains 4 to 5 shRNAs per gene of interest, which were tested in parallel to increase the chance of obtaining efficient knockdown and to minimize the risk of scoring unrelated off-target effects (247;293). With these shRNA constructs 368 lentiviral vectors were produced to transduce SupT1 cells. The cellular targets are listed in Table 6.1 and ranked according to the impact of HIV-1 infection on their expression level as determined in the proteomic analysis. Cell proliferation was strongly reduced or no viable cells were obtained in 28 cultures, which were excluded from follow-up analysis (Table 6.1, grey box). This includes all knockdown cells for endoplasmisin/HSP90 beta protein (target 13) and tubulin alpha chain protein (target 17), but also heterogeneous nuclear ribonucleoprotein K HNRPK (target 66) that yielded only a single viable culture. The remaining 340 cultures were challenged with HIV-1 and virus replication was monitored by measuring HIV-1 capsid protein (CA-p24) production in the supernatant and by
microscopic inspection of the culture for syncytia formation. The replication efficiency was scored relative to HIV-1 replication in the parental SupT1 cell line. An example of scoring decreased virus replication is presented in Figure 6.2A and increased virus replication in Figure 6.2B. All replication experiments were performed at least twice and the results of independent experiments were reproducible (Figure 6.2C, left and right panel). We observed decreased HIV-1 replication in 194 cultures, no change in 104 cultures and increased replication in 42 cultures (Table 6.1). Microscopic inspection of the knockdown cultures for targets 12, 21 and 30 demonstrated reduced cell proliferation. As reduced cell growth may indirectly reduce virus replication rates, these cases were not further analyzed (Table 6.1, grey boxes). A stringent consensus score per target protein was determined as follows: 1) at least two shRNAs per target should exhibit an effect and 2) all shRNAs should work in the same direction, that is either to inhibit or stimulate virus replication (or behave neutrally). This leaves 38 targets for which knockdown reproducibly resulted in decreased viral replication (Table 6.1, blue coded targets). For 3 targets an increase in viral replication was consistently observed. Knockdown of 29 genes produced no or no consistent effect on HIV-1 replication (Table 6.1, red coded targets). It was not a realistic option to verify the mRNA knockdown efficiency for each shRNA. Thus, the absence of an effect on virus replication may be caused by insufficient gene knockdown. In fact, the criterion that at least 2 shRNAs should induce the same phenotype may lead to an underestimation of the number of interesting targets, e.g. when only a single shRNA is able to reduce the protein expression level to such an extent that an effect on virus replication is apparent.

Figure 6.2. HIV-1 replication in stable shRNA-knockdown T cells
HIV-1 replication assays were performed in the 96-well format. All infections were performed in duplicate. A. Example of CA-p24 production post-infection of cells expressing shRNAs against target 31 (OXCT1). HIV-1 replication was scored as -2 and -1 in the shRNA31-A and shRNA31-B cultures, respectively. B. For 3 targets an increase in HIV-1 replication was scored, with target 35 (BDH1) shown as an example with a +1 score. C. HIV-1 replication was analyzed in at least 2 independent experiments, and the reproducibility is demonstrated for target 32 (VDAC2) in the left and right panels.

Table 6.1
<table>
<thead>
<tr>
<th>Protein</th>
<th>Symbol</th>
<th>HIV-1 effect</th>
<th>shRNA effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Beta actin</td>
<td>ACTB</td>
<td>4.83</td>
<td>-0.3</td>
</tr>
<tr>
<td>GDP dissociation inhibitor 2</td>
<td>GDI2</td>
<td>3.88</td>
<td>-1.5</td>
</tr>
<tr>
<td>Proteasome beta type 4 subunit</td>
<td>PSMB4</td>
<td>3.72</td>
<td>-2.0</td>
</tr>
<tr>
<td>Mt heat shock protein 60</td>
<td>HSPD1</td>
<td>3.47</td>
<td>0.0</td>
</tr>
<tr>
<td>Single stranded mitochondrial DNA-1 binding</td>
<td>SSBP1</td>
<td>2.96</td>
<td>0.0</td>
</tr>
<tr>
<td>Enoyl coenzyme A hydratase</td>
<td>ECH1</td>
<td>2.92</td>
<td>-1.0</td>
</tr>
<tr>
<td>MN-1 superoxid dismutase</td>
<td>SOD2</td>
<td>2.87</td>
<td>0.0</td>
</tr>
<tr>
<td>ATPase beta chain</td>
<td>ATP1B1</td>
<td>2.76</td>
<td>0.0</td>
</tr>
<tr>
<td>Prohibitin</td>
<td>PHB</td>
<td>2.75</td>
<td>-0.3</td>
</tr>
<tr>
<td>NADH2 dehydrogenase (ubiquinone) chain</td>
<td>NDUF53</td>
<td>2.67</td>
<td>0.0</td>
</tr>
<tr>
<td>Ubiquinol-1cytochrome-1c reductase core 1</td>
<td>UQRC1</td>
<td>2.62</td>
<td>-2.0</td>
</tr>
<tr>
<td>Endoplasm/HSP90 beta</td>
<td>HSP90B1</td>
<td>2.59</td>
<td>-0.1</td>
</tr>
<tr>
<td>Peroxiredoxin 4c</td>
<td>PRDX4</td>
<td>2.58</td>
<td>-1.0</td>
</tr>
<tr>
<td>ATP synthase alpha chain</td>
<td>ATP5A1</td>
<td>2.51</td>
<td>-2.0</td>
</tr>
<tr>
<td>NADH dehydrogenase (ubiquinone) Fe-15</td>
<td>NDUF51</td>
<td>2.49</td>
<td>-2.0</td>
</tr>
<tr>
<td>Tubulin alpha chain</td>
<td>TUBA1B</td>
<td>2.44</td>
<td>-1.0</td>
</tr>
<tr>
<td>Protein disulfide isomerase 3</td>
<td>PDI3</td>
<td>2.43</td>
<td>-0.5</td>
</tr>
<tr>
<td>Purine nucleoside phosphorylase</td>
<td>NP</td>
<td>2.41</td>
<td>0.0</td>
</tr>
<tr>
<td>hVDAC1</td>
<td>VDAC1</td>
<td>2.40</td>
<td>-1.7</td>
</tr>
<tr>
<td>Hydroxyacyl-1coenzyme A dehydrogenase</td>
<td>HADH2</td>
<td>2.38</td>
<td>-1.3</td>
</tr>
<tr>
<td>Cyclophilin B</td>
<td>PPIB</td>
<td>2.36</td>
<td>2.0</td>
</tr>
<tr>
<td>Serine hydroxymethyltransferase</td>
<td>SHMT1</td>
<td>2.33</td>
<td>-0.8</td>
</tr>
<tr>
<td>Fumarate hydratase</td>
<td>FH</td>
<td>2.30</td>
<td>0.0</td>
</tr>
<tr>
<td>ATP synthase D chain</td>
<td>ATP5H</td>
<td>2.29</td>
<td>-0.3</td>
</tr>
<tr>
<td>BIP/GRP78</td>
<td>HSPA5</td>
<td>2.25</td>
<td>0.0</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH1</td>
<td>2.24</td>
<td>-0.5</td>
</tr>
<tr>
<td>Dihydrioloamide dehydrogenase</td>
<td>DLD</td>
<td>2.20</td>
<td>0.0</td>
</tr>
<tr>
<td>P4HB</td>
<td>P4HB</td>
<td>2.15</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycoprotein gC1qBP/P33</td>
<td>C1QBP</td>
<td>2.14</td>
<td>-0.5</td>
</tr>
<tr>
<td>hVDAC2</td>
<td>VDAC2</td>
<td>2.12</td>
<td>-1.5</td>
</tr>
<tr>
<td>Succinyl-1CoA 3-1ketocacid-1CoA transferase 1</td>
<td>OXCT1</td>
<td>2.06</td>
<td>-2.0</td>
</tr>
<tr>
<td>3-1methylcrotonyl-1CoA carboxylase subunit</td>
<td>MCCC2</td>
<td>2.05</td>
<td>-0.3</td>
</tr>
<tr>
<td>49 kDa TBP-1Interacting protein</td>
<td>RUVBL1</td>
<td>2.05</td>
<td>0.0</td>
</tr>
<tr>
<td>3-1hydroxybutyrate dehydrogenase</td>
<td>BDH1</td>
<td>2.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 3 (NAD1) alpha</td>
<td>IDH3A</td>
<td>2.01</td>
<td>-0.3</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>CS</td>
<td>1.96</td>
<td>0.0</td>
</tr>
<tr>
<td>Dihydrioloamide S-1succinytransferase</td>
<td>DLST</td>
<td>1.94</td>
<td>-0.3</td>
</tr>
<tr>
<td>Ts translation elongation factor, mitochondrial</td>
<td>TSFM</td>
<td>1.90</td>
<td>-0.5</td>
</tr>
<tr>
<td>Glucosidase II alpha subunit</td>
<td>GANAB</td>
<td>1.90</td>
<td>-1.5</td>
</tr>
<tr>
<td>Peroxiredoxin 3</td>
<td>PRDX3</td>
<td>1.88</td>
<td>1.0</td>
</tr>
<tr>
<td>Aconinate hydratase</td>
<td>ACO2</td>
<td>1.88</td>
<td>-1.0</td>
</tr>
<tr>
<td>Electron transfer flavoprotein (beta ETF)</td>
<td>ETFB</td>
<td>1.87</td>
<td>0.0</td>
</tr>
<tr>
<td>HSP75</td>
<td>TRAP1</td>
<td>1.86</td>
<td>-0.3</td>
</tr>
<tr>
<td>Stomatin</td>
<td>STOM</td>
<td>1.85</td>
<td>0.3</td>
</tr>
<tr>
<td>Annexin A11</td>
<td>ANXA11</td>
<td>1.84</td>
<td>-1.3</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein H</td>
<td>HNRPH1</td>
<td>1.75</td>
<td>-0.3</td>
</tr>
</tbody>
</table>
The Cyclophilins A and B have opposing roles in HIV-1 replication

We decided to focus on the Cyclophilin B (PPIB) protein that is upregulated upon HIV-1 infection (286). Interestingly, knockdown of this protein enhanced HIV-1 replication (Table 6.1, target 22), although a reduction of cell proliferation was also apparent. We focused on PPIB to study whether it acts as a cellular restriction factor to block HIV-1 replication. Indeed, PPIB has been implicated in innate immune responses (263;396;397) and appears to mediate intercellular communication in response to inflammatory stimuli, similar to the action of cytokines (reviewed in (375)). Replication experiments with HIV-1 were repeated in freshly transduced PPIB knockdown SupT1 cells in the more regular format of T25 culture flasks. The experiments confirm the initial results that HIV-1 replicates faster in cells that express a shRNA against PPIB. In fact, this was observed in all four cell cultures that express a unique shRNA against PPIB (Figure 6.3A).

Grey boxes: Cellular targets were excluded from further experimentation due to large impact on cell proliferation
Blue: Knockdown of cellular targets decreases HIV-1 replication
Red: Knockdown of cellular targets increases HIV-1 replication
A: Fold up or down regulation of cellular target as analyzed in the proteomics screen
B: Effect of individual shRNAs on HIV-1 replication

**PPIB knockdown enhances HIV-1 replication**

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDHB</td>
<td>Pyruvate dehydrogenase (lipoamide)</td>
<td>1.73</td>
</tr>
<tr>
<td>HYO1</td>
<td>Oxygen-regulated 15K protein</td>
<td>1.72</td>
</tr>
<tr>
<td>AHCY</td>
<td>S-1adenosylhomocysteine hydrolase chain B</td>
<td>1.68</td>
</tr>
<tr>
<td>PDI4</td>
<td>Protein isosulfide isomerase A4 (ERP72)</td>
<td>1.67</td>
</tr>
<tr>
<td>GOT2</td>
<td>Transaminase A</td>
<td>1.66</td>
</tr>
<tr>
<td>TP-1beta</td>
<td>Ribosomal protein S5</td>
<td>1.65</td>
</tr>
<tr>
<td>PSMD13</td>
<td>26 proteasome subunit (PSMD13)</td>
<td>1.34</td>
</tr>
<tr>
<td>PSMD13</td>
<td>Activator of HSP90 ATPase homolog 1</td>
<td>-1.38</td>
</tr>
<tr>
<td>VI2</td>
<td>Ezrin</td>
<td>-1.44</td>
</tr>
<tr>
<td>FABP5</td>
<td>Fatty acid binding protein</td>
<td>-1.46</td>
</tr>
<tr>
<td>HNRPK</td>
<td>Heterogenous nuclear ribonucleoprotein K</td>
<td>-1.48</td>
</tr>
<tr>
<td>E1F5A</td>
<td>Translation initiation factor eIF-5A</td>
<td>-1.50</td>
</tr>
<tr>
<td>LCP1</td>
<td>L-1plasmin/65K phosphoprotein</td>
<td>-1.50</td>
</tr>
<tr>
<td>HSPA4L</td>
<td>Heat-shock 70K-related protein APG-2</td>
<td>-1.52</td>
</tr>
<tr>
<td>ARHGDIB</td>
<td>Rho-1GDP-1dissociation inhibitor beta</td>
<td>-1.54</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-1mobility group box 1</td>
<td>-1.58</td>
</tr>
<tr>
<td>UBE1</td>
<td>Ubiquitin activating enzyme E1</td>
<td>-1.65</td>
</tr>
<tr>
<td>PAK2</td>
<td>Serine/threonine-1protein kinase PAK2</td>
<td>-1.67</td>
</tr>
<tr>
<td>YWHAE</td>
<td>14-13-13 protein epsilon</td>
<td>-1.75</td>
</tr>
<tr>
<td>ACTN1</td>
<td>Cytoskeletal alpha-1actinin</td>
<td>-1.89</td>
</tr>
<tr>
<td>STMN1</td>
<td>Stathmin 1</td>
<td>-2.31</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNRPK</td>
<td>Heterogenous nuclear ribonucleoprotein K</td>
<td>-1.48</td>
</tr>
<tr>
<td>E1F5A</td>
<td>Translation initiation factor eIF-5A</td>
<td>-1.50</td>
</tr>
<tr>
<td>LCP1</td>
<td>L-1plasmin/65K phosphoprotein</td>
<td>-1.50</td>
</tr>
<tr>
<td>HSPA4L</td>
<td>Heat-shock 70K-related protein APG-2</td>
<td>-1.52</td>
</tr>
<tr>
<td>ARHGDIB</td>
<td>Rho-1GDP-1dissociation inhibitor beta</td>
<td>-1.54</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-1mobility group box 1</td>
<td>-1.58</td>
</tr>
<tr>
<td>UBE1</td>
<td>Ubiquitin activating enzyme E1</td>
<td>-1.65</td>
</tr>
<tr>
<td>PAK2</td>
<td>Serine/threonine-1protein kinase PAK2</td>
<td>-1.67</td>
</tr>
<tr>
<td>YWHAE</td>
<td>14-13-13 protein epsilon</td>
<td>-1.75</td>
</tr>
<tr>
<td>ACTN1</td>
<td>Cytoskeletal alpha-1actinin</td>
<td>-1.89</td>
</tr>
<tr>
<td>STMN1</td>
<td>Stathmin 1</td>
<td>-2.31</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDHB</td>
<td>Pyruvate dehydrogenase (lipoamide)</td>
<td>1.73</td>
</tr>
<tr>
<td>HYO1</td>
<td>Oxygen-regulated 15K protein</td>
<td>1.72</td>
</tr>
<tr>
<td>AHCY</td>
<td>S-1adenosylhomocysteine hydrolase chain B</td>
<td>1.68</td>
</tr>
<tr>
<td>PDI4</td>
<td>Protein isosulfide isomerase A4 (ERP72)</td>
<td>1.67</td>
</tr>
<tr>
<td>GOT2</td>
<td>Transaminase A</td>
<td>1.66</td>
</tr>
<tr>
<td>TP-1beta</td>
<td>Ribosomal protein S5</td>
<td>1.65</td>
</tr>
<tr>
<td>PSMD13</td>
<td>26 proteasome subunit (PSMD13)</td>
<td>1.34</td>
</tr>
<tr>
<td>PSMD13</td>
<td>Activator of HSP90 ATPase homolog 1</td>
<td>-1.38</td>
</tr>
<tr>
<td>VI2</td>
<td>Ezrin</td>
<td>-1.44</td>
</tr>
<tr>
<td>FABP5</td>
<td>Fatty acid binding protein</td>
<td>-1.46</td>
</tr>
<tr>
<td>HNRPK</td>
<td>Heterogenous nuclear ribonucleoprotein K</td>
<td>-1.48</td>
</tr>
<tr>
<td>E1F5A</td>
<td>Translation initiation factor eIF-5A</td>
<td>-1.50</td>
</tr>
<tr>
<td>LCP1</td>
<td>L-1plasmin/65K phosphoprotein</td>
<td>-1.50</td>
</tr>
<tr>
<td>HSPA4L</td>
<td>Heat-shock 70K-related protein APG-2</td>
<td>-1.52</td>
</tr>
<tr>
<td>ARHGDIB</td>
<td>Rho-1GDP-1dissociation inhibitor beta</td>
<td>-1.54</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-1mobility group box 1</td>
<td>-1.58</td>
</tr>
<tr>
<td>UBE1</td>
<td>Ubiquitin activating enzyme E1</td>
<td>-1.65</td>
</tr>
<tr>
<td>PAK2</td>
<td>Serine/threonine-1protein kinase PAK2</td>
<td>-1.67</td>
</tr>
<tr>
<td>YWHAE</td>
<td>14-13-13 protein epsilon</td>
<td>-1.75</td>
</tr>
<tr>
<td>ACTN1</td>
<td>Cytoskeletal alpha-1actinin</td>
<td>-1.89</td>
</tr>
<tr>
<td>STMN1</td>
<td>Stathmin 1</td>
<td>-2.31</td>
</tr>
</tbody>
</table>

Grey boxes: Cellular targets were excluded from further experimentation due to large impact on cell proliferation
Blue: Knockdown of cellular targets decreases HIV-1 replication
Red: Knockdown of cellular targets increases HIV-1 replication
A: Fold up or down regulation of cellular target as analyzed in the proteomics screen
B: Effect of individual shRNAs on HIV-1 replication
The protein knockdown efficiency obtained by the different shRNAs was determined by Western blot analysis. We measured a profound PPIB knockdown for shRNAs 22B, 22C and 22D, but almost no reduction for 22A (Figure 6.3B). The previous proteomics study in PM1 cells revealed increased PPIB expression upon HIV-1 infection (286). We thus determined PPIB expression in infected versus uninfected SupT1 cells that were analyzed by intracellular staining and flow cytometry. We used the mean fluorescence intensity (MFI) of the PPIB specific signal in the live cell population as a measure for PPIB expression, which is somewhat increased in SupT1 cells upon HIV-1 infection (Figure 6.3C).
We also infected the 4 PPIB knockdown cultures. A modest reduction in PPIB expression was observed compared to infected wild-type SupT1 cells, which results in a level similar to that of uninfected SupT1 cells. Intriguingly, we observed increased PPIB expression in all individual cells of the culture, independent of the level of infection (30-90%) as determined by intracellular expression of CA-p24. Already 5h after infection, when the infected cells do not yet express CA-24 protein, all cells in the culture already show increased PPIB expression (data not shown). These results support the notion that PPIB expression is induced upon virus infection as an innate antiviral response that also reaches the uninfected bystander cells. Together these results indicate that the shRNA-induced PPIB knockdown is counteracted by PPIB upregulation during HIV-1 infection.

We noticed in these experiments that the PPIB knockdown cultures proliferated slower than the parental untransduced SupT1 cells. To analyze this in more detail we performed a competitive cell growth assay (Eekels et al, Chapter 4). To do so, the puromycin selection marker in the lentiviral vector was replaced for the GFP gene, such that a mixture of transduced GFP⁺ and untransduced GFP⁻ cells is obtained after transduction. When transduced cells grow slower than untransduced control cells in that mixed culture, one should observe a gradual decrease in the percentage of GFP⁺ cells over time (Figure 6.3D, left panel). We compared the PPIB-knockdown cells with the SHC2-transduced control cells that express a scrambled control shRNA. The percentage of GFP⁺ cells remained stable over time in this control SHC2 culture and the relative doubling time of SHC2 cells was set at 100%. All PPIB knockdown cultures showed a relatively fast decrease in the percentage of GFP⁺ cells. We calculated a 1.5 to 2 fold increased doubling time compared to the control cells (Figure 6.3D, right panel). Thus, expression of shRNAs against PPIB is indeed toxic for SupT1 cells. Interestingly, cell cultures in the presence of HIV-1 did not show such a dramatic effect on cell viability (see above). As demonstrated in Figure 6.3C, PPIB expression is restored to almost wild type levels during HIV-1 infection, which will minimize the adverse effects.

**PPIB knockdown enhances an early HIV-1 replication step**

We next analyzed which step of the HIV-1 replication cycle is inhibited by PPIB and how HIV-1 replication enhances PPIB expression. To determine the PPIB effect on the late virus production steps, we used a SupT1 clonal cell line that contains an integrated doxycycline (dox)-inducible provirus (165). These cells start producing virus upon dox addition to a maximum of 45% positive cells after 30h. This experiment was performed in the presence of the fusion inhibitor T1249 to prevent new rounds of infection. PPIB expression and CA-p24 production was analyzed over time by flow cytometric analysis (Figure 6.4A). No PPIB increase was detected in this virus-production assay. A small increase in PPIB expression was measured shortly after the start of the experiment, but this was similar to what happened in the control culture without dox. In fact, we observed no difference in PPIB MFI level of the dox-induced and the control culture along the experiment. Thus, although HIV-1 infection of SupT1 cells induces increased PPIB expression, this is not occurring during the late phase of virus production.

To analyze the effect of PPIB knockdown on early steps of viral replication, we used a single-round infection assay (165;239;328). SupT1 cells were infected with HIV-1 for 3h, excess virus was washed away and new rounds of infection were blocked by addition of the potent fusion inhibitor T1249. Intracellular staining for CA-p24 was used to quantitate
productive infection after 48h. The four PPIB knockdown cells produced a significantly larger number of CA-p24 positive cells compared to control SupT1 cells, consistent with an impact on the early phase of the replication cycle (Figure 6.4B, left panel). Extracellular virus production was determined by measuring the CA-p24 concentration in the supernatant and - concomitant with the increased number of infected cells - the CA-p24 levels displayed the same trend, although this difference did not reach statistical significance (Figure 6.4B, middle panel). When the extracellular CA-p24 concentration was normalized for the increased percentage of CA-p24 positive cells, a negative trend on virus production upon PPIB knockdown may become apparent, although these differences were not statistically significant (Figure 6.4B, right panel). These results indicate that a reduction of PPIB expression enhances an early step of virus replication, but that virus production per infected cell is not increased. These combined results indicate that PPIB acts as a cellular restriction factor at an early HIV-1 replication step.

**Figure 6.4. PPIB and HIV-1 CA-p24 expression levels during HIV-1 replication**

A. PPIB expression after 5h, 22h and 28h dox induction (closed squares) and a no-dox control (open squares) was determined as in Figure 6.3B. The percentage of CA-p24 positive cells in the same culture was determined by flow cytometry with a RD1 labeled CA-p24 antibody (KC57-RD1) and plotted on the secondary y-axis (closed and open triangles). B. The infectivity of HIV-LAI was analyzed in the parental SupT1 cell line and four different cultures that express an anti-PPIB shRNA. Equal amounts of cells were infected with HIV-1 LAI for 3h and excess virus was washed away and new rounds of HIV-1 infection were blocked with the fusion inhibitor T1249. The data was normalized for experimental variation by the factor correction program (299). The mean and SEM of 7 experiments are plotted. Cells were collected 48h after infection and used for intracellular staining of the HIV-1 CA-p24 protein (left panel). The supernatant was harvested 48h after infection and used for CA-p24 ELISA to determine virus production (middle panel). To determine virus production per cell, the extracellular CA-p24 concentration was divided by the percentage of CA-p24 positive cells (right panel).

**PPIA and PPIB have opposing roles in HIV-1 replication**

It is well documented that a close family member of PPIB, the Cyclophilin A protein or PPIA, is required for early HIV-1 replication. Thus, PPIA and PPIB seem to exhibit opposing activities with respect to HIV-1 infection. We therefore decided to test the effect of the
CsA, a drug that binds and inactivates all Cyclophilin family members. Although CsA has a higher affinity for PPIA, CsA can bind to PPIB as well (48;123;226). CsA blocks HIV-1 replication in different cell types (50;240;350), which has been attributed to inhibition of PPIA during early replication. In addition, CsA blocks HIV-1 Envelope protein incorporation in the virion (327) and this late inhibitory effect is PPIA-independent (328).

We tested if CsA did also influence HIV-1 replication in the PPIB knockdown SupT1 cells. Similar to what has been reported for the H9 cell line (392) and the HeLa-P4 cell line (387), we did not observe any antiviral effect when CsA was added during virus infection at a final concentration of 5 mM (results not shown). Increased concentrations of 7.5 and 10 mM were also tested, but were toxic for the SupT1 cells. When fresh CsA was added daily to the cultures in concentrations of 2.5-5 µM, inhibition of viral replication was observed in the control SupT1 cells (Figure 6.5A). Very similar results were obtained for the PPIB-knockdown cells (Figure 6.5B).

Figure 6.5. Effect of CsA on HIV-1 replication

A. The effect of CsA on HIV-1 in SupT1 T cells was analyzed by infecting $1 \times 10^6$ cells in a 5 ml culture with HIV-1 (5 ng CA-p24). Daily fresh CsA was added to a concentration of 5 µM, and the same volume of 70% EtOH was used as a control for effects induced by the solvent. HIV-1 replication was monitored by daily CA-p24 measurement in the culture supernatant. CsA inhibits HIV-1 replication. B. CsA inhibits HIV-1 replication in PPIB knockdown cells.
To distinguish the late CsA effect on virion assembly from the early effect we used the single-round infection assay, which addresses the PPIA-dependent effect of CsA on the early steps of infection. As expected, CsA addition just prior to virus infection significantly decreased the percentage of CA-p24 producing cells from 6.5% to 5.0% (t-test, p=0.028) (Figure 6.6A, left panel).

Surprisingly, when we tried to enhance the potency of CsA treatment by pre-incubating the cells for 2h before infection, an increase in the percentage of CA-p24 positive cells was scored from 6.1% to 9.1% (t-test, p=0.021). Also adding CsA post-infection increased the percentage of positive cells to 9.3% (t-test, p=0.047), which implies a post-entry event. These results indicate that CsA can influence early and late steps of HIV-1 replication in a negative and positive manner, respectively.

We reasoned that these complex CsA effects might relate to the opposing roles of PPIA and PPIB in HIV-1 replication. Both PPIA and PPIB will be bound and inactivated by CsA pre-incubation such that cells effectively become negative for PPIA and PPIB, which increases viral infectivity similar to what happens upon shRNA-mediated knockdown of PPIB. When CsA is added just before infection, inhibition of PPIB has to compete against PPIB upregulation caused by viral infection but PPIA can still be downregulated. This results in a decreased infectivity, similar to what was described for PPIA-knockdown cell lines (80;104;328). Adding CsA after infection (thus after removal of excess virus and addition of T1249) will result in similar downregulation of PPIA and PPIB since no induction of PPIB is observed during this virus production phase (Figure 6.4A).

Based on this reasoning, the pleiotropic effects of CsA in these single-round infections should disappear in PPIB knockdown cells. With reduced PPIB levels, there should be no difference between treated and untreated samples. This is indeed what was observed when the CsA time of addition experiments were performed with the PPIB knockdown cells (Figure 6.6A, right panel). No significant differences were measured between the infections in the presence or absence of CsA. It appears that the effect of CsA addition in SupT1 cells is closely related to the expression of PPIB.

These data prompted us to analyze the opposing roles of PPIA and PPIB in further detail. We generated 4 double-knockdown cell cultures that express shRNAs against PPIA and PPIB. For the PPIA-shRNA a good knockdown efficiency was obtained (85% knockdown at mRNA level, result not shown). Cytotoxicity tests indicated that PPIA knockdown is not cytotoxic in SupT1 cells, in contrast to PPIB knockdown. Replication of HIV-1 in the single and double knockdown cells and the parental SupT1 cell line was monitored (Figure 6.6B). As expected, HIV-1 replication was enhanced upon PPIB knockdown and diminished upon PPIA knockdown when compared to control SupT1 cells. Interestingly, HIV-1 replication in the double knockdown cells was similar to replication in the control cells. Thus, knockdown of PPIB is able to compensate for the negative effect on virus replication that is caused by PPIA knockdown.
Figure 6.6. Time of addition experiments with CsA demonstrate pleiotropic effects on HIV-1 infectivity
A. The effect of CsA addition on the infectivity of HIV-LAI was analyzed in single-round infection assays in the SupT1 cell line. The cultures were infected with HIV-1 LAI for 3h and excess virus was washed away and new infections were blocked with the T1249 fusion inhibitor. Cells were left untreated or CsA was added as follows: 2h pre-infection (CsA pretreatment), at the moment of infection (CsA) or added after removal of excess virus (CsA post infection). Cells were collected 48h after infection and used for intracellular staining of HIV-1 CA-p24 protein. The data was normalized for experimental variation by the factor correction program (299). The mean and SEM of 7 experiments are plotted (left panel). The same was done in the PPIB knockdown cells, results for shRNA 22B are shown as an example (right panel). B. The effect on HIV-1 replication in SupT1 cell cultures with a double knockdown of PPIA and PPIB (A+B-22A to A+B-22D) was determined in comparison to the wild-type SupT1 cell line and the single knockdown of PPIA (A) and the 4 different cell lines with knockdown of PPIB (B-22A to B-22D). Replication was initiated with equal amounts of virus (5 ng CA-24) in 1×10^6 cells in 5 ml culture volume of the indicated cell culture. Each day supernatant samples were taken for ELISA to determine the amount of CA-p24 produced. Two independent infections were performed per experiment and the average concentration of CA-p24 per culture is plotted. The experiment was repeated 3 times.
Discussion

In this study, we analyzed the effect of RNAi-mediated knockdown of cellular factors on the replication of HIV-1 in SupT1 T cells. The cellular targets were chosen based on a previous proteomic screen for cellular proteins of which the expression level is altered during HIV-1 infection (286). These changes may reflect the induction of defensive mechanisms that prevent the host cell against virus infection. Alternatively, these changes may represent a viral strategy to induce certain cellular co-factors that facilitate specific steps of the replication cycle. For 76 cellular targets the effect on HIV-1 replication was studied upon gene knockdown using shRNAs from the Mission™ library (247). For each target gene we used 4 to 5 shRNAs to generate stably transduced T cells, thereby reducing the chance of scoring a shRNA-specific off-target effect. We designed a stringent scoring system for evaluation of the shRNA-induced effects. Knockdown of 38 targets resulted in decreased virus replication, possibly because a viral co-factor was suppressed. Of these, 27 proteins were upregulated during HIV-1 infection in the proteomic screen, which nicely fits the co-factor concept. For 3 targets an increase in viral replication was observed, raising the possibility that a viral restriction factor was hit.

We decided to focus on Cyclophilin B (PPIB) as putative restriction factor for several reasons. First, HIV-1 replication was consistently induced in cells upon knockdown of PPIB. Second, PPIB protein is upregulated during HIV-1 infection, a property that is consistent with restriction factor action. Third, PPIB has already been implicated in the innate immune response (263;396;397). Fourth, we were intrigued to analyze the possible interplay between PPIA as viral co-factor (80;104;328) and PPIB as putative restriction factor. We first confirmed the phenotype in regular replication assays with an independent set of PPIB knockdown SupT1 T cells. The level of PPIB protein knockdown is modest in all four cells that express a different anti-PPIB shRNA, which is apparently sufficient to boost HIV-1 replication. In single-round infection assays, we demonstrated that the positive effect of PPIB knockdown on HIV-1 replication affects an early, post-entry step of virus replication.

PPIB knockdown affects the growth rate of SupT1 T cells, which was confirmed in a direct competition assay between GFP-expressing PPIB knockdown cells and GFP-minus parental cells. This could explain the relative modest knockdown efficiency as cells with relatively weak shRNA expression may have been selected. In fact, we noticed that prolonged culturing of the PPIB knockdown cells gradually decreased the HIV-1 boosting phenotype (results not shown). This is likely to be a PPIB-specific effect and not an unrelated off-target effect because all 4 anti-PPIB shRNAs yielded similar results.

Flow cytometry analyses demonstrated that the upregulation of PPIB expression upon HIV-1 infection is a rather quick cellular response. Surprisingly, this upregulation is not only apparent in the infected cells, but does also affect all other cells in the culture, suggesting a trans-cellular signaling response to protect bystander cells against HIV-1 infection. We did not observe this effect in a clonal SupT1 T cell line with an inducible HIV-1 provirus that mimics only the late production phase of the replication cycle, suggesting that early events are required to induce PPIB expression.

Cyclosporine A (CsA) inhibits HIV-1 replication at two steps in the replication cycle. An early block is PPIA-dependent and occurs during virus uncoating or another step before nuclear import (328;355). A late block prevents Envelope protein incorporation in virion
The Cyclophilins A and B have opposing roles in HIV-1 replication

particles (327;328). The extent of these effects is cell type dependent (240). In our hands, CsA was able to inhibit HIV-1 replication in SupT1 cells, similar to what has been reported for the Jurkat T cell line and CD4^+ HeLa cells (240;392), but only with repeated CsA administration. With the intensified treatment, CsA inhibited HIV-1 replication to the same extent in the PPIB knockdown cells as in the parental SupT1 cells. Addition of CsA in single round infections with the knockdown cells did not show inhibition of the early steps of replication, thus indicating that the inhibition observed in virus replication assays is mainly due to the effects of CsA on the late phase. As reported, this late CsA effect is PPIA and PPIB independent (327).

Although it has been known for a long time that PPIA is important for HIV-1 replication, its precise role is still not completely understood (120;226;238;366). Part of the confusion is due to the fact that CsA was assumed to act exclusively as specific PPIA inhibitor, but we now know that CsA also impacts virion assembly in a PPIA-independent manner (327;328). It has recently been demonstrated that deletion of the PPIA gene in Jurkat cells results in inhibition of viral replication (51) and that the interaction of PPIA with CA is needed at an early step of infection before reverse transcription (50;226). However, although a clear relation between PPIA expression and HIV-1 infectivity exists (392), the level of PPIA expression in the cells is not the only factor that determines the effect on infectivity (240). In addition, PPIA interacts with the viral vpR protein and this interaction is required for its functional expression (398).

In this study we demonstrate that HIV-1 does not require PPIA when PPIB is absent. This suggests an active interplay between these two proteins, which have a very high structural and functional similarity and binding studies indicate similar CA-binding efficiencies (48;109;227). The main difference is the signal sequence in PPIB that directs the protein to the ER. Thus, binding of PPIB to CA early after infection could result in redirection of the pre-integration complex leading to its degradation, whereas binding to PPIA would result in correct nuclear localization.

An intriguing remaining question is why wild-type HIV-1 is dependent on the PPIA interaction with CA. HIV-2 and several SIV viruses that do not employ this interaction are fully replication competent in cells that lack PPIA (50;355). Also, HIV-1 escape variants have been isolated that are PPIA independent (4;47;387). These variants have been isolated in cells that do not express PPIA (either by CsA treatment or PPIA knockdown). Some of these viruses carry CA mutations that prevent binding to PPIA. Our results with the PPIB knockdown cells are consistent with these observations on escape variants as it is very likely that interference with PPIA binding to CA will also influence the PPIB-CA interaction (48;226). Other escape variants have CA mutations that do not alter the interaction with PPIA (4;51;387;392). Remarkably, some of these mutations make the virus CsA-dependent in some cell lines. It is possible that this CsA-dependency of some mutants is caused by a residual need to decrease PPIB. It thus seems fairly easy for HIV-1 to evolve to a PPIA-independent phenotype. Yet, primary HIV-1 isolates are usually more sensitive to CsA than lab-adapted strains (51), indicating that primary isolates are more dependent on the interaction with PPIA. This finding suggests a direct effect of the PPIA-CA interaction on virus replication, rather than an indirect effect through preventing the PPIB-CA interaction. For instance, PPIA may assist in migration of the HIV-1 pre-integration complex to the nucleus, and this effect could be cell type dependent and more critical in
primary cells. In conclusion, we identified the cellular PPIB protein as a candidate restriction factor, whose anti-HIV-1 action is counteracted by the cellular PPIA protein.

Acknowledgements

We thank Stef Heynen for performing the CA-p24 Elisa experiments and Hans Langedijk for providing T1249. We thank Renée van der Sluis and Dave Speijer for useful discussions. This research was supported by grants from the Dutch AIDS Fund (AIDS Fonds 2007028, 2008014 and 2006006).
Chapter 7
The RNA helicase DDX3 is involved in trans-activation of the HIV-1 Long Terminal Repeat promoter

Julia J.M. Eekels, Rienk E. Jeeninga and Ben Berkhout

Manuscript in preparation

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam
Abstract
The RNA helicase DDX3 has a plethora of cellular functions and has been implicated as a co-factor or inhibitor in some types of cancer. DDX3 can also influence the replication of human pathogenic viruses, including human immunodeficiency virus type 1 (HIV-1). We show that stable RNA interference (RNAi) mediated knockdown of DDX3 inhibits HIV-1 replication with minor effects on cell proliferation. As DDX3 is involved in the transcriptional control of different promoters, we studied the effect of DDX3 overexpression and knockdown on the activity of the HIV-1 Long Terminal Repeat (LTR) promoter. Knockdown of DDX3 reduces both the basal and Tat-induced LTR transcription levels. Overexpression of DDX3 has no effect on basal transcription, but enhanced Tat-induced transcription from the HIV-1 LTR. The DDX3 protein thus seems to facilitate optimal LTR transcription and may play a role as cellular co-factor of the viral Tat protein.

Introduction
The acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus type 1 (HIV-1) remains a serious global health threat, although combined anti-retroviral therapy (cART) is highly active against the virus. Despite the clinical success of cART, the emergence of drug-resistant viral strains is another major problem. To avoid viral drug resistance, it has been proposed to target cellular co-factors that HIV-1 requires for its replication cycle.

One of the many possible therapeutic targets is the RNA helicase DDX3. DDX3 is a member of the family of DEAD box RNA helicases (DEAD = AspGluAlaAsp) that possesses RNA-dependent ATPase and helicase activity (394). Members of this family are involved in a wide range of cellular processes that deal with RNA; e.g. splicing, mRNA export, transcription and translation (289). DDX3 has been implicated in many of these cellular functions as summarized in Figure 7.1 and reviewed in (308). DDX3 interacts with the transcription factor Sp1 and binds to several promoters, e.g. the interferon-β promoter to execute a transcriptional role (70;309). DDX3 shuttles between the cytoplasm and nucleus and nuclear export is mediated via interaction with the CRM1 protein, which also implicates DDX3 in the nuclear export of other RNAs (388). A function in mRNA translation is executed via an interaction with the translation initiation factor eIF3 (196). In most cases the exact molecular mechanism of DDX3 action remains unclear.

DDX3 also plays a role in innate immunity. DDX3 is involved in the intracellular signaling upon activation of pathogen recognition receptors (PPRs) such as Toll-like receptors and cytoplasmic viral RNA sensors like RIG-I. When PPRs sense the presence of a pathogen, several signaling pathways are triggered, eventually resulting in activation of the transcription factors NF-κB and interferon regulatory factors (IRF) 3 and 7. NF-κB activation leads to the expression of anti-inflammatory cytokines, IRF3 and 7 activation leads to the induction of type I interferons. Cytokines and type I interferons are known to be potent antiviral mediators. Several specific DDX3-virus interactions have been reported in literature as summarized in Figure 7.1. Vaccinia virus (VACV) inhibits the induction of interferon-β (IFNβ) by the viral protein K7, which binds to DDX3, to inhibit the DDX3-mediated induction of the IFNβ promoter (309). DDX3 plays a similar role in hepatitis B virus (HBV) replication. The HBV polymerase protein binds to DDX3 and blocks IFNβ
production (369;395). Interestingly, interaction with the viral polymerase leads to incorporation of DDX3 into the virion particle and DDX3 inhibits the first step of reverse transcription in the newly infected cell, thus augmenting the antiviral effect (368). In contrast, other viruses such as hepatitis C virus (HCV) use DDX3 as a cellular co-factor for its replication, although the precise mechanism remains unclear. DDX3 interacts with the HCV structural core protein and may modulate viral or cellular gene expression (17;23;265).

**Figure 7.1. Different functions of the DDX3 protein**

DDX3 has been implicated in many cellular processes and we grouped the specialized functions in innate immunity, virus replication and cancer. “↑” indicates a process positively influenced by DDX3, “↓” indicates processes negatively influenced by DDX3. Functions of DDX3 that are linked are depicted in the same colour. Red: nuclear RNA export. Blue: Upregulated transcription. Green: Downregulated transcription. Purple: Interferon-β induction. The effect of DDX3 on Tat-mediated transcription is the focus of this study (grey box).

DDX3 binds CRM1 and this interaction is important in HIV-1 replication as unspliced and incompletely spliced viral transcripts require nuclear export via the CRM1 pathway that is controlled by the viral Rev-RRE axis (388). Furthermore, DDX3 expression was found to be induced by the HIV-1 protein Tat. It has been reported that DDX3 does not influence transcription from the HIV-1 LTR promoter, but these experiments were performed exclusively in the absence of Tat (388). Two recent studies confirmed that DDX3 expression is enhanced by Tat and that the two proteins physically interact (62;129).

Opposing roles have also been described for DDX3 in cancer. DDX3 acts as an oncogene in breast cancer cells by reducing transcription from the E-cadherin promoter, which has been associated with progression to cancer and metastasis (44). DDX3 acts as a tumor suppressor in hepatocellular carcinoma cells, where it upregulates the expression of the tumor suppressor p21 via an interaction with the transcription factor Sp1 (70).

As DDX3 has been implicated in transcriptional control in diverse settings, we wanted to readdress a putative role in HIV-1 transcription (see box in Figure 7.1). We and others have previously shown that stable knockdown of DDX3 inhibits HIV-1 replication (103;158). This result was confirmed in this study, with additional experimentation to
document the mRNA knockdown level and the impact on cell growth. We subsequently tested the effect of DDX3 overexpression or knockdown on transcription from the HIV-1 LTR promoter. DDX3 overexpression enhances transcription from the LTR in the presence of the viral Tat protein, while knockdown of DDX3 has the opposite effect. The ATPase and helicase activities of DDX3 are critical for this role as HIV-1 co-factor. We thus propose that DDX3 functions at two steps of the HIV-1 replication cycle, via Tat in transactivation of the HIV-1 LTR promoter and via CRM1/Rev in the nuclear export of HIV-1 RNAs. This double action as co-factor makes DDX3 a promising target for anti-HIV-1 therapy, of which the impact may be broader as DDX3 is also a co-factor for HCV, which is frequently seen as co-infection in HIV-1 infected individuals.

Materials and methods

Plasmids

ShRNA-expressing constructs (pLKO.1) are from the MISSIONTM TRC-Hs 1.0 library (Sigma-Aldrich) and were obtained as bacterial clones (293). Target sequences can be found on the website of Sigma-Aldrich [http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html]. Plasmid DNA was isolated with Nucleobond Midi prep columns according to the manufacturer’s protocol (Macherey-Nagel). For the competitive cell growth assay it was necessary to replace the gene for the puromycin selection marker in pLKO.1 for the GFP gene, as described earlier (Eekels et al, Chapter 4). pCMV-Myc-DDX3 encodes DDX3 with a Myc tag and pCMV-Myc-K230E encoding a mutant form of DDX3. Both plasmids were kindly provided by dr. M. Schröder (National University of Ireland, Maynooth). pSuper-shDDX3 was constructed as described (292). pBluescript3’LTRLuc (164) was used to study HIV-1 LTR activity and the empty vector pBluescript KS(-) was used to control for input DNA amounts in transfections. pTat-exon contains the Tat coding sequence under control of the constitutive CMV promoter (360).

Cell culture, DNA transfection, lentiviral vector production and transduction

Human embryonic kidney 293T (HEK293T) adherent cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human SupT1 suspension T cells were grown in Rosewell Park Memorial Institute medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. For puromycin selection the final concentration in the culture medium was 1 μg/ml. Cell lines were cultured in a humidified chamber at 37°C and 5% CO2. DNA transfections were performed with Lipofectamine 2000 according to manufacturer’s instructions (Invitrogen). In short, 2 x 10^5 cells per well (24-wells) were seeded a day before transfection. The next day cells were transfected, where necessary the empty plasmid pBluescript KS (-) was used to obtain an equal amount of DNA per well. Cells were harvested 24 hours post transfection. All transfections were performed in four-fold, of which two wells were analyzed for luciferase activity and two wells were harvested for RNA isolation. Luciferase activity was measured using the DualGlo Luciferase kit (Promega).
Lentiviral vectors were produced as previously described (292). Briefly, HEK293T cells were co-transfected with the shRNA-construct and the packaging plasmids (pRSV-Rev, pMDLg/pRRE and pVSV-G) using Lipofectamine 2000 (Invitrogen). One day after transfection the medium was refreshed and the supernatant was harvested the next day. The virus containing supernatant was centrifuged and aliquots were stored at −80°C. A sample was taken for CA-p24 enzyme-linked immunosorbent assay (ELISA) to monitor lentiviral particle production. SupT1 T cells were seeded in a 24-wells plate (1 x 10^5 cells/well), lentiviral vector was added and incubated overnight. Excess virus was washed away the next day. Stably transduced cells were selected for either by addition of 1 µg/ml puromycin to the culture medium or by FACS sorting based on GFP expression, depending on the selection marker encoded by the lentiviral vector.

**CA-p24 ELISA**

Culture supernatant was heat-inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as the capture antibody and the alkaline phosphatase-conjugated anti-CA-p24 monoclonal antibody EH12-AP (International Enzymes) as the detection antibody. Detection was performed with the Lumiphos plus system (Lumigen) in a LUMIstar Galaxy luminescence reader (BMG Labtechnologies). Recombinant CA-p24 produced in a baculovirus system was used as reference standard.

**CCG assay and FACS analysis**

To study the effects of shRNA expression on cell proliferation, the CCG assay was used as described earlier (Eekels et al, Chapter 4). Briefly, cells were transduced with 1 or 10 µl of lentiviral vector encoding a shRNA and the GFP selection marker. The percentage of GFP-expressing cells was monitored over time with FACS analysis. To do so, a sample was taken from the cell culture and cells were collected by centrifugation (4 min, 4000 rpm, Eppendorf centrifuge). The cell pellet was resuspended in FACS solution (Phosphate buffered saline (PBS) + 2% FCS). FACS analysis was performed with the FACScanto (BD Biosciences). Live cell population was based on forward and side scatter parameters and GFP-positive population was determined based on unstained and untransduced cells.

**HIV-1 replication and single cycle infection experiments**

HIV-1 was produced by transfection of HEK293T cells with the molecular clone HIV-1 LAI (270) and virus production was measured by CA-p24 ELISA. SupT1 T cell cultures (5 ml in T25 format, 2.5 x 10^6 cells/flask) were infected with HIV-1 (0.5 ng of CA-p24). Every day virus replication was monitored by scoring syncytia formation and supernatant samples were taken for CA-p24 ELISA. For single cycle infection experiments, 1.5 x 10^5 SupT1 cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away. Cells were subsequently cultured in complete medium supplemented with 0.1 µg/ml fusion inhibitor T1249 to block new infections (Eggink et al., 2008). Cells were analyzed 48 hours post infection for intracellular CA-p24 by FACS. Therefore cells were collected by centrifugation (as above) and fixated in 500 µl 4% formaldehyde for 5 minutes at room temperature. 500 µl Perm/Wash (BD Biosciences) was added and cells were again pelleted by centrifugation. Cells were stained
The RNA helicase DDX3 is involved in trans-activation of the HIV-1 Long Terminal Repeat promoter for at least 1 hour at 4°C in 50 µl BD Perm/WashTM buffer containing 5 µl 1:100 diluted antibody against CA-p24 conjugated with PE (monoclonal mouse, clone KC57, Coulter). Excess antibody was washed away by washing twice with 500 µl FACS solution, cells were resuspended in 250 µl FACS solution and analyzed on a FACScanto. Uninfected and unstained samples were used as negative controls.

**Western blot**

Cell lysates that were prepared for luciferase measurement were mixed 1:1 with SDS-PAGE loading buffer, boiled for 5 minutes and run in a 10% SDS-PAGE gel. Proteins were transferred to an Immobilon-P membrane (Millipore) via semi-dry transfer. Membranes were treated for 1 hour at room temperature in blocking solution (PBS + 5% milk + 0.1% Tween-20) and detection was performed with anti-DDX3 (Abcam, ab50703, mouse, 1:1000), anti-β-actin (Sigma, clone AC-74, mouse, 1:5000) and HRP-labeled goat anti-mouse IgG (1:5000). Luminometric detection of proteins was performed with Western Lightning ECL (PerkinElmer Life Sciences) and membranes were analyzed on a LAS4000 imager (GE Healthcare).

**RNA isolation and RT-qPCR**

RNA was isolated from 0.5 x 10^6 cells with the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions, including the optional DNase I treatment on column. 1 µg RNA was reverse transcribed with the Thermoscript kit (Invitrogen) and oligo-dT primers. cDNA synthesis was performed at 50°C for 1 hour. The resulting cDNA was diluted 100 times and 5 µl of the dilution was used as template in a SYBR Green based RT-qPCR assay (Qiagen). Primers for DDX3 and the internal control β-actin were obtained from Quantitect Primer Assays (Qiagen). Primers for Firefly luciferase were Forward 5’-GGATTACCCAGGGATTTCACT-3’ and Reverse: 5’-CTCAAGCAGGCGAATTC-3’. The levels of luciferase, DDX3 and β-actin mRNA were measured in duplo using Ct (threshold cycle) for every sample and the equation 2^ΔΔCt was used for relative quantification of luciferase and DDX3 mRNA expression levels (222).

**Results**

**DDX3 knockdown inhibits HIV-1 replication**

We previously demonstrated inhibition of HIV-1 replication by stable RNAi-mediated knockdown of cellular proteins in the human T cell line SupT1 (103). One of the tested candidate co-factors was the DDX3 helicase and 5 anti-DDX3 shRNAs were tested for their impact on HIV-1 replication. In this study we used shRNA1 that gave the best results. The SupT1 T cell line was transduced with a lentiviral vector encoding the anti-DDX3 shRNA and control cell lines were generated with the empty lentiviral vector SHC1 to test for potential effects of vector integration and SHC2 that expresses a scrambled shRNA molecule. The three cell lines were challenged with HIV-1 and virus replication was monitored over a period of 13 days (Figure 7.2A). Both controls cells support HIV-1 replication with peak infection at 9 days post infection, while HIV-1 replication is inhibited in the DDX3 knockdown cell line (shDDX3).
To test whether the knockdown of DDX3 has any effect on cell proliferation, a competitive cell growth (CCG) assay was performed (Eekels et al, Chapter 4). This assay is performed directly on the lentivirus transduced culture that contains transduced GFP-expressing cells and non-transduced cells. The percentage of GFP-positive cells was analyzed longitudinally by simple FACS analysis. A reduced cell growth rate due to shRNA expression is scored as a gradual decrease in the fraction of GFP-positive cells. Knowing the doubling time of untransduced SupT1 cells, the relative proliferation rate of transduced cells can be calculated. The relative proliferation rate of SHC1 cells was set at 100% and DDX3 knockdown resulted in a moderate loss of 10% (Figure 7.2B).

The DDX3 mRNA level was measured in the three cell lines using RT-qPCR. A knockdown percentage of 55% was scored in the shDDX3 cells compared to the empty SHC1 control. The scrambled shRNA in SHC2 had no effect on DDX3 mRNA expression level (Figure 7.2C). It has in fact been reported that a knockdown percentage above 50% is difficult to achieve (309;388). Nonetheless, this relative modest knockdown efficiency has a clear effect on HIV-1 replication.

![Figure 7.2. Stable knockdown of DDX3 delays HIV-1 replication without inducing cytotoxicity](image)

**A.** Stable knockdown SupT1 cells were generated with a shRNA against DDX3 (shDDX3). Two control cells were generated in parallel: SHC1, with an empty lentiviral vector and SHC2, expressing a scrambled shRNA molecule. The cell lines were infected with HIV-1LAI and viral replication was monitored in the culture supernatant by ELISA for CA-p24 over a period of 13 days. **B.** The relative cell growth rate was measured to score adverse effects of DDX3 knockdown. Cellular proliferation rate of SHC1 was set at 100%. A minor reduction of 10% was observed in the DDX3 knockdown cell line. **C.** DDX3 mRNA levels were measured with RT-qPCR. Three independent cell samples were analyzed and the data was pooled. The mean relative gene expression levels are shown (SHC1 set at 100%), error bars represent standard deviation.

**DDX3 knockdown in single cycle infection experiments**

To assess which step of the replication cycle HIV-1 is blocked by DDX3 knockdown, we performed single cycle infection experiments in stably transduced SupT1 cells. To do so, cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away and the fusion inhibitor T1249 was added to prevent new rounds of infection. After 48 hours, the cells are harvested and intracellularly stained for viral gene expression in the form of CA-p24 protein. With flowcytometry the percentage of cells positive for CA-p24 can be analyzed, as well as the mean production of CA-p24 per positive cell, expressed as the mean fluorescence intensity (MFI). Extracellular virus production was analyzed by CA-p24 ELISA in the culture supernatant.

When DDX3 expression is reduced, we observe a reduction in the percentage of cells that become positive for intracellular CA-p24 (Figure 7.3, left panel), which indicates that less cells were productively infected. The amount of CA-p24 produced by these infected cells is...
also modestly reduced upon DDX3 knockdown (Figure 7.3, middle panel). As less cells become productively infected and those cells produce less CA-p24, the reduction of extracellular CA-p24 in the culture supernatant is expected (Figure 7.3, right panel). The fact that less cells are productively infected upon DDX3 knockdown indicates that an early step in HIV-1 replication is inhibited. However, the small reduction in CA-p24 production by infected cells indicates an additional effect on a late HIV-1 replication step.

**Figure 7.3. DDX3 knockdown in single round HIV-1 infections**
Control and DDX3 knockdown cells were incubated with HIV-1 for 4 hours, after which excess virus was washed away. Cells were further cultured in the presence of the fusion inhibitor T1249 to prevent new rounds of infection. The percentage of cells positive for intracellular CA-p24 production was measured by FACS analysis 48 hours post infection (left panel). The mean fluorescence intensity (MFI) is plotted as a measure of CA-p24 production per infected cell (middle panel). The CA-p24 concentration in the culture supernatant was measured by ELISA (right panel). The average of 2 independent experiments that were performed in duplicate are shown, error bars represent the standard deviation.

**DDX3 acts in synergy with Tat to transactivate the HIV-1 LTR**

Because DDX3 plays multiple roles in the regulation of cellular transcription, we tested the effect of DDX3 knockdown on the HIV-1 LTR promoter. To monitor the exclusive effects on LTR activity, experiments were performed with a luciferase reporter gene under control of the LTR promoter (LTR-luc) in HEK293T cells. We initially transduced these HEK293T cells with the shRNA-expressing lentiviral vectors but did not obtain significant DDX3 knockdown. We therefore decided to use transient DNA transfection. Cells were co-transfected with LTR-luc and increasing amounts of the pSuper plasmid encoding the shRNA against DDX3. The effect of DDX3 knockdown on LTR-luc activity was analyzed in the absence and presence of the viral protein Tat that activates transcription from the LTR (Figure 7.4A). The basal luciferase activity without Tat was set at 1.

In the absence of Tat, an increasing amount of shDDX3-expression construct and thus increased knockdown level gradually decreased the basal Luciferase activity. When the LTR-promoter is activated by Tat, DDX3 knockdown again decreased Luciferase activity. DDX3 has been reported to be important in many cellular mechanisms such as nuclear export of RNAs and translation. In fact, the firefly luciferase mRNA requires the CRM1 RNA export pathway, in which DDX3 has been implicated (174). To show that the observed effect was due to decreased transcription activity rather than e.g. nuclear RNA export, the luciferase mRNA level was measured by RT-qPCR. Both in the presence and absence of Tat a gradual decrease of luciferase mRNA expression was observed when DDX3 is knocked down (Figure 7.4B). The knockdown of DDX3 protein was confirmed by Western blot analysis, on which the cellular β-actin protein acts as loading control (Figure 7.4C).
We next tested the impact of DDX3 overexpression by co-transfection with a CMV-driven DDX3 expression vector (Figure 7.4D). DDX3 overexpression further boosted Tat-activated Luciferase activity, but it has no significant effect on the basal Luciferase activity. The latter finding is similar to those of Yedavalli et al, but that study did not test the DDX3 effect with Tat (388). RT-qPCR analysis confirmed that the increase in Luciferase activity is due to increased luciferase mRNA expression (Figure 7.4E), and the overexpression of DDX3 was confirmed by Western blot analysis (Figure 7.4F).

Figure 7.4. DDX3 influences LTR transcription in a Tat-dependent manner
A. HEK293T cells were co-transfected with 25 ng LTR-luciferase construct and increasing amounts of pSuper- shDDX3 (10, 50, 100 and 500 ng), in the absence and presence of Tat (5 ng). Knockdown of DDX3 decreases both basal and Tat-inducted LTR activity. The basal Luciferase activity without Tat was set at 1.  B. RT-qPCR was performed to measure luciferase mRNA levels. Basal luciferase mRNA level without Tat was set at 1.C. Knockdown of DDX3 was confirmed by Western blot analysis, with β-actin as loading control. D. HEK293T cells

110
The RNA helicase DDX3 is involved in trans-activation of the HIV-1 Long Terminal Repeat promoter

were co-transfected with 25 ng LTR-luciferase construct and increasing amounts of pCMV-DDX3-Myc (10, 50, 100 and 500 ng), in the absence and presence of Tat (5 ng). Overexpression of DDX3 has no effect on basal LTR activity, but enhances Tat-induced LTR activity. E. The increase in luciferase mRNA levels was confirmed by RT-qPCR. F. The overexpression of DDX3 was confirmed by Western blot analysis.

The ATPase and helicase capacity of DDX3 is necessary for LTR activation

To test whether the catalytic functions of DDX3 are required for the effect on HIV-1 LTR transcription, we used the K230E mutant. This DDX3 mutant has a single amino acid substitution in the Walker A motif and is not able to hydrolyse ATP and to unwind RNA (388). DDX3-K230E overexpression did not increase the Luciferase activity (Figure 7.5A) and the level of luciferase mRNA (Figure 7.5B), but expression of the K230E DDX3 mutant was confirmed by Western blotting (Figure 7.5C). Parallel experiments with wild-type DDX3 reproduced the activation phenotype (Figure 7.5D and 7.5E) and protein expression levels were similar to that of the mutant DDX3 (Figure 7.5F). The ATPase and helicase activity of DDX3 are therefore important for the stimulatory effect on Tat-mediated transcription from the HIV-1 LTR promoter.

Figure 7.5. The ATPase and helicase activity of DDX3 are required for modulation of the HIV-1 LTR
A and B. HEK293T cells were co-transfected with 25 ng LTR-luciferase reporter construct and increasing amounts of DDX3-K230E expression construct (100 and 500 ng), in the absence or presence of Tat (5 ng). No effect was observed on basal and Tat-induced LTR Luciferase activity (A) and the luciferase mRNA levels (B). C. Overexpression of the mutant K230E protein was confirmed by Western blot, with β-actin as loading control. D and E. Overexpression of wild-type DDX3 (100 and 500 ng) has no effect on basal LTR transcription, but enhanced Tat-induced LTR transcription, as apparent in Luciferase activity assays (D) and luciferase mRNA assays (E). F. Overexpression of wild-type DDX3 was confirmed by Western blot analysis.
Discussion

HIV-1 requires many cellular co-factors to complete its replication cycle and one of these co-factors is the RNA helicase DDX3. This protein has been described as a co-factor of the HIV-1 Rev protein, which facilitates the export of HIV-1 transcripts from the nucleus via the CRM1 pathway. DDX3 has also been implicated in many cellular mechanisms, including transcription and translation (Figure 7.1). We first tested the effect of DDX3 knockdown in T cells on HIV-1 replication. A relatively modest knockdown of DDX3 did significantly inhibit HIV-1 replication, with only a minor effect on cell proliferation. We next tested the effect of DDX3 knockdown and overexpression on transcription from the HIV-1 LTR promoter. We measured transcription levels of a luciferase reporter construct under control of the HIV-1 LTR and show that LTR-driven transcription is decreased when DDX3 is knocked down, both in the presence and absence of the Tat protein. This result is consistent with a supportive role of DDX3 in HIV-1 gene expression.

Overexpression of DDX3 leads to enhanced levels of LTR transcription, but only in the presence of Tat. This result could indicate that endogenous DDX3 expression is sufficient for basal LTR transcription, but that more DDX3 is beneficial for high-level Tat activated LTR transcription. RT-qPCR analyses of luciferase mRNA expression indicated that the observed DDX3 effects are due to regulation at the transcriptional level. It was obviously important to rule out that DDX3 acts on nuclear RNA export or mRNA translation, mechanisms known to be influenced by DDX3.

To test if the enzymatic ATPase and associated helicase activity of DDX3 are involved in the enhancement of Tat-mediated transcription, we used the previously described K230E mutant. This mutant has a single amino acid substitution in the Walker A motif that abrogates ATPase and helicase activity (367). When overexpressed, the K230E mutant had no effect on both basal and Tat-induced transcription from the HIV-1 LTR, as opposed to the wild-type DDX3 protein. Because the mutant is stably expressed in cells, we conclude that the enzymatic helicase activity is important for enhancement of HIV-1 LTR transcription levels. Interestingly, it has been shown that the K230E mutant is fully able to enhance transcription from the IFNβ promoter (309), indicating that different molecular interactions and mechanisms are at play during transcriptional activation of these promoters. For instance, HIV-1 LTR transcription may be special because of the multiple binding sites for the Sp1 transcription factor, which interacts directly with DDX3. Sp1 is required for both basal and Tat-mediated activation of the LTR promoter (38;332).

DDX3 has recently been the focus of many studies because of its role in cancer development and the replication of at least two pathogenic human viruses (HIV-1 and HCV). Inhibition of such a cellular co-factor has obvious therapeutic potential and different antiviral strategies can be proposed. We and others showed that RNAi mediated DDX3 knockdown inhibits HIV-1 replication, with only minor effects on cell proliferation. DDX3 could thus be placed on the shortlist of candidate targets for a gene therapy approach to silence critical co-factors of pathogenic viruses, although the CCR5 receptor remains the primary target for anti-HIV therapies (13). Other studies have used small-molecules inhibitors of DDX3 to achieve potent inhibition of HIV-1 replication (229;230). The notion that DDX3 contributes to two important steps of HIV-1 replication, that is Tat-mediated LTR transcription and Rev-mediated RNA export, makes it an even more attractive therapeutic target.
Acknowledgements

This research was supported by the Dutch AIDS fund (grant 2006006 and 2007028). We thank Berend Hooibrink for FACS sorting, Stephan Heynen for CA-p24 ELISA experiments, Dirk Geerts for providing the shRNA-expression constructs and Dr. Martina Schöder for the DDX3-expression constructs. We also thank the Belgian Federal Government for financial support through the Inter-University Attraction Pole grant P6/41.
Chapter 8
General discussion
Lessons from RNAi silencing of HIV-1

RNA interference (RNAi) is an evolutionary conserved mechanism induced by double-stranded RNA (dsRNA) that triggers sequence-specific gene silencing at the post-transcriptional level. The dsRNA-inducer molecule consists of 19-23 nucleotides with one strand complementary to the target mRNA (116). Intracellularly expressed short hairpin RNAs (shRNAs) as well as transfected small interfering RNAs (siRNAs) have been successfully used against target sequences in the HIV-1 RNA genome (76;345). However, resistance mutations can be selected when a single viral RNA sequence is targeted in a monotherapy setting. Furthermore, since the RNA genome from infecting virion particles is not a target for RNAi attack, the virus can only be inhibited at the level of the de novo synthesized RNA transcripts, thus after integration of the provirus into the host cell genome. Therefore, targeting of the cellular co-factors that are important for virus replication may provide several advantages. First of all, it has been hypothesized that it is more difficult for the virus to gain resistance against such inhibitors, unless HIV-1 can evolve to use a related co-factor (276). Second, targeting of cellular co-factors that are important in early replication steps can prevent the integration of HIV-1 DNA into the cellular genome and thus the establishment of the stable provirus reservoir. The best example is the HIV-1 CCR5 co-receptor. Persons who are homozygous for a 32-bp deletion in the CCR5 gene (CCR5Δ32) are healthy and resistant to HIV-1 infection as in most cases CCR5-tropic HIV-1 variants are transmitted (89;156). CCR5 facilitates the first step of HIV-1 replication; entry of the virus into the target cell. Targeting of CCR5 is therefore often presented as the perfect candidate in a therapy based on targeting of cellular co-factors. However, HIV-1 may switch to use the CXCR4 co-receptor. Third, it is possible that different viral pathogens use the same cellular co-factor, which could thus be a broad-spectrum therapeutic target.

In this thesis we tested whether RNAi against cellular co-factors can be used to inhibit HIV-1 replication. Therefore we choose 30 candidate co-factors based on a literature survey and generated stable knockdown T cell lines. This enabled the long-term analysis of the effects of host protein silencing on viral replication and, perhaps equally important, on cell viability. In any setting where long-term culturing of cells is involved, a small delay in cell proliferation can eventually have a major impact on the experimental outcome. A major impact on cell proliferation can easily be scored by visual inspection of the cell cultures and by the practical observation that some cultures need to be passaged less frequently than others. However, subtle differences in cell growth are not easily detected. Several methods exist to determine cell viability and proliferation, but we demonstrated that these assays are usually not as sensitive as one would like. To detect smaller deficiencies in cell proliferation, we designed the competitive cell growth or CCG assay, which exhibits a higher sensitivity than currently available assays.

With the CCG assay we were able to determine the effect of co-factor silencing on cell growth. In some cases fairly small defects were measured (1-5% reduced cell growth), which is a difference that easily will go unnoticed during culturing. For other co-factors we measured a higher impact on cell growth. One can ask whether this poses a problem in a gene therapy setting. The answer obviously depends on the cell types that are targeted in the RNAi therapy and their proliferation capacity. Another critical issue is whether the cell function is altered by co-factor silencing, which requires additional experimentation.
However, when cell growth is affected by a treatment that makes the cells non-susceptible for HIV-1 replication, one could hypothesize that the therapeutic value outweighs the negative effect on cell growth.

To obtain a prolonged inhibition of virus replication as shown in Chapter 3, a high amount of lentiviral vector input was needed and eventually only breakthrough viruses and no true viral escape were observed. This can be explained by the fact that no complete knockdown was achieved for any of the cellular co-factors tested in this thesis. Although this may also be the case for HIV-1 RNA knockdown, the situation changes when the virus replication cycle is taken in account. In case of targeting an early viral mRNA, the cascade of viral gene expression may actually result in an enhanced reduction of late viral mRNAs such that viral replication is seriously affected. One can of course boost the antiviral effect when targeting cellular co-factors by the use of additional shRNAs against the same co-factor (intensified RNAi). This approach was used for the cellular co-factor LEDGF/p75, for which a nearly complete knockdown is needed to score an effect on HIV-1 replication (223). The antiviral effect can also be boosted by combining shRNAs against multiple cellular co-factors. We show in Chapter 5 that simultaneous targeting of 2 autophagy factors increases the inhibitory effect on HIV-1 replication. However, it appears that the knockdown efficiency required for HIV-1 inhibition differs significantly per co-factor. For the DDX3 helicase a modest knockdown of 55% at the mRNA level was sufficient to score inhibition of virus replication.

Targeting cellular co-factors of pathogenic viruses

In this thesis we describe the use of RNAi against cellular co-factors of HIV-1 replication. However, this approach can be used for the therapeutic silencing of other viruses as well. RNAi screens have recently been performed for several pathogens to identify candidate co-factors that are critically involved in the pathogen replication cycle. We will present those RNAi screens and antiviral RNAi approaches and will start with influenza A virus, for which several RNAi screens have been reported.

Influenza A virus

Influenza A virus is a member of the genus Orthomyxoviridae and is an enveloped, negative-stranded RNA virus. It is well known for causing the flu in humans and can infect e.g. birds and pigs as well. Influenza viruses cause annual epidemics and sporadic pandemics, with the potential of severe pathogenicity in humans. The last pandemic in 2009 was caused by the swine-derived H1N1 virus and caused around 60 million infections worldwide, although most cases reported only relatively mild symptoms. Several drugs are available for prophylaxis and treatment: oseltamivir and zanamivir (both inhibitors of the viral neuraminidase protein) and amantidine and rimantadine (blocking uncoating of the virus via inhibition of the viral M2 protein). Most human influenza variants are resistant against the latter class of drugs (59) and several strains are resistant against oseltamivir (351).

Influenza encodes only 10 proteins and therefore requires the cellular machinery and many of its proteins as co-factors in the replication cycle. Although the function of the viral proteins has been studied extensively, many of the important co-factors remained elusive. Several genome-wide studies have been conducted to identify candidate co-
factors and for influenza no less than 6 RNAi studies were performed. The first study by Hao et al was done in Drosophila cells as mammalian cell systems were not yet available (149). Although RNAi-based screening works well in insect cells, the virus had to be modified to allow replication in Drosophila cells. Over 100 Drosophila genes were identified as being involved in influenza virus replication. Next, three genome-wide RNAi studies were performed in mammalian systems. Brass et al used human osteosarcoma cells (55), while König et al and Karlas et al used the human lung cell line A549 (172;179). The studies of Brass and König were designed to identify cellular co-factors involved in the early to mid-replication cycle, while the experimental set-up of the Karlas study assessed the complete replication cycle up to budding of new virions. Many possible co-factors were described; Brass listed 133, König 295 and Karlas 287 candidates. Two studies used quite different approaches to identify co-factors of virus replication. Shapira et al combined yeast-2-hybrid screening with gene expression profiling and validated the resulting 1745 hits with siRNA-mediated silencing in virus replication assays. Subsequently the effect of siRNA-mediated knockdown on the interferon-β response was measured in experiments using transfection of viral RNA and infection with a ΔNS1 virus (317). This last virus does not express the NS1 protein that would normally block the interferon response. These validation experiments produced a shortlist of 616 genes. A new technique was employed by Sui et al called “random homozygous gene perturbation” or RHGP (208). This silencing strategy uses antisense RNA generated from an integrated genetic element with a promoter. This genetic element integrates via lentiviral mediated transduction at a single allelic site in the genome, either in sense or antisense orientation. In sense orientation, insertion of a promoter element upstream of the gene will lead to overexpression, such that gain-of-function experiments can be performed. In antisense orientation, the antisense transcript can silence the other allele in trans, such that knockdown studies can be performed. The major advantage of this system is that no prior knowledge or annotation is needed about candidate genes. After screening for the desired phenotype, the cell clone can be analyzed to identify the affected gene. This screen revealed 110 human genes that render host cells resistant to influenza infection (331).

It may come as a surprise that there is hardly any overlap between these 6 studies. Pairwise comparisons revealed between 0 to 32 common co-factor candidates. The largest overlap of 32 hits concerned the similar König and Karlas analyses that employed siRNA-mediated silencing in the same cell line. A mere 128 genes were found in common in at least 2 studies. The overlap in common cellular pathways is much higher, e.g. eukaryotic translation initiation, processing of capped pre-mRNAs, and Golgi-to-ER retrograde transport were among the overrepresented pathways (373).

Many studies have tested the use of RNAi therapeutics against the influenza A RNA targets. Several siRNAs against conserved viral targets such as the mRNAs encoding the NP, PA, PB1, PB2, M and NS proteins have been tested. Targeting the HA and NA sequence was also attempted, but a single siRNA could only silence a few viral strains due to sequence variability (130).

**Hepatitis B virus**

Globally some 390 million persons carry hepatitis B virus (HBV) and chronic infection leads to an increased risk for developing cirrhosis and hepatocellular carcinoma (HCC) (21). Between 25 and 40% of individuals who are chronically infected with HBV will develop one
of these complications. Clinical treatment of HBV consists of interferon alpha (IFNα) and nucleoside/nucleotide analogs, which are only partially effective. Prolonged treatment is necessary and often complicated by the emergence of resistant virus variants, and thus new therapy options are needed. As for HIV-1, RNAi against viral targets has been developed and promising results have been obtained by targeting the polymerase gene, including the variants thereof with resistance mutations against the antiviral drug lamivudine (242).

Several host proteins and cellular pathways have been described as being essential for HBV replication, e.g. heterogenous ribonucleoprotein K, heat shock protein 70, the sphingolipid biosynthesis pathway and the DNA damage signaling pathway (257;341;371;403). Although RNAi has been used against these host factors as a research tool to test the impact on virus replication, no therapeutic approach based on RNAi against host proteins has been described yet.

**Hepatitis C virus**

Hepatitis C virus (HCV) is frequently detected in HIV-1 infected individuals, with estimates of 30% double infections among the HIV-1 infected individuals (320). Most acute infections are asymptomatic or cause only mild symptoms. However, similar to HBV infection, the virus can cause a persistent infection that eventually may cause cirrhosis and HCC. Treatment options include a combination of pegylated INFα and ribavirin, but treatment responses range from 45 to 80%. RNAi against viral targets leads to inhibition of viral replication, with prime targets in the 5'-non-translated region (5'NTR) and non-structural genes such as the one encoding the polymerase (186;338). Not surprisingly, HCV can rather quickly develop resistance against RNAi targeting the viral NS5B gene, although the resistant viruses remained sensitive to siRNAs that target other viral sequences (382). RNAi has been used as a research tool to identify cellular co-factors for HCV replication in two genome-wide siRNA screens (210;336) and three smaller siRNA screens against more specific sets of candidate co-factors (282;283;333). Again, very little overlap in the genome-wide siRNA screens was apparent; none of the 9 hits identified by Li et al were scored by Tai et al, while similar experimental settings were used. Only a single co-factor from a genome-wide screen was also scored in the smaller screen performed by Randall et al (75). Interestingly, DDX3 represents the strongest hit in one of the small siRNA screens, and other studies showed that DDX3-knockdown reduces viral replication (23). It has been suggested that DDX3 interacts with the HCV core protein (265), although this has become the focus of discussion (17). As shown in chapter 7, DDX3 has two functions in HIV-1 replication and stable knockdown did only moderately affect cell proliferation. This makes DDX3 an attractive broad spectrum therapeutic target as it plays important roles in the replication cycle of at least two human pathogenic viruses that frequently cause co-infections.

RNAi against cellular co-factors has been described in therapeutic settings; siRNAs and adenovirus delivered siRNAs against the La autoantigen, polypyrimidine tract binding protein and VAMP-associated protein of 33 kDA were tested (96;401). Another class of proteins that is important for HCV replication concerns the Cyclophilins, and siRNAs against several proteins of this family reduced viral replication (253). Currently, analogues of cyclosporine A such as Debio-025 are being tested for treatment of HCV infection (118). Worth mentioning is that HCV is unique in that it has another special cellular co-factor, the
cellular microRNA miR-122 (166). This miRNA binds to two sites in the 5’NTR of HCV RNA and upregulates viral replication, in part by stimulating HCV translation. A candidate drug in the form of an antisense molecule has been tested in chimpanzees, and a significant reduction in viral load and HCV-induced liver pathology was observed (107). However, viral resistance against the miR-122 antagonist could occur in cell culture by introducing mutations in the target site of miR-122 (212). Long-term tests with miRNA antagonist should reveal whether drug-resistant HCV variants will be selected.

**Herpes simplex virus type 2**

Herpes simplex virus type 2 (HSV-2) causes genital herpes and HSV-2 infection can increase the risk for HIV-1 transmission by disruption of the mucosal membranes in the genital tract. A microbicide against HSV-2 could thus help in the prevention of sexually transmitted diseases (STDs). Intravaginal administration of siRNAs targeting two HSV-2 genes (UL27 and UL29) complexed in a transfection lipid transiently protected mice from HSV-2 infection (124). However, the transfection lipid itself enhanced viral infection, and the authors therefore switched to cholesterol-conjugated siRNAs. In this second study, the viral UL29 gene and the cellular Nectin-1 receptor were targeted with siRNAs. Intravaginal protection against HSV-2 infection was observed for up to a week when mice were treated with these 2 siRNAs (384). This case shows that transient RNAi against a cellular co-factor can be used as a prevention measure against viral infections. A particularly useful property in STD prevention is the fact that this RNAi-based microbicide does not have to be applied immediately prior to sexual intercourse.

**Using RNAi against bacterial infections**

Not only viruses are intracellular pathogens, some bacteria replicate inside host cells as well. Although most of these bacteria do not rely on host proteins for their life cycle as much as viruses do, bacteria do modulate several cellular pathways to make the host cell more permissive for bacterial propagation. Many RNAi studies have been performed to identify cellular co-factors important for infection with Listeria monocytogenes, *Mycobacterium fortuiitem* and *tuberculosis*, *Legionella pneumophila* and *Chlamydia spp* (6;74;92;97;272). We will focus here on research performed on *M. tuberculosis* as tuberculosis (TB) is the leading cause of death among HIV-1 infected persons in Africa. *M. tuberculosis* is an obligate human pathogen that primarily targets macrophages, where the bacterium resides in early and late phagosomal compartments. After infection, mostly via inhalation of contaminated aerosols, the bacteria are internalized by alveolar macrophages via phagocytosis (24). Normally, activated macrophages will transfer the phagocytosed bacteria to the lysosomes in order to degrade the pathogen. However, some bacteria escape from lysosomal delivery and survive within the macrophage. Only 10% of infected individuals eventually develop tuberculosis as healthy people can keep *M. tuberculosis* in check. But the combination of HIV-1 and *M. tuberculosis* infection forms a deadly combination, leading to a higher mortality rate than a single infection with either TB or HIV-1. This is further complicated by the fact that many *M. tuberculosis* strains are resistant against the first line antibiotics rifampin and isoniazid and annually 440,000 cases are reported to be multi-drug resistant (MDR) (415). Another challenge in the treatment of co-infections concerns the possible interactions between antiretrovirals and TB drug
regimens. For example, the anti-TB drug rifampin can lead to subtherapeutic levels of HIV-1 protease inhibitors, and thus trigger virus escape and therapy failure (190). The Bacillus Calmette-Guérin vaccine is available for TB, but conflicting studies have shown an efficacy of 0 to 80% (77;290). A new therapeutic approach could include the targeting of host factors.

*M. tuberculosis* has evolved to modulate several host mechanisms in order to survive in the hostile cellular environment. RNAi gene knockdown has played a significant role in elucidating the cellular co-factors that are involved. Two studies have been performed in Drosophila cells with Mycobacterium strains that replicate in insect cells; a genome-wide siRNA screen with *M. fortuiitem* (272) and a 1000 target gene RNAi study with *M. marinum* (183). Another genome-wide study was performed in a human macrophage-like cell line with a virulent *M. tuberculosis* strain (187), this study used siRNA pools against more than 1100 genes with antibacterial activity in the initial screen, and subsequent screens followed by individual siRNA validation resulted in 275 candidate co-factors. Interestingly, when siRNAs against the 275 candidate co-factors were tested for inhibition of other virulent and MDR *M. tuberculosis* strains, only 74 showed robust activity against all 8 strains tested. Half of these targets (44 out of 74) are involved in negative regulation of the autophagy mechanism, which may make sense as autophagy is the cellular defense mechanism against intracellular pathogens. Treatment with autophagy-inducing drugs has therefore been proposed as a novel treatment option for MDR *M. tuberculosis* (259). In this thesis we have shown that silencing of individual protein components of the autophagy pathway presents a therapeutic option in the treatment of HIV-1. Thus, extreme caution is warranted to avoid the development of an antiviral strategy (autophagy inhibition) that will boost *M. tuberculosis* or an antibacterial strategy (autophagy induction) that may facilitate HIV-1 replication.

**Concluding remarks**

The examples mentioned above demonstrate the usefulness of RNAi as a research tool to identify candidate co-factors in the replication of viruses, intracellular bacteria and other pathogens. Although not discussed here, these techniques have also been used in other research fields such as cancer and metabolic diseases to elucidate host proteins that are important for e.g. tumor growth or insulin resistance (153;277;319). When more than a single study was performed, the overlap in the identified co-factors was always reasonably small, even when the same cells and virus strains were used. This can largely be attributed to the manner in which such large data sets are analyzed, such as the definition of what is a “hit”. It is clear that the results of such large screens need to be carefully validated. The use of stable knockdown cells instead of short-term gene suppression with siRNAs is important in this respect. On a positive note, several pathogens turn out to use the same cellular pathways or even the same protein co-factors, and this opens the possibility of developing a broad spectrum therapy against multiple pathogens.

For clinical application many hurdles need to be taken, such as the targeted delivery of the RNAi inducer molecule. Several possibilities have been presented in literature, from nanoparticle delivery of siRNAs for transient therapies to the transduction of progenitor target cells with a viral vector with an inducible promoter for the expression of an antiviral shRNA. Several RNAi-based therapies are currently being tested in clinical trials (86;168).
&

Addendum
Samenvatting

HIV-1 en AIDS

Het humaan immuun deficiëntie virus type 1 (HIV-1) is bekend om het veroorzaken van de ziekte “acquired immune deficiency syndrome” ofwel AIDS. Eind 2009 waren wereldwijd 33 miljoen mensen geïnfecteerd en in dat jaar zijn er 2,5 miljoen nieuwe besmettingen bijgekomen. HIV-1 infecteert cellen van het immuunsysteem, wat ertoe leidt dat patiënten uiteindelijk geen werkend immuunsysteem meer hebben. Tegenwoordig bestaat er een effectieve combinatietherapie met meerdere soorten drugs waarmee de virusreplicatie doeltreffend geremd kan worden. Helaas kleven er ook vele nadelen aan deze therapie; patiënten moeten dagelijks medicijnen slikken voor de rest van hun leven, chronisch gebruik kan tot toxiciteit leiden, de therapie is relatief duur en de medicijnen kunnen de ziekte niet genezen. Vanwege de hoge mutatiesnelheid van HIV-1 kunnen resistentie virusvarianten ontstaan waardoor sommige virus remmers niet meer werken. Een vaccin is nog niet in beeld en het is daarom nodig om nieuwe therapeutische strategieën te ontwikkelen om HIV-1 te bestrijden.

DNA, RNA, eiwitten en RNA interferentie

Het menselijk lichaam bestaat, net zoals alles wat leeft, uit cellen. Deze cellen kunnen verschillende vormen en functies hebben, spiercellen doen heel wat anders dan T cellen, maar de bouw van alle cellen komt grotendeels overeen. De buitenkant wordt gevormd door een membraan, in de cel bevinden zich allerlei compartimenten die bijvoorbeeld de cel van energie voorzien. In de kern van elke cel bevindt zich de genetische blauwdruk; het DNA. DNA bestaat uit een lange ketting die uit twee strengen bestaat en bevat alle informatie om eiwitten te kunnen produceren. Eiwitten zijn de bouwstenen van het leven, de werkpaarden die biologische processen uitvoeren. Om van DNA naar eiwit te kunnen gaan is een tussenstap nodig; dit is het messenger (boodschapper) RNA of mRNA. RNA lijkt op DNA, maar bestaat slechts uit één streng. Dit proces wordt het centrale dogma van de moleculaire bioogie genoemd: DNA wordt vertaald in mRNA, mRNA in eiwit. Als HIV-1 een cel infecteert, dan wordt de virale genetische blauwdruk ingebouwd in het DNA genoom van de geïnfecteerde cel. Daarna vindt de productie van nieuwe HIV-1 eiwitten op dezelfde manier plaats als voor alle andere eiwitten in de cel; er wordt mRNA gemaakt en dat wordt vertaald in nieuwe HIV-1 eiwitten. De geïnfecteerde cel wordt zodanig gemanipuleerd dat er voornamelijk nieuwe HIV-1 deeltjes gemaakt gaan worden.

In dit proefschrift beschrijven we het gebruik van RNA interferentie als antivirale methode. De naam zegt het al; met deze techniek wordt de RNA stap verhinderd, om precies te zijn wordt het mRNA afgebroken zodat er geen eiwit meer gemaakt kan worden. Deze techniek is pas onlangs ontwikkeld, maar werd al snel een standaard techniek in de wetenschap. Het berust op sequentie-specifieke genregulatie door dubbelstrengs RNA, een wat vreemde vorm van RNA. Wanneer één streng van dit RNA precies past op een mRNA wordt dit molecuul in stukken geknipt, of de translatie naar eiwitten wordt geremd (Zie ook figuur 1.3). Zulk dubbelstrengs RNA kan in het lab in de cel gebracht worden via transfectie met small interfering RNAs (siRNAs). Deze kleine stukjes dubbelstrengs RNA worden in de cel herkend en in een eiwitcomplex geladen, de RISC
Addendum

vernietiger. Als het geladen RISC een mRNA vindt met een sequentie complementair aan de siRNA, dan wordt het mRNA kapot geknipt en dit betekent geen eiwitproductie. We kunnen met deze techniek dus doelgericht de expressie van bepaalde eiwitten remmen.

**Lentivirussen en RNAi tegen HIV-1**

Het is ook mogelijk om een continué productie van dubbelstrengs RNA in de cel tot stand te brengen. Daarvoor worden lentivirus vectoren gebruikt, welke zijn afgeleid van HIV-1. Een bruikbare eigenschap van HIV-1 is dat het zijn genetisch materiaal in het genoom van de gastheercel kan inbouwen. Virussen worden zo gemodificeerd dat ze deze eigenschap behouden, maar zich niet meer kunnen vermenigvuldigen en ziekte veroorzaken. Via zo’n gentherapie kan genetisch materiaal nodig voor het maken van dubbelstrengs RNA in de cel gebracht worden, waarna de cel continue dubbelstrengs RNA gaat produceren die shRNAs (short hairpin RNA) worden genoemd. shRNAs werken bijna hetzelfde als siRNAs. shRNAs zijn met succes in het laboratorium tegen HIV-1 gebruikt; menselijke T cellen werden behandeld met lentivirussen, de cellen producerden continue shRNAs tegen HIV-1 mRNA waardoor de cellen niet meer bevattelijk zijn voor HIV-1. Echter, de hoge mutatiesnelheid van HIV-1 zorgt ervoor dat het virus resistentie kon ontwikkelen, vooral als er maar één shRNA remmer wordt gebruikt. Eén enkele mutatie in het HIV-1 mRNA was genoeg om de RNAi remming te omzeilen.

Een antwoord hierop zou kunnen zijn om niet het virus aan te vallen, maar de RNAi methode te gebruiken tegen bepaalde eiwitten van de gastheercel. HIV-1 is een klein virus en maakt zelf maar 15 eiwitten. Dit betekent dat het veel eiwitten uit de gastheercel gebruikt om zicht te kunnen vermenigvuldigen en al deze eiwitten zijn een potentieel therapeutisch doelwit. De hypothese is dat het moeilijker voor HIV-1 zal zijn om hier resistentie tegen te ontwikkelen, aangezien het virus zich in dat geval zou moeten aanpassen aan een ander gastheereiwit. Het beste voorbeeld is het CCR5-eiwit, een receptor aan de buitenkant van T-cellen waaraan HIV-1 moet binden om de cel binnen te komen. Wanneer we de expressie van CCR5 kunnen remmen, blokkeren we ook het vermogen van HIV-1 om de T-cel te kunnen infecteren. Eén van de grootste zorgen bij deze aanpak is de mogelijke toxiciteit, aangezien componenten van de gastheercel aangevallen worden. In het geval van CCR5 weten we dat mensen zonder dit eiwit gezond zijn, want een deel van de bevolking bezit een inactief CCR5-gen. Deze mensen zijn praktisch immuun voor HIV-1 varianten die CCR5 gebruiken. Er zij wellicht nog meer eiwitten in de cel die heel belangrijk zijn voor de HIV-1 infectie, maar waarvan uitschakeling geen grote bijwerkingen veroorzaakt.

**Onderzoek beschreven in dit proefschrift**

In dit proefschrift beschrijven we het gebruik van RNAi tegen dertig verschillende cellulaire eiwitten (Hoofdstuk 3). Ook hier werden de menselijke T-cellen behandeld met lentivirale vectoren die zorgen voor de aanmaak van shRNA-remmers van cellulaire eiwitten. Allereerst werd geanalyseerd of de cellen deze ingreep wel tolererden. Daartoe hebben we de groeisnelheid van de cellen gemeten. In elf van de dertig gevallen bleek het zeer nadelig om dat specifieke eiwit uit te schakelen; de cellen deelden niet meer of gingen dood. Uiteraard werden deze shRNA remmers uitgesloten van verder onderzoek. De behandeldelcellen waar geen effect of slechts een klein effect op de groeisnelheid
Addendum

werd gemeten werden vervolgens geïnfecteerd met HIV-1. Als controle dienden cellen behandeld met een lege vector of een vector die een controle-shRNA maakt. In deze controlecellen meten we na ongeveer 8 dagen de piek van HIV-1 productie. In sommige shRNA-cellen maten we maar weinig of geen HIV-1 productie en dat betekent dat HIV-1 geremd werd in zijn vermenigvuldiging. Het lijkt dus mogelijk om RNAi tegen cellulaire eiwitten te gebruiken om HIV-1 te remmen.

Omdat RNAi tegen cellulair co-factoren veel bijwerkingen kan hebben, is er een nauwkeurige methode nodig om kleine effecten op de groeisnelheid van cellen te kunnen meten. De methodes die momenteel beschikbaar zijn, zoals het elke dag tellen van het aantal cellen en daar de groeisnelheid mee berekenen zijn erg omslachtig en niet nauwkeurig. Wij hebben daarom een nieuwe methode ontwikkeld die berust op competitie in een kweek tussen behandelde en onbehandelde cellen (Hoofdstuk 4). Wanneer cellen behandeld worden met het shRNA-lentivirus dan wordt er ook een selectiemarker ingebouwd. In veel gevallen is dat het GFP-eiwit, ofwel het Green Fluorescent protein, dat ervoor zorgt dat behandelde cellen groen oplichten in UV-licht. Na behandeling heeft men dus een mengsel van twee soorten cellen: behandelde shRNA-cellen die groen zijn en de onbehandelde controle cellen. Als de behandelde cellen langzamer groeien dan onbehandelde cellen, bijvoorbeeld omdat de shRNA toxisch is, dan zal de verhouding van groene versus gewone cellen gestaag afnemen in de tijd en dit is erg gemakkelijk te meten. Hoe sneller het percentage groene cellen afneemt, hoe groter het negatieve effect van de shRNA is. We hebben laten zien dat deze methode gevoeliger is dan de gebruikelijke methode en deze methode is dan ook toegepast in alle verdere hoofdstukken.

Eén van de beste antivirale shRNAs uit hoofdstuk 3 had als doelwit een eiwit dat deel uitmaakt van de autofagie proces. Autofagie is een cellulair mechanisme dat ervoor zorgt dat de cel kan overleven als er een tijdelijk gebrek aan voedingsstoffen is door componenten af te breken en de stoffen die daarbij vrijkomen te hergebruiken. In dit proces worden membraanstructuren in de cel gevormd en die structuren worden door sommige virussen gebruikt tijdens hun replicatie. Het was al bekend dat autofagie ook een rol speelt tijdens HIV-1 infectie, maar nadere details ontbreken. We hebben in Hoofdstuk 5 verschillende autofagie-eiwitten getest via dezelfde shRNA-methode als in Hoofdstuk 3. Het bleek dat meerdere autofagie-eiwitten belangrijk zijn voor HIV-1 en wellicht dat stoffen die autofagie remmen gebruikt kunnen worden om HIV-1 te behandelen. Om de HIV-1 remming te verbeteren hebben we ook cellen behandeld met twee shRNAs tegen verschillende autofagie-eiwitten.

Dat cellulair cofactoren een complexe rol kunnen spelen tijdens de HIV-1 replicatie blijkt in Hoofdstuk 6. Eerder was een studie uitgevoerd naar veranderingen in het expressieniveau van eiwitten in HIV-1 geïnfecteerde cellen. Sommige eiwitten worden na infectie verhoogd tot expressie gebracht, bijvoorbeeld omdat ze een antivirale werking hebben (een respons van de cel) of omdat het virus meer van dat bepaalde eiwit nodig heeft (een respons van HIV-1). Veel van deze interessante eiwitten zijn in Hoofdstuk 6 nader geanalyseerd via shRNA-remming en HIV-1 replicatie testen. Al snel is de aandacht verlegd naar het eiwit Cyclophilin B. De expressie van dit eiwit gaat omhoog als HIV-1 de cel infecteert, maar als we dit eiwit remmen gaat HIV-1 juist beter repliceren. Dit suggereert dat Cyclophilin B een antivirale functie heeft. Interessant is dat een naast familielid, namelijk Cyclophilin A, precies een tegenovergestelde werking heeft. HIV-1
heeft Cyclophilin A juist nodig om zich te kunnen vermenigvuldigen. Beide eiwitten binden aan een onderdeel van een HIV-1 virusdeeltje en doen dat op precies dezelfde plaats. We veronderstellen dat het virus Cyclophilin A heeft leren te gebruiken om de antivirale werking van Cyclophilin B te verhinderen.

In Hoofdstuk 7 hebben we de functie van het DDX3-eiwit tijdens de HIV-1 replicatie geanalyseerd. Van DDX3 was al langer bekend dat het ervoor zorgt dat HIV-1 RNA moleculen uit de celkern getransporteerd worden. In andere cellen is ook aangetoond dat DDX3 ook een invloed kan hebben op het proces van genexpressie. Wij tonen aan dat DDX3 ook invloed heeft op HIV-1 genexpressie. DDX3 gaat een interactie aan met het virale Tat eiwit en we meten verhoogde HIV-1 genexpressie dan ook alleen als zowel DDX3 en Tat tot overexpressie worden gebracht. Dit maakt DDX3 een aantrekkelijk aangrijpingspunt voor de ontwikkeling van nieuwe medicatie, omdat het meerdere functies vervult in de HIV-1 replicatiecyclus.

In Hoofdstuk 8 beschrijven we verschillende RNAi studies die zijn verricht om te bepalen welke cellulaire eiwitten belangrijk zijn voor verschillende virussen. Zo zijn er zes studies uitgevoerd om te bepalen welke cellulaire eiwitten het griepvirus gebruikt. Vele kandidaat-eiwitten zijn gerapporteerd, maar de overlap tussen de verschillende studies is gering. Dit toont aan dat het nodig is om resultaten van deze studies nauwkeurig te valideren in vervolgonderzoek. Een ander interessant punt is dat virussen die vaak samen gezien worden in dezelfde patiënt, zoals hepatitis C virus en HIV-1, soms dezelfde cellulaire eiwitten gebruiken. Het zou mogelijk zijn om met één medicijn daardoor meerdere virusinfecties tegelijkertijd te behandelen. Wel is voorzichtigheid geboden, bijvoorbeeld bij de bestrijding van tuberculose en HIV-1 in dezelfde patiënt. Voor de behandeling van tuberculose is gesuggereerd om het autofagiemechanisme te activeren, maar het tegenovergestelde is nodig om HIV-1 te remmen (Hoofdstuk 5). Cellulaire eiwitten als aangrijpingspunt voor de ontwikkeling van antivirale medicatie blijft een reële optie, maar men moet bedacht zijn op dit soort complicaties.
Summary

The human immunodeficiency virus type 1 (HIV-1) is known for causing the disease “acquired immune deficiency syndrome” or AIDS. At the end of 2009 around 33 million people were infected worldwide and in the same year some 2.5 million people became newly infected. The disease can nowadays successfully be treated with a combination drug therapy, but this therapy comes with drawbacks; patients have to take drugs daily for the rest of their lives, the drugs can have severe side-effects, the medication is relatively expensive and they cannot cure the disease. Due to the high mutation rate of HIV-1 resistant virus variants emerged, rendering some drugs ineffective. With no vaccine in sight, new therapies need to be developed to combat HIV-1 infection.

In this thesis we developed RNA interference (RNAi) as an antiviral method. RNAi is a sequence-specific cellular mechanism that is induced by double-stranded RNA and that leads to gene silencing. RNAi has been already used successfully against HIV-1 RNA targets. T cell lines were transduced with lentiviral vectors encoding shRNAs against HIV-1 RNA and virus replication was inhibited in these cells. However, when a single HIV-1 sequence was viral targeted, resistance was observed, often by a point mutation in the targeted sequence. One of the solutions to prevent viral escape is to target the cellular proteins that HIV-1 requires to complete its replication cycle. Since HIV-1 encoded only 15 proteins, it relies on many cellular proteins, all representing possible therapeutic targets. The best example is the co-receptor CCR5. It is known that a minority of the human population has a deletion in the CCR5-gene and thus do not produce an active CCR5 protein. These individuals are completely healthy, but virtually immune to infection by CCR5-tropic HIV-1 strains. There may be many more cellular co-factors that are indispensable for the virus, while their RNAi-mediated knockdown is harmless for the cell.

In this thesis we describe the use of RNAi against 30 different cellular co-factors (Chapter 3). T cells were treated with lentiviral vectors that encode a shRNA for co-factors silencing. First the cells were analyzed for negative effects of the transduction or shRNA expression on cell proliferation. To do so, the doubling time was measured and in 11 of 30 cases the knockdown was moderately to severely toxic for the cells; cells exhibited an increased doubling time or died. The remaining cells were infected with HIV-1 and the effects on HIV-1 replication were analyzed by measuring the production of HIV-1 in the culture supernatant. In some cases we could inhibit virus replication up to 2 months, indicating that RNAi against cellular cofactors can be used to strongly delay HIV-1 replication.

Since RNAi against a cellular co-factor can be potentially toxic, a sensitive method is necessary to score even small effects on cell growth. Available methods, such as counting cells, are labor-intensive and unreliable. We therefore developed a new and sensitive method that is based on competition in a culture between transduced and untransduced cells (Chapter 4). When cells are transduced with a lentiviral vector encoding a shRNA, a selection marker gets integrated as well. In most cases this is the GFP (green fluorescent protein) marker. When cells are transduced, the culture contains both transduced GFP-positive cells and untransduced GFP-negative cells. If transduced cells grow slower than normal cells (due to toxicity of the shRNA), this will result in a gradual decrease of the GFP+/GFP- ratio. We show that this method, the competitive cell growth (CCG) assay is more sensitive than existing methods and it was used in all further chapters.
One of the top shRNAs in Chapter 3 targets a protein that is involved in the autophagy pathway. Autophagy is a cellular process that helps the cell to survive in cases of nutrient starvation. Many viruses rely on this pathway for their replication and although it already has been shown that autophagy plays a role in HIV-1 replication, the details remain elusive. In Chapter 5 we tested twelve autophagy proteins and found several of those to be important for HIV-1 replication. To improve the potency of virus inhibition, cells were transduced with lentiviral vectors encoding shRNAs against two autophagy factors.

It became clear in Chapter 6 that cellular proteins can play complex roles in HIV-1 replication. Earlier, a proteomics study was performed to analyze the up- and downregulation of protein expression in HIV-1 infected cells. Many of the hits from this study were further analyzed by assessing the effect of RNA-mediated knockdown of these proteins on HIV-1 replication. The focus of this chapter was on the cellular protein Cyclophilin B. The proteomics screen revealed that this protein is upregulated during HIV-1 infection, and knockdown enhances HIV-1 replication, suggesting that Cyclophilin B has an antiviral function. Interestingly, a closely related family member, Cyclophilin A, has an opposite function; HIV-1 requires this protein for replication. Both cyclophilins bind to the same part of the HIV-1 capsid protein. We propose that HIV-1 binds Cyclophilin A to prevent the antiviral activity of Cyclophilin B.

In Chapter 7 we analyzed the function of the cellular DDX3 protein in HIV-1 replication. DDX3 was already known to be involved in HIV-1 replication, as it helps in the nuclear export of HIV-1 RNAs. We show that DDX3 also influences HIV-1 gene expression by activating the viral Long Terminal Repeat promoter in a Tat-dependent manner. This makes DDX3 an attractive therapeutic target as it has multiple supportive roles in HIV-1 replication.

In Chapter 8 we describe different RNAi studies that have been described in literature to determine which cellular co-factors are used by a variety of viruses. For example, for influenza virus six studies have been done to determine the required host proteins. All studies report many candidate co-factors, but the overlap between these studies is surprisingly small. This shows that it is necessary to validate the initial results. Interestingly, viruses frequently present in the same patient, such as HIV-1 and hepatitis C virus, may require the same cellular co-factors. It may thus be possible to treat two viruses with a single drug. Caution is however warranted, e.g. for the treatment of both HIV-1 and tuberculosis, as it has been suggested to treat tuberculosis by induction of the autophagy pathway, whereas the opposite action seems needed to treat HIV-1 infection (Chapter 5). Cellular co-factors as novel antiviral therapeutic targets remain a viable option, but one has to be vigilant for these potential complications.
Over de auteur

‘Zou jij dat willen doen, broertje?’
...was de vraag voor het schrijven van deze cv voor haar eindpublicatie. ‘Ja’, antwoordde ik enthousiast, ‘Maar wat moet er dan in komen?’, vroeg ik twijfelend. Echt inhoudelijk heb ik (en de rest van ons thuis) namelijk niet echt een clou van wat ‘ons Juul’ precies doet. Bij een inzage van een meegebracht onderzoeksresultaat keken wij steevast met enige egards naar de ‘mooie grafiekjes’ en afbeeldingen. Hier en daar kwam een opmerking over het lettertype van ons mam, ‘Schon lettertiep, Juul!’ Maar inhoudelijk waren wij het spoor al snel bijster.

Het was vanaf haar geboorte (18 juli 1982) meteen duidelijk, mijn zus leefde het liefst in haar ‘eigen wereldje’. Ikzelf had dat nog niet meteen zo in de gaten. Als baby was ik dol op mijn grote zus Julia en probeerde van alles om haar aandacht te trekken. Dolgelukkig met een brede grijns zat ik naast haar. Dit in tegenstelling tot mijn zus. Zij had het liefst zo min mogelijk ogen op haar gericht, van aandacht moest zij niets hebben. Ook toen ze ouder werd veranderde dit niet.


Ook toen ze op 17-jarige leeftijd slaagde voor haar gymnasium-eindexamen op het Dr. Mollercollege te Waalwijk trok ze wederom haar eigen plan. Julia ging op kamers in Wageningen om te beginnen aan haar studie biotechnologie. Na een droomstart in het verenigingsleven aldaar en een wat mindere start op de studie verhuise ze nog regelmatig. Ook toen ze de smaak van het studeren te pakken kreeg en een grote liefde voor virologie ontwikkelde. Dit kwam door de mooie colleges van prof. Goldbach, waar ze ook haar eerste stage liep; het ontwikkelen van een testsysteem voor virale supressor eiwitten van RNAi. De familie heeft regelmatig met een geleend busje de inboedel van zuslief mee verhuizen. Vier verhuizingen binnen Wageningen, daarna Leiden voor een stage aan het LUMC bij prof. Hoeben (nu lid van haar promotiecommissie). Hier werkte ze aan mutanten van het adenoviruspolymerase voor “directed evolution” doeleinden. Door naar Dublin voor een nieuwe stage aan Trinity College, waar ze haar favoriete eiwit DDX3 (zie ook hoofdstuk 7) leerde kennen. En via Utrecht naar Diemen voor haar promotie-onderzoek aan de Universiteit van Amsterdam, waarvan het resultaat nu voor u ligt. En de spullen zijn alweer ingepakt, klaar voor vertrek richting Zwitserland.

Concluderend, Julia trekt haar eigen plan maar vindt ook overal haar draai!

Mart Eekels, Waalwijk, augustus 2011
Dankwoord

Promoveren is niet bepaald makkelijk en onmogelijk om alleen te doen. Daarom zijn er heel wat mensen die ik hier, in het meest gelezen stuk van het proefschrift, wil bedanken.

Allereerst Ben. Dank voor de kans de je me gegeven hebt door me in jouw lab te laten promoveren. Het was niet altijd makkelijk, het duurde even voor het onderzoek op gang kwam en ik had al je hulp nodig bij het schrijven. Gelukkig hebben we ook de jaarlijkse IAP uitjes om op terug te kijken.

Rienk, mijn dagelijks begeleider. Een beetje per ongeluk kreeg je me op je dak en je hebt me heel erg geholpen om mijn projecten weer op de rails te krijgen. Wie zegt dat Friezen en Brabanders niet samen gaan? Zo kwam ik ook per ongeluk bij de latencygroep terecht, waar ik veel geleerd heb tijdens de werkbesprekingen; Renée; dank voor de nuttige discussies en veel succes met je eigen promotie. Dave, het was een waar genoegen om elke week naar je monologen te mogen luisteren.

Dirk Geerts: dank voor de vele, vele constructen die je voor me uitgezocht hebt, de nuttige commentaren op de manuscripten, de aanbevelingsbrieven, hardloopadviezen en je gevoel voor humor, door jouw mailtjes rolde ik regelmatig van mijn stoel van het lachen. Ik zie je in mei 2012 aan de start van de halve marathon van Winterthur.

Carolien, dank voor al je hulp met de bureaucratie die met promoveren gepaard gaat.

Dr. Sophie Sagnier et Prof. Martine Biard-Piechaczycy, merci pour la coopération! Martina Schröder, thanks again for all your help and for introducing me to my favorite protein. To all HIV-STOP people, thanks for all the interesting meetings and the delicious Belgian beers afterwards. Sebastiaan Bol, van die andere HIV-groep; het was leuk om samen een beetje te frunniken!


Gelukkig ook genoeg vrouwelijke tegenhang: Karin, voor het enthousiasme in alles wat je doet. Gisela (Bambi), die snapt dat je af en toe gewoon even naar mooie schoenen moet staren op het internet en de gezelligheid. Ying Poi, (1 kom soep is geen soep) voor de expertise in het RNAi-veld. Gözde: for being there. Singing. Loudly. Fatima: at last somebody who is also interested in cellular co-factors! Steffi, for sharing the love for hopelessly romantic movies. Nancy, keep on running! Nikki, misschien zie ik je nog in een
berghutje in Zwitserland? En Emily, fijn om te weten dat er nog steeds iemand Brabant van “ons Guus” foutloos kan meezingen. En Elly, voor het goede voorbeeld.

En de rest van Experimentele Virologie, natuurlijk Mo; dank voor de heerlijke baksels en alle uitjes die je hebt georganiseerd samen met Alex (die dus ook bedankt!), de primers, het joehoe en je vrolijkheid. Jeroen, voor alle spullen die ik op stel en sprong nog wilde bestellen en gezelligheid tijdens wintersport. En Ilja, dank voor alle keren dat je me weer met beide benen op de grond hebt gezet. Je bent waarlijk een superanalist, maar ook buiten het lab een fantastisch mens! En de rest van Experimentele Virologie; bedankt!

De studenten die ik heb mogen begeleiden, ik hoop dat het voor jullie net zo leerzaam was als voor mij. Joyce Blokker, Anne Marieke Schut en kleine Ronald Derking (time-share student), ik wens jullie alle goeds.

Niels, het was fijn een huis te delen met iemand die he-le-maal niks weet van PCRs, ELISAs of Western blots, maar wel van dezelfde kwaliteits-TV houdt als ik.

Klaske, het is fijn iemand te kennen die zo open en lief is als jij. Wij twee hebben het toch maar mooi voor elkaar gekregen. Dank voor alle etentjes en wijntjes, dat er maar vele mogen volgen, waar we ook uithangen. Nienke, dank je voor je nuchterheid, die eigenschap van jou heeft me vaak genoeg weer laten zien dat het leven niet zo moeilijk is als het soms lijkt. En dan zijn er nog meer mensen die ik veel te weinig zie, maar als ik ze zie, dan is het goed; Joeri, Ronald (Turf) en Rianne, Marcel.

En natuurlijk de familie! Ons pa en ons ma, dank voor wat jullie van me gemaakt hebben. Het is goed om te weten dat wát ik ook doe, er altijd twee mensen zullen zijn die onvoorwaardelijk van me houden. Ons Mart en ons Paula, lieve broer en zus, het is een goed gevoel dat juist jullie twee naast me zullen staan op deze dag. En de “kouwe kaant”, die gelukkig heel warm is: Marcela en Koos , dank jullie wel voor alle gezelligheid. De volgende spelletjesavond in Zwitserland dan maar?

Ook dank voor de schoonfamilie, die zich zo open hebben gesteld in zo’n moeilijke tijd. Els, het is altijd fijn om met jou in de keuken te staan. René en Simone, Freddy, Petra en lieve Joyce, hopelijk snel wat dichterbij?

En Ronald, lief, dank voor je steun en liefde. Je bent als stugge Noordeling een rots in de branding voor deze af en toe emotionele Brabantse. Een goede combinatie die heel lang meegaat, dat weet ik zeker. Eerst Zwitserland en dan zien we wel wat de toekomst ons brengt. Als we maar samen zijn.

Diemen, 2011
References


impact on viral replication. J Biol Chem 282, 13456-13467


ribonuclease in the initiation step of RNA interference. Nature 409, 363-366


Addendum


75 Cherry, S. (2009) What have RNAi screens taught us about viral-host interactions? Curr.Opin.Microbiol. 12, 446-452


Addendum


148 Hannon, G. J. (2002) RNA interference. Nature 418, 244-251


HIV-1 transmission and disease progression. Nat.Med. 2, 1240-1243


experimental approach predicts human microRNA targets. Genes Dev. 18, 1165-1178


178 Kok, K. H., Lei, T., and Jin, D. Y. (2009) siRNA and shRNA screens advance key understanding of host factors required for HIV-1 replication. Retrovirology 6, 78


Addendum


219 Liu, Y. P., Vink, M. A., Westerink, J. T., Ramirez de A. E., Konstantinova, P., ter, B. O., and Berkhout, B. (2010) Titers of lentiviral vectors encoding shRNAs and miRNAs are reduced by different mechanisms that require distinct repair strategies. RNA 16, 1328-1339


230 Maga, G., Falchi, F., Radi, M., Botta, L., Casaluce, G., Bernardini, M., Irunnejad, H., Manetti, F., Garbelli,
Addendum


genotoxicity of lentiviral vector integration. Nat.Biotechnol. 24, 687-696


Addendum

T-lymphoid cells and derived cell lines. Cancer Res 44, 5657-5660


360 Verhoef, K., Koper, M., and Berkhout, B. (1997) Determination of the minimal amount of Tat activity required for human immunodeficiency virus type 1 replication. Virology 237, 228-236


363 von Eije, K. J., ter Brake, O., and Berkhout, B. (2008) Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. J.Virol. 82, 2895-2903


Addendum

susceptible cells by human immunodeficiency virus type 1. Blood 72, 1904-1910


412 Zielske, S. P. and Stevenson, M. (2005) Importin 7 may be dispensable for human immunodeficiency virus type 1 and simian immunodeficiency virus infection of primary macrophages. J.Virol. 79, 11541-11546

