Silencing of HIV-1 co-factors
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Chapter 5
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

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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 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 pi50 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.
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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 × 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 × 10⁵ 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 × 10⁵ 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
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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 FACSscanto. 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).
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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 WIPI1-1 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 WIPI1 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, WIPI1 and Atg16) induce a more significant cytotoxicity with a 10-20% reduced cell growth rate.
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

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>
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<tr>
<td>WIPI1</td>
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<tr>
<td>Beclin1</td>
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<td>PIK3R4</td>
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<td>Atg4A-1</td>
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<td>Atg16</td>
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\(^{a}\) 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 (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.

**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).
Inhibition of HIV-1 replication with stable RNAi-mediated knockdown of autophagy factors

**A**

Days post infection vs. CA-p24/ng/ml.

**B**

Relative expression of Atg16 and Atg5.

**C**

Cell growth defect vs. % CA-p24 positive.

**D**

MFI vs. CA-p24/ng/ml.

**E**

Fold LC3-II/GAPDH.

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Starvation: - + + + - + + + + + +

Protease inhibitors: - + + - + + - + - + +
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.

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