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

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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.

1. 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 (Arhel and Kirchhoff, 2009; Adamson and Freed, 2009).

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 (Hannon, 2002; Fire et al., 1998). 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 (Gitlin et al., 2005; Kanda et al., 2005; Moore et al., 2005; Wilson and Richardson, 2005; Tan et al., 2008; Schubert et al., 2005; Phipps et al., 2004; Ge et al., 2003). 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 (Coburn and Cullen, 2002; Lee et al., 2002). 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 (ter Brake et al., 2006). 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 (Das et al., 2004). Second, the complete or part of the target region could be deleted, especially when non-essential viral genes are targeted (Das et al., 2004). Indeed, no such deletion-based escape was observed when essential and well-conserved viral sequences were targeted (von Eije et al., 2008). 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 (Westerhout et al., 2005). 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 (ter Brake et al., 2008).

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

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can only escape by evolving to use a different cellular co-factor (Nair et al., 2005; Zhou et al., 2004). 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 (Huang et al., 1996; Liu et al., 1996). 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.

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 (Rato et al., 2010; Zhou et al., 2008; Brass et al., 2008; Konig et al., 2008; Yeung et al., 2009; Kok et al., 2009). Proteomics analysis of HIV-1 infected cells also revealed numerous host proteins that could participate in viral replication (Toro-Nieves et al., 2009; Ringrose et al., 2008; Chan et al., 2007, 2009; Wang et al., 2008). 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 (Zielske and Stevenson, 2005; Ao et al., 2007). 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.

2. Materials and methods

2.1. shRNA constructs

pLKO.1 constructs expressing shRNA candidates and with the puromycin-resistance marker were from the MISSION™ TRC-Hs 1.0 library (Root et al., 2006). 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, 4–5 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].

2.2. 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% CO_2.

2.3. Generation of stable knockdown cell lines

The shRNA-expressing lentiviral vectors were produced as previously described (ter Brake et al., 2006). 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 (1×10^5 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.

2.4. RT-qPCR

For selected cell lines the knockdown efficiency of the target mRNA was measured by RT-QPCR. RNA was isolated from 1×10^6 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 h 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 $\Delta\Delta Ct$ 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 $\Delta\Delta Ct$ 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 $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.5. HIV-1 infection

HIV-1 was produced by transfection of HEK293T cells with the molecular clone HIV-1 LAI (Peden et al., 1991) and virus production was measured by CA-p24 ELISA. SupT1 cultures (2 ml cultures in 6 wells plate, 2×10^5 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.

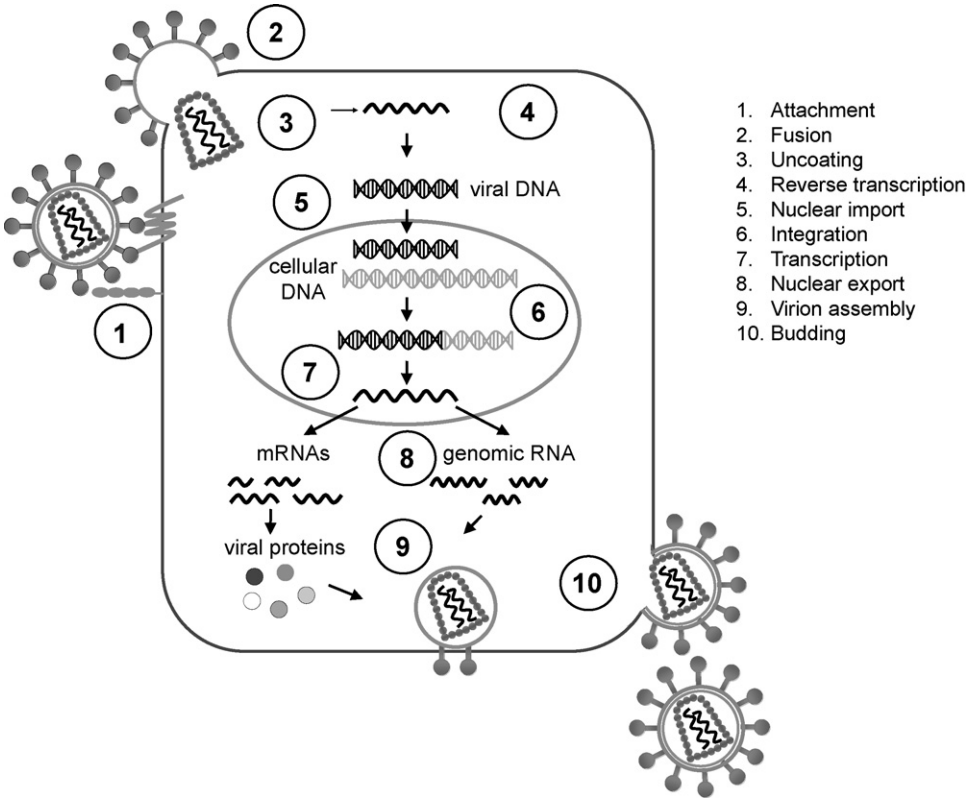

Fig. 1. Schematic of the HIV-1 replication cycle. Ten important steps of the replication cycle are numbered from attachment (1) to budding (10).

2.6. CA-p24 ELISA

Culture supernatant was heat-inactivated at 56 °C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem, La Jolla, CA). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom, Berlin, Germany) 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, Southfield, MI) in a LUMIstar Galaxy (BMG Labtechnologies, Offenburg, Germany) luminescence

Table 1
Cellular co-factors that support HIV-1 replication.

	Co-factor	Function in HIV-1 replication	Replication step[a]	Reference
1	CD4	Receptor	1	Dalgleish et al. (1984)
2	CXCR4	Co-receptor	1	Feng et al. (1996)
3	CD81	Membrane fusion	2	Gordon-Alonso et al. (2006)
4	CYPA	Function not clear, assists in early replication	3	Luban et al. (1993)
5	IPO7	Nuclear import of reverse transcription complex	5	Ao et al. (2007)
6	EED	Interactor of HIV-1 integrase	5	Violot et al. (2003)
7	ATM	Provirus integration	6	Lau et al. (2005)
8	INI1	DNA joining activity of HIV-1 integrase	6	Kalpana et al. (1994)
9	WRN	DNA repair after provirus integration	6	Sharma et al. (2007)
10	EP300	Tat-mediated transcription	7	Benkirane et al. (1998)
11	ATF3	Transcription factor	7	Shaheduzzaman et al. (2002)
12	TRBP	Binds TAR-hairpin and acts in synergy with Tat	7	Gatignol et al. (1991)
13	PRKRA	Binding partner of TRBP	7	Bennasser et al. (2006)
14	SUPT5H	Tat-mediated transcription	7	Wu-Baer et al. (1998)
15	PP2A	Tat-mediated transcription	7	Ruediger et al. (1997)
16	PPP1CA	Tat-mediated transcription	7	Bharucha et al. (2002)
17	PCAF	Tat-mediated transcription	7	Benkirane et al. (1998)
18	DHX9	Nuclear export of HIV-1 RNA via RRE	8	Li et al. (1999)
19	DDX3	Nuclear export of HIV-1 RNA via CRM1	8	Yedavalli et al. (2004)
20	DDX1	Proper cellular colocalization of Rev	8	Fang et al. (2004)
21	DHPS	Biosynthesis of eIF-5a, co-factor for Rev	8	Ruhl et al. (1993)
22	AMD	Biosynthesis of eIF-5a, co-factor for Rev	8	Ruhl et al. (1993)
23	AGFG1	Co-factor for Rev	8	Bogerd et al. (1995), Fritz et al. (1995)
24	PACS1	Localization of furin, which cleaves gp160 in Golgi	9	Piguet et al. (2000)
25	KIF4A	Transport of Gag to cell membrane	9	Martinez et al. (2008)
26	HSPD1	Incorporated into virion though Gag-interaction	9	Gurer et al. (2002)
27	M6PRBP1	Env incorporation into virions	9	Lopez-Verges et al. (2006)
28	ABCE1	HIV-1 capsid assembly	9	Zimmerman et al. (2002)
29	ALIX	Viral budding machinery	10	Strack et al. (2003),
30	ATG16	Autophagy factor	?	Brass et al. (2008)

[a] Numbers refer to the HIV-1 replication step (Fig. 1).

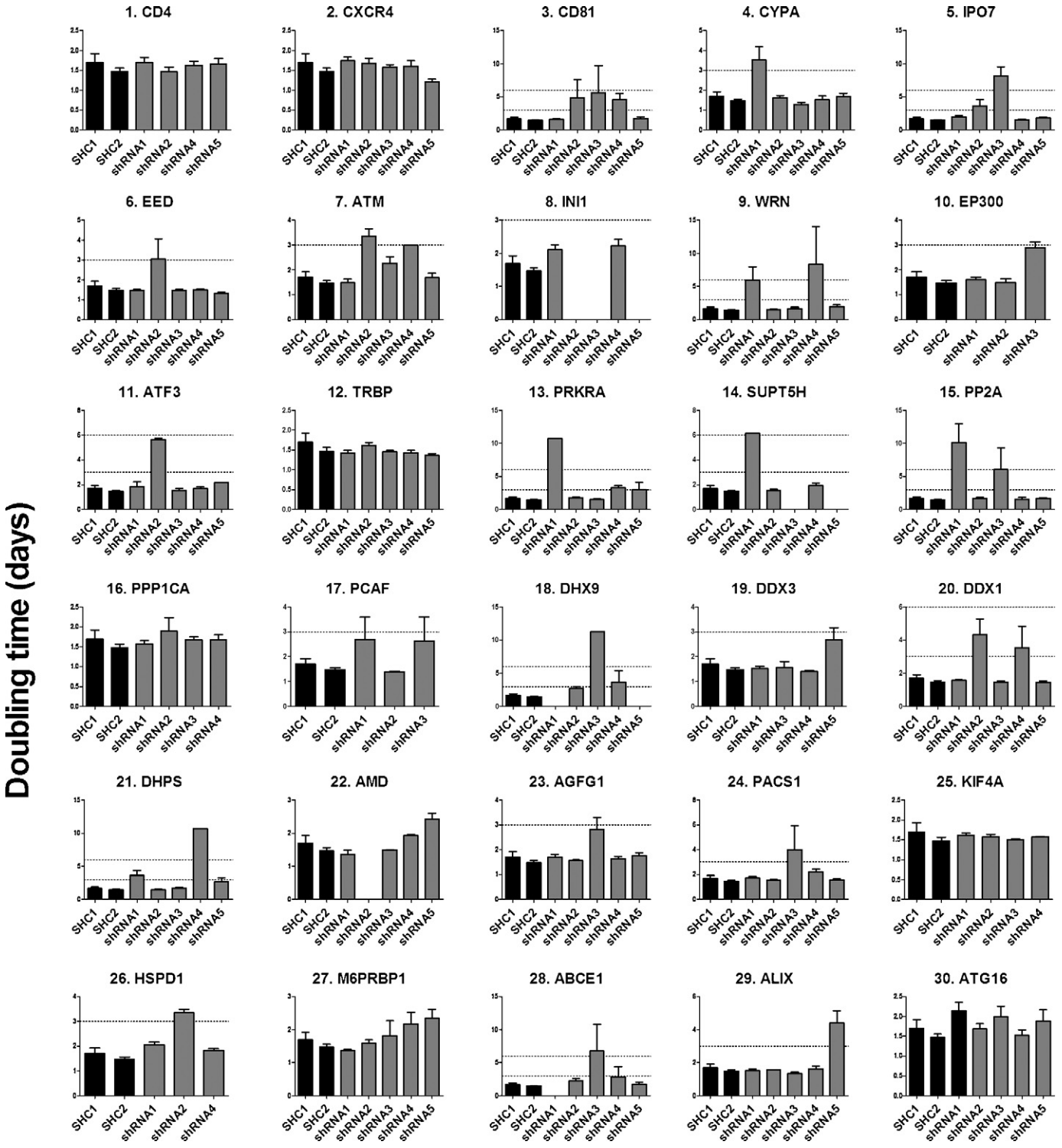

Fig. 2. Effect on cell proliferation in shRNA-expressing cell lines. shRNA cell lines for thirty cellular co-factors were counted by FACS over a period of 8 days. Each graph shows the doubling time in days (*y*-axis) per co-factor. Results for the controls SHC1 and SHC2 (black bars) and shRNA cell lines (grey bars) are shown. Data shown is from three independent experiments, error bars represent the standard error of the mean.

reader. Recombinant CA-p24 produced in a baculovirus system was used as the reference standard.

3. Results

3.1. 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 (Fig. 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 1). Some of these co-factors were previously demonstrated to support HIV-1 repli-

Table 2
Cytotoxicity and HIV-1 inhibition by co-factor shRNAs.

		Cytotoxicity per shRNA					HIV-1 inhibition per shRNA				
		1	2	3	4	5	1	2	3	4	5
1	CD4										
2	CXCR4										+
3	CD81		I	I	I						
4	CYPA	I							+	+	+
5	IPO7		I	S			+ +			+ +	+
6	EED		I						+		+
7	ATM		I				+ +				
8	INI1		S	S		S	+ +				
9	WRN	I			S				+		+ +
10	EP300										
11	ATF3		I						+	+	nd
12	TRBP									+ +	+
13	PRKRA	S			I	I					+
14	SUPT5H	S		S		S					
15	PP2A	S		S							
16	PPP1CA								+		
17	PCAF							+ +	+ +		
18	DHX9	S		S	I	S				+	
19	DDX3				nd		+ +		+ +		
20	DDX1		I		I				+ +		
21	DHPS	I			S		+ +	+			+ +
22	AMD		S								
23	AGFG1						+				
24	PACS1			I			+ +		+		
25	KIF4A						+	+			
26	HSPD1		I	nd					+ +	+ +	
27	M6PRBP1						+ +	+	+		
28	ABCE1	S		S							nd
29	ALIX					I	+ +	+			
30	ATG16							+		+ +	+ +

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.

cation 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.

3.2. 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 (Fig. 2 and Table 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).

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 (CYPA, 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 (Fig. 2.8, 2.14 and 2.18, respectively).

3.3. 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.

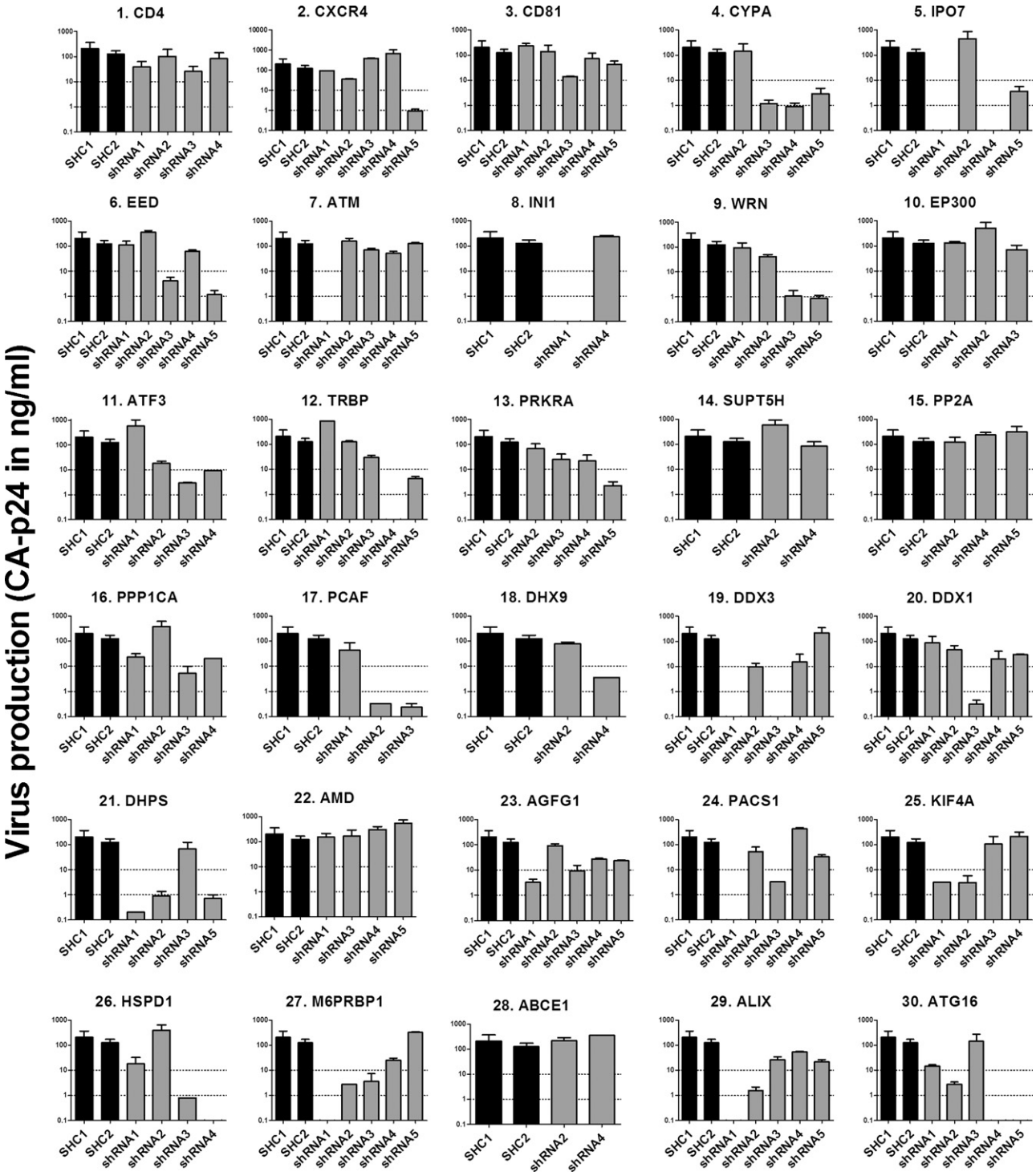

Fig. 3. Inhibition of HIV-1 replication in shRNA-expressing cell lines. shRNA cell lines for thirty different cellular co-factors were tested for inhibition of HIV-1 replication. Each graph shows the CA-p24 levels (*y*-axis) in the culture supernatant 7 day post infection per cellular co-factor. Results 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.

The CA-p24 measurements for the three infections are summarized in Fig. 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 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 2) and strong inhibition of viral replication (CA-p24 values below 1 ng/ml or undetectable, marked + + in Table 2).

Virus replication showed substantial inhibition for 15 of the 30 co-factors tested (CYPA, 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

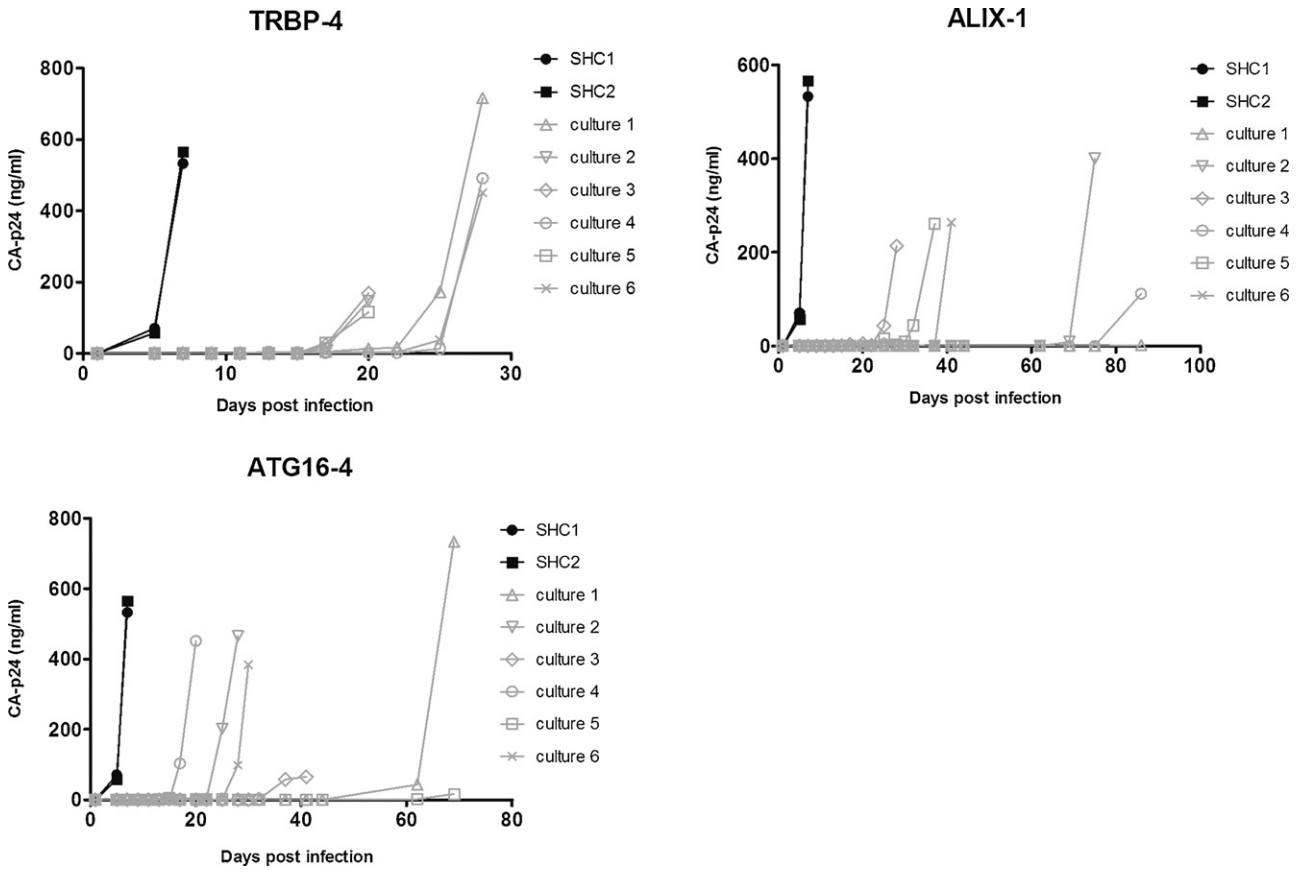

Fig. 4. Long term inhibition of HIV-1 replication and testing for viral escape. Prolonged inhibition was observed in a single cell line with a shRNA against the cellular co-factor TRBP, ALIX and ATG16. Each graph shows the result for six individual cultures per cell line and for the control cell lines SHC1 and SHC2. CA-p24 levels in the supernatant were monitored in time.

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 (Fig. 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 (Fig. 3.2), 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.

3.4. 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 (Fig. 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%) (Fig. 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.

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 (Fig. 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

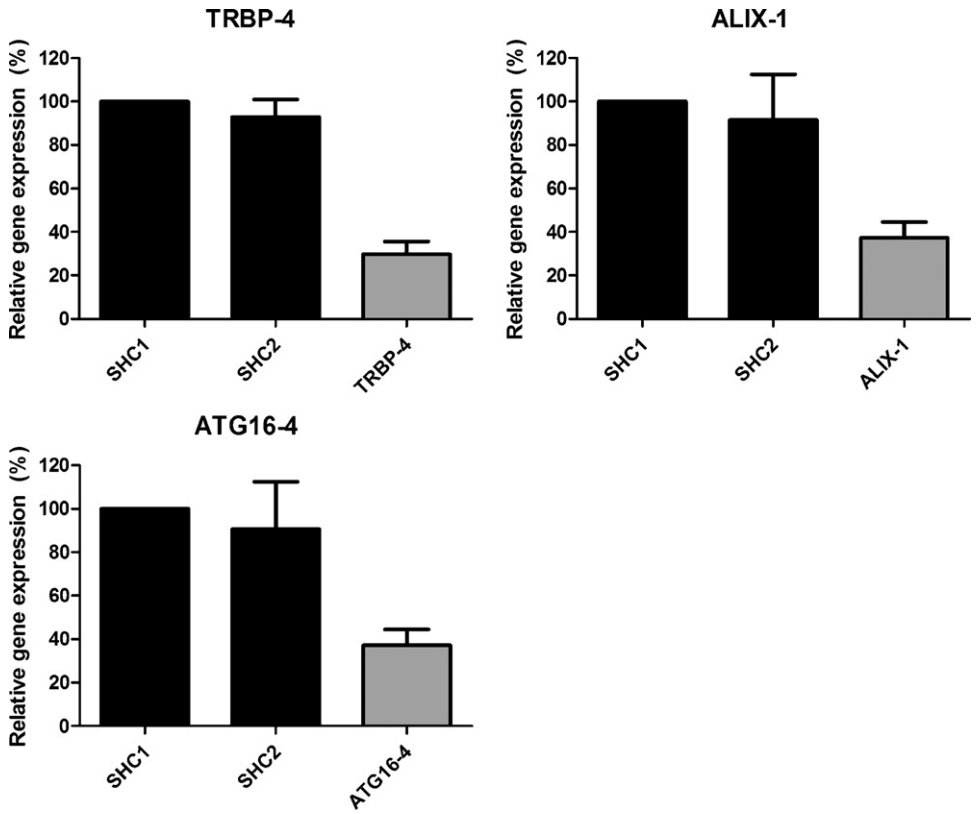

Fig. 5. mRNA expression levels for TRBP, ALIX and ATG16 in shRNA cell lines. Target mRNA expression levels were measured by RT-qPCR. Target mRNA expression was normalized based on the target and β-actin mRNA expression levels measured in the control SHC1 cell line. The mean and standard error of the mean of the three different time points are shown.

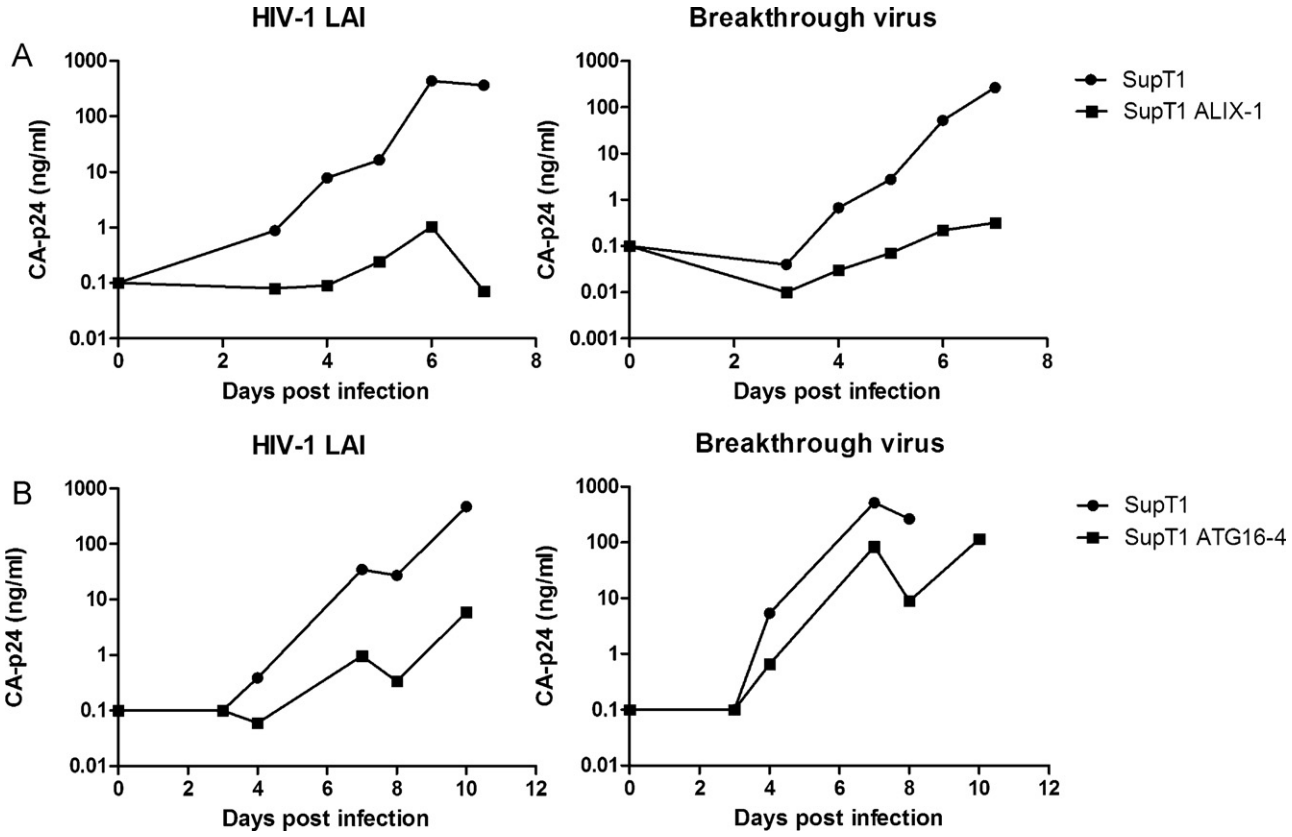

Fig. 6. Testing of the breakthrough viruses for viral escape. The parental SupT1 and shRNA cell lines ALIX-1 (A) and ATG16-4 (B) were infected with HIV-1_{LAI} (left panels) and corresponding breakthrough virus (right panels) harvested from previously infected shRNA cultures. CA-p24 levels in the culture supernatant were monitored over time.

such pseudo-escape in HIV-1 evolution studies (ter Brake et al., 2008). 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 Fig. 6. Wild-type HIV-1_{LAI} was restricted on the shRNA-cell line, but rapid viral replication could be measured in the parental cells (Fig. 6A, left). The breakthrough virus obtained in the evolution experiment was inhibited when the shRNA-expressing cell line is infected (Fig. 6A, right).

A single breakthrough virus showed the pattern predicted for a true escape virus, the virus harvested from ATG16-4 culture 3 (Fig. 6B). Wild-type HIV-1 was restricted on the ATG16-4 shRNA-cell line (Fig. 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 (Fig. 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.

4. 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-lived proteins and organelles, and was recently identified as candidate HIV-1 co-factor in one of the previous transient RNAi-screens (Brass et al., 2008). 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 (Denizot et al., 2008). 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 (Strack et al., 2003). 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 (Gatignol et al., 1991).

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 (Hutter et al., 2009). 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 (Holt et al., 2010). 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.

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