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Novel lentiviral vectors with mutated reverse transcriptase for mRNA delivery of TALE nucleases

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TAL-effector nucleases (TALENs) are attractive tools for sequence-specific genome modifications, but their delivery still remains problematic. It is well known that the presence of multiple sequence repeats in TALEN genes hampers the use of lentiviral vectors. We report that lentiviral vectors readily package full-length vector mRNAs encoding TALENs, but recombination during reverse transcription prevents successful delivery. We reasoned that preventing reverse transcription of lentiviral-vector RNA would allow transfer of TALENs as mRNA. We demonstrate that lentiviral particles containing genetically inactivated reverse transcriptase (RT) mediated efficient transduction of cultured cells and supported transient transgene expression. For proof-of-principle, we transferred CCR5- and TCR-specific TALEN pairs for efficient targeted genome editing and abrogated expression for each of the receptor proteins in different cell lines. Combining the high specificity of TALENs with efficient lentiviral gene delivery should advance genome editing *in vitro* and potentially *in vivo*, and RT-deficient lentiviral vectors may be useful for transient expression of various other genes-of-interest.

Genome engineering with designer nucleases is a very potent technology for both, basic research as well as therapeutic applications. The development of different synthetic nucleases, e.g. zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENs), or CRISPR/Cas9 nucleases, has opened up the possibility to easily design and clone specific sets of designer nucleases to edit any gene of choice[1–3]. The recently established CRISPR/Cas9-technology is particularly attractive due to its easy use and the possibility of targeting multiple genes in parallel[4], whereas off-target activity of that systems can still be relatively high[5,6]. In comparison, cloning of individual TAL effector nucleases is more labour-intensive, but they have been shown to stand out for their excellent specificity[7]. Importantly, besides specificity, efficient delivery of designer nucleases into respective target cells remains a major bottleneck in genome engineering[8,9].

Viral vectors, in general, and lentiviral vectors, in particular, are very attractive tools for the transfer of nucleic acids to a variety of mammalian cells[10,11]. In contrast to microinjection, electroporation or chemical transfection, gene transfer by lentiviral vectors is an active, receptor-dependent process and therefore not only efficient, but also less cytotoxic. Recently it has been shown that delivery of TALENs by lentiviral vectors is hampered by repetitive elements[12]. To avoid this problem, adenoviral vectors, a well-established and efficient viral vector system, were proposed as gene-transfer vehicles[12,13].

Importantly, short-term expression of designer nucleases in the target cells might not only be sufficient, but even desirable to ensure specificity. Vice versa, excessively high-level as well as prolonged expression of nucleases might result in increased off-target activity[14], but also re-mutation of the targeted locus. These preconditions may limit the use of DNA-based systems such as adenoviral and adeno-associated vectors or plasmids, since they transiently provide very high protein levels. Moreover, expression mediated by such vectors is relatively stable in non-dividing cells, and there is a finite risk of undesired transgene integration into the host genome, potentially mediating long-term expression[15]. To address these limitations we aimed to develop a delivery system that combines the benefits of efficient viral transgenesis with the safety features of mRNA-based systems. To further minimise the risk of off-target cutting, we made use of TALENs with high intrinsic specificity based on long genomic recognition sites (18 bp each arm)[7,16].

In the present study, we have been able to show that lentiviral vectors encoding TALENs deliver still intact TALEN-mRNA and that recombination occurs during reverse transcription after entering the target cell. These findings led us to hypothesize that mRNA TALEN transfer might be feasible by reverse-transcriptase incompetent viral particles. To address this question, we developed an enzymatically inactive reverse transcriptase, which was incorporated into lentiviral particles. After introducing several modifications into the vector construct and the transduction procedure, we were able to provide proof-of-concept for efficient mRNA delivery of different TALENs by these novel, non-reverse transcribable lentiviral vectors (NRTLV).

Results

Recombination of TALEN-sequences in lentiviral systems occurs during reverse transcription. It is well established that sequence repeats result in recombination events during reverse transcription in retroviral vectors[17,18]. However these are very attractive delivery systems for designer nucleases, given their high transduction efficiency for many cell types and the potential for targeted delivery into defined cell populations[19]. To investigate the likelihood of recombination events in lentiviral vectors encoding TALEN, we cloned a pair of lentiviral vectors containing the respective subunits (L & R) of a well-characterised CCR5-TALEN-pair[16] (Fig. 1a). As control vectors, we made use of similar lentiviral constructs harbouring a corresponding CCR5-ZFN[20] with the obligate heterodimeric FokI variants[21]. Both nucleases facilitated editing of their target sequence in the CCR5 gene resulting in decreased CCR5 expression upon transient transfection of vector plasmids into CCR5-positive 293T reporter cells (Fig. 1b and not shown). However, we did not detect any evidence of CCR5 knockout in reporter cells after transduction with non-integrating (Fig. 1c) or integrating (Fig. 1d) lentiviral vectors encoding the CCR5-TALENs. On the contrary, comparable amounts (as based on p24 titration) of non-integrating lentiviral vectors encoding the CCR5-ZFNs mediated efficient editing of the target locus in CCR5+-GHOST cells (Fig. 1c).

To address whether the lentiviral particles containing TALEN-vector mRNAs retained infectivity, we made use of two vector constructs linking the left TALEN to an eGFP and the right TALEN to a tdTomato marker (Fig 1a). Using these vectors, we observed expression of eGFP/tdTomato in transduced CCR5$^+$ 293T cells (not shown). However, despite successful transduction with both TALEN arms (=eGFP/tdTomato double positive cells), no change in CCR5 expression was observed (Fig. 1d). In keeping with this, no DNA-fragment of the expected size was detectable upon PCR amplification of integrated proviruses using TALEN-specific primers on DNA from transduced 293T-cell mass cultures (Fig. 1e, Suppl. Fig. 1a). On the contrary, sequencing of 15 subcloned PCR products indicated the presence of multiple different deletions within the TALEN repeat domain (Suppl. Fig. 1b). To quantify recombination frequency we derived clones of transgene-positive cells by single-cell sorting, and integrated proviral sequences were amplified for 25 clones (13 TALEN-L, 12 TALEN-R). Sequence analysis revealed occurrence of a variety of different recombination events in all 25 clones (Fig. 1g). Notably, in 39 of 40 analysed clones recombination was found between identical nucleotides of different repeats, thus always resulting in the in-frame-elimination of complete repeat modules (Fig. 1g and Suppl. Fig. 1b). Together, these results strongly suggest an underlying mechanism of template switch-mediated recombination during reverse transcription[17,22,23].

To further prove that recombination occurred during reverse transcription and, in consequence, the initially packaged TALEN-coding mRNA had still been intact, we isolated and reversely transcribed genomic vector RNA from lentiviral particles *in vitro*. As shown by RT-PCR on the vector genome (Fig. 1f) and confirmed by DNA-sequencing (not shown), full-length intact vector RNA was present in the majority of vector particles.

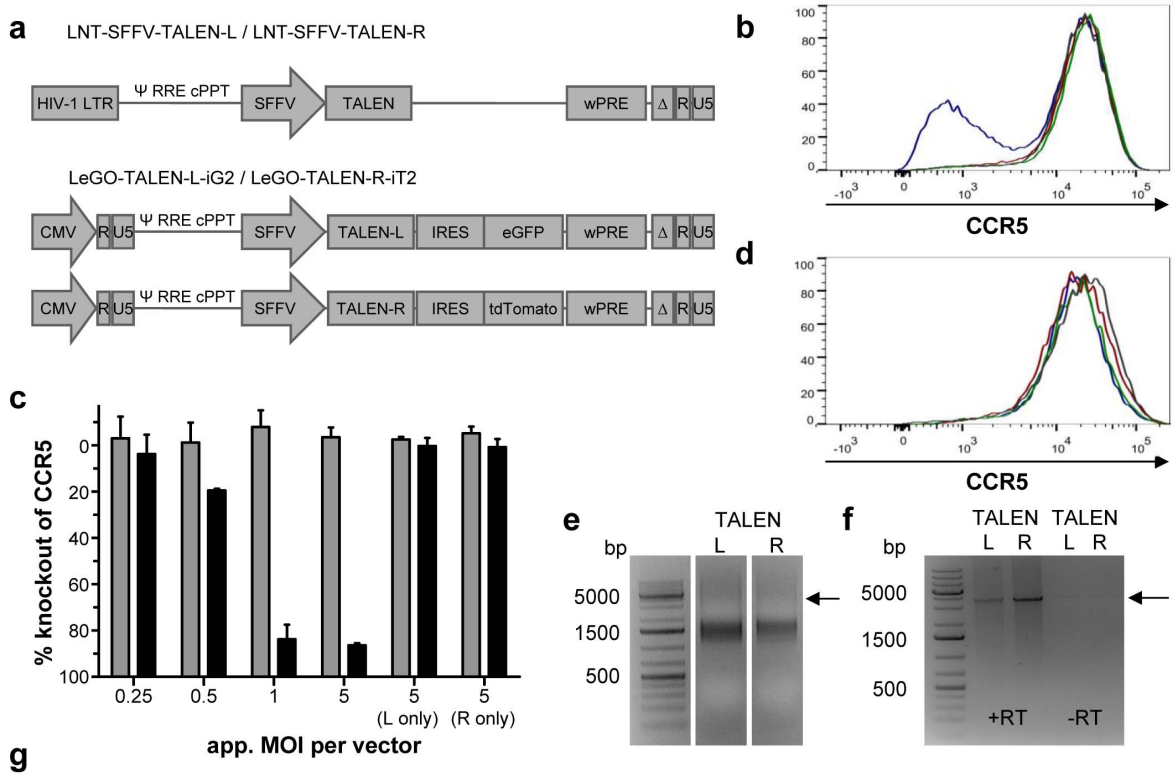
Altogether our data proves that, in agreement with a recent publication[12], lentiviral delivery of TALENs is strongly impaired, most likely due to template switch-mediated recombination during reverse transcription[22,23]. Importantly, our results demonstrated that packaging of intact full-length RNA vector genomes is retained. We therefore hypothesized that mRNA TALEN transfer by lentiviral particles lacking functional reverse transcriptase could enable lentiviral-mediated and transient TALEN delivery.

Generation and optimisation of non-reverse transcribable lentiviral vectors (NRTLV) by incorporation of mutated HIV-1 reverse transcriptase. To further investigate the idea of functional TALEN-delivery with a viral mRNA-vector system we generated an enzymatically inactive reverse transcriptase (RT) by directed mutagenesis to destroy the active centre of HIV-1 RT[24,25] changing the amino acid sequence YMDD to YMVV (Fig. 2a). We used the resulting mutated RT in place of standard RT during production of the eGFP-expressing vector LeGO-G2, and named the RT-deficient vectors non-reverse transcribable lentiviral vectors (NRTLV) to distinguish them from both integrating lentiviral vectors (ILV) and non-integrating lentiviral vectors (NILV). Lentiviral mRNA genomes are plus stranded and capped and therefore capable of direct translation initiation. However, in lentiviral vectors direct translation initiation is very inefficient, most probably due to the large 5′ untranslated region (leader sequence). Accordingly, we observed low levels of NRTLV-mediated (cap-dependent) eGFP expression by flow cytometry in 293T cells (Figure 2b, G2). To overcome this restriction we introduced an internal ribosomal entry site (IRES) 5′ to the transgene thus facilitating cap-independent translation initiation. Indeed, the 5′ IRES resulted in strongly increased fluorescence levels (Fig. 2b–c, iG2).

We next assessed kinetics of eGFP expression in NRTLV-transduced cells. By flow cytometry eGFP expression was detectable as early as 16 hours after transduction, peaked at 40 to 48 hours and disappeared around one week post transduction (Fig. 2d, Suppl. Fig. 2). This data not only indicates the absence of integrated vector copies, but also suggests *de-novo* translation rather than protein transfer by the vector. To address the latter point directly, we transduced 293T cells with NRTLV-iG2 in the presence or absence of different concentrations of the protein-biosynthesis inhibitor cycloheximide (CHX)[26,27]. As illustrated in Suppl. Fig. 3, eGFP mean fluorescence intensities (MFIs) were strongly (app. 10-fold) decreased in the presence of CHX 24 hours after transduction, independent of the CHX concentration. Together our data proves that *de-novo* translation is the main mechanism of TALEN transfer by NRTLV. Our findings are in line with observations for γ-retroviral pseudo-transduction, where reverse transcription was blocked by a mutated primer-binding site in the retroviral genome[28,29].

To improve NRTLV-based lentiviral mRNA delivery we tested different conditions for transduction and cultivation of cells. We found strongly improved eGFP expression under hypothermic conditions (48 hours at 30°C or 32°C, Fig. 2e). Hypothermic conditions have been proposed to prolong half-life of viral particles[30]. However, in this setting it is more likely that either cell-cycle arrest or increased stability facilitated accumulation of mRNA/protein. Notably, enhanced activity of zinc-finger nucleases has also been shown under hypothermic conditions[29,31].

We next asked whether introduction of mutations into the reverse transcriptase had any effect on particle formation. To do so, we titrated numbers of physical NRTLV particles using an established p24 ELISA. An integrating LeGO-G2 vector with a known infectious titre was measured in parallel. We determined particle numbers for four different concentrated vector preparations of CCR5-TALEN and iG2 NRTLVs. In all cases, particle concentrations were above



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proviral DNA - single cells			N-terminus	monomers 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	linker	FokI
TALEN L	in frame	#1.1		- 0						7 - 9								15					
	in frame	#2.1													- 12				18				
	in frame	#3.2			- 1												12 - 14		17				
	in frame	#6.2			- 1				16						12								
	in frame	#7.1				- 2													16				
	in frame	#11.3			- 1						7- 9								16				
	frameshift	#12.2			- 1				6											17			
	in frame	#24.2				- 2											13						
	in frame	#25.1		- 0									10							18			
	in frame	#28.1		- 0				5	6											17			
	in frame	#29.1		- 0											6 - 14				17				
	in frame	#35.1			- 2								9 - 13					16					
	in frame	#35.2		- 0																18			
TALEN R	in frame	#1.1			- 1								10										
	in frame	#2.1			- 1	3		4-6 / 14-16												17			
	in frame	#5.2			- 1	3 - 4											13						
	in frame	#6.2		- 0					6-7			16-17						6/16		18			
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	in frame	#25.2					- 4										10 - 15			17			
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	in frame	#47.1								- 6		9 - 11				14							

Figure 1 | High-frequency recombination in TALEN-encoding sequences during reverse transcription impedes transfer by lentiviral vectors. (a) Schematic vector design of LNT-SFFV-TALEN-L/R 2nd-generation LVV derived from Demaison *et al.*[53] and LeGO-TALEN-L-iG2/LeGO-TALEN-R-iT2 3rd-generation LVV derived from LeGO-system[11]. HIV-1 LTR=HIV-1 long-terminal-repeat; CMV=CMV-ie promoter; Δ (ΔU3), R, U5=elements of SIN-LTR, self-inactivating LTR; Ψ=*Psi*, packaging signal; RRE=Rev response element; cPPT=central polypurine tract; SFFV=promoter of spleen focus-forming virus; wPRE=Woodchuck hepatitis virus posttranscriptional regulatory element; IRES=internal ribosome entry-site; tdTomato=tandem-dimer Tomato (red fluorescent protein); eGFP=enhanced green fluorescent protein. (b) Functionality of CCR5-TALEN-plasmids assessed by transfection of CCR5+/293T with vectors depicted in (a): LeGO-TALEN-L-iG2 (encoding TALEN-L and eGFP; green), LeGO-TALEN-R-iT2 (TALEN-R and tdTomato; red), co-transfection with both constructs (blue) and no vector (grey). (c) Knockout of human CCR5. ZFNs (black bars) and TALENs (grey bars) targeting human CCR5 were delivered by non-integrating LVV (NILV) at the depicted multiplicity-of-infection (MOI). Relative knockout was determined by measuring expression of CCR5 by flow cytometry and normalized to nontransduced CCR5+-GHOST-cells. As controls either left-only (L only) or right-only (R only) ZFN/TALEN arms were delivered (n=2). (d) LeGO-TALEN-L-iG2 and R-iT2 were transduced with integrating LVV in CCR5+/293T (MOI 5). CCR5 expression depicted for cells expressing the respective fluorescent proteins (see 1b for colour codes). Fluorescence and CCR5-expression were measured 4 days post transduction. (e)–(g) Molecular analysis of recombination events in TALEN constructs (for PCR design see Suppl. Fig. 1a). (e) PCR on proviral DNA. Smear of unexpectedly small PCR product was subcloned and analysed for recombination (see Suppl. Fig.1b). (f) PCR on viral mRNA after first-strand synthesis revealed full length mRNA-genomes in viral particles. Shown are the results for non-integrating LVV coding for either the left (L) or right (R) TALEN arms (+RT). Samples without addition of RT (-RT) served as controls. Arrows indicate expected PCR-product size for full-length TALENs (3765 bp). (g) Frequency and appearance of recombination events in proviral TALEN-constructs after reverse transcription. Proviral DNA of transduced single-cell clones was isolated, subcloned and sequenced. Recombination pattern (dark grey) for either left (TALEN-L) or right (TALEN-R) arms are depicted. Light grey blocks represent monomers that could not be identified unambiguously.

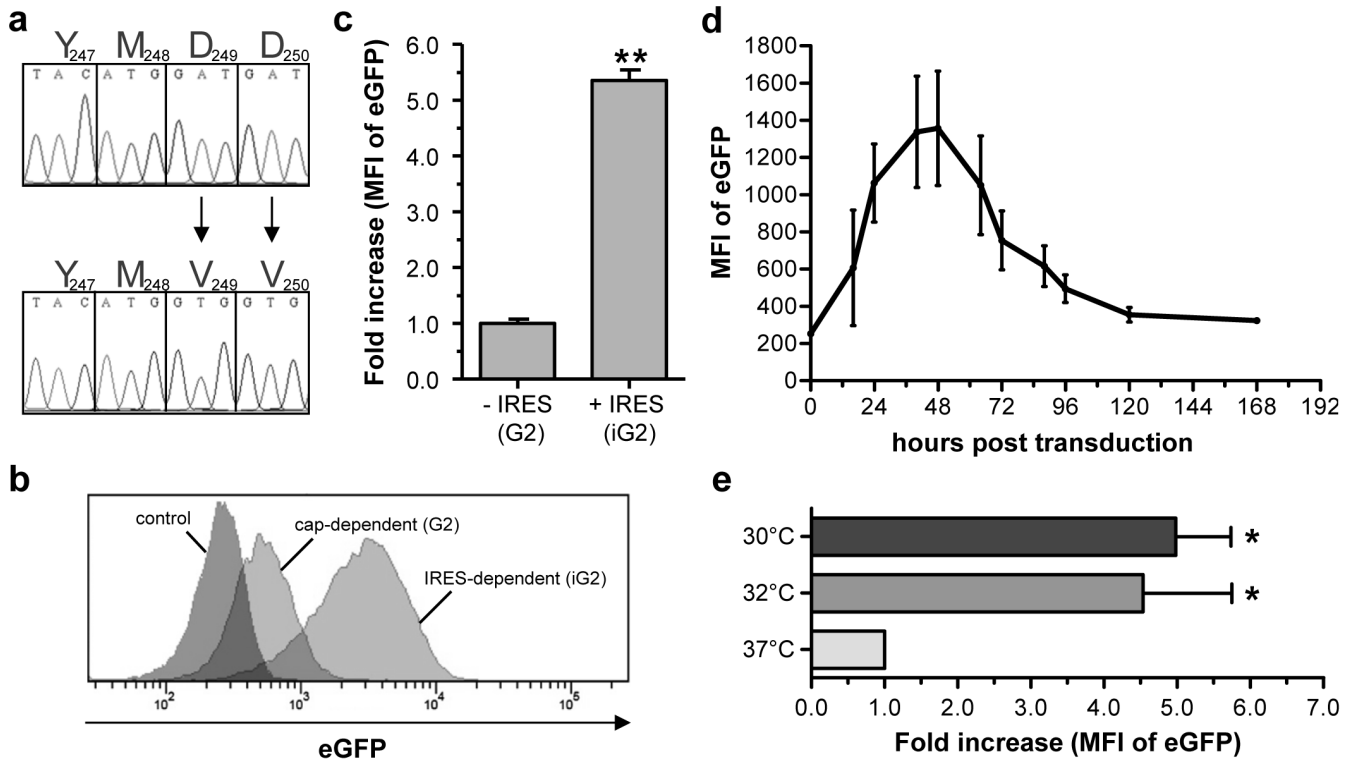

Figure 2 | Generation and characterisation of the non-reverse-transcribable lentiviral vector (NRTLV). (a) Targeted amino acid exchange in the active centre of HIV-1 reverse transcriptase (RT). (b+c) Cap-dependent versus internal ribosomal entry site (IRES)-dependent translation of NRTLV-delivered mRNA. (b) Representative histogram flow-cytometry plots depicting eGFP expression levels by untransduced cells (grey), cells transduced with NRTLV-G2 (without IRES), and NRTLV-iG2 (with IRES). (c) eGFP-encoding lentiviral mRNA was delivered by NRTLV either without (G2) or with (iG2) an IRES in front of eGFP. IRES-dependent translation led to a 5-fold increase in eGFP expression, as determined by flow cytometry based on mean fluorescence intensities (MFI) normalized to G2 (measured in quadruplicates for each of 2 independently produced vector preparations). (d) Kinetics of eGFP expression. Transduction of 293T cells was performed with NRTLV-iG2, and eGFP-expression was measured at indicated time points by flow cytometry. Summarised data for 3 independently produced vector preparations is shown. (e) Hypothermia improves eGFP expression after transduction with NRTLV. 293T cells were transduced with NRTLV-iG2, and eGFP expression was measured after 48 hours of cultivation at indicated temperatures. MFI was normalized to 37°C; $n = 3$. Error bars indicate SD; * $p < 0.01$; ** $p < 0.005$. G2 = LeGO-G2; iG2 = LeGO-iG2.

10^9 per ml with very little variations between different preparations (mean: $1.25 \pm 0.15 \times 10^9$ ml^{-1}, range: $1.10–1.40 \times 10^9$ ml^{-1}). Although the p24 ELISA is not indicative of actual infectivity of viral particles, it is notable that particle titres of NRTLV were in the same range as titres we regularly obtain after concentration of standard vector preparations with proven functionality. It is therefore safe to conclude that there was no negative impact of the introduced RT mutation on the formation of vector particles. Moreover, a significant proportion of NRTLV particles were able to mediate efficient mRNA transfer (see below).

TALEN-mediated knock out after lentiviral delivery. We next applied the NRTLV-system for delivery of a CCR5-specific TALEN[16]. With the initial IRES constructs (Fig. 3a) we achieved up to 12% CCR5 knockout in CCR5+/293T reporter cells with non-concentrated lentiviral supernatants (Fig. 3b). In order to obtain higher gene-editing rates, we set out to further improve transgene expression by NRTLVs. It is well known that self-inactivating (SIN) vectors suffer from weak polyadenylation[32]. Since efficient polyadenylation of mRNA is crucial for strong transgene expression, we next tested the effect of introducing much stronger internal poly(A)-signals. Importantly, the presence of a strong poly(A)-signal prior to the 3′ LTR drastically impairs transcription of full-length RNA vector genomes and consequently reverse transcription thus resulting in strongly reduced titres of both integrating and non-integrating lentiviral vectors[33]. We reasoned that for NRTLV, on the contrary, a strong internal poly(A)-signal would be beneficial for transgene expression and titre, as the lentiviral RNA serves as mRNA only. Indeed, introduction of poly(A)-signals (Fig. 3a, constructs II and III) led to an up to 2.5x increase in CCR5-knock out (31.6% for BGH-p(A), 30.5% for SV40-p(A); Fig. 3b). As expected, this effect was most pronounced when the poly(A)-signal was introduced 3′ of the post-transcriptional regulatory element (wPRE), an element which improves transgene expression and titre in retroviral vectors[34,35] (Suppl. Fig. 4). We then assessed expression kinetics for NRTL-vectors utilising different internal poly(A)-signal as compared to the initial iG2 vector (Fig. 3c). During an observation period of 96 hours after transduction, eGFP levels were higher for constructs with internal p(A)-signals at all time points, but interestingly there was no difference in duration of expression.

We applied concentrated vector supernatants to transduce CCR5+/293T cells at increasing multiplicities-of-infection (MOI) for each TALEN subunit. Two days after transduction, rates of eGFP-positive cells reached up to 94%. By flow cytometry five days post-transduction we detected the loss of CCR5 expression in a dose-dependent manner and in direct correlation with eGFP levels measured three days earlier. Importantly, for the highest MOI of 250 a high proportion (58%) of cells showed the CCR5 knockout (Fig. 3d, Suppl. Fig. 5).

To test this mRNA-based lentiviral delivery system in primary cells, we transduced primary T-lymphocytes with the CCR5-specific TALEN-NRTLVs. Based on the eGFP-expression on d2 post transduction we could transduce most of the primary T cells (Suppl. Fig. 6,

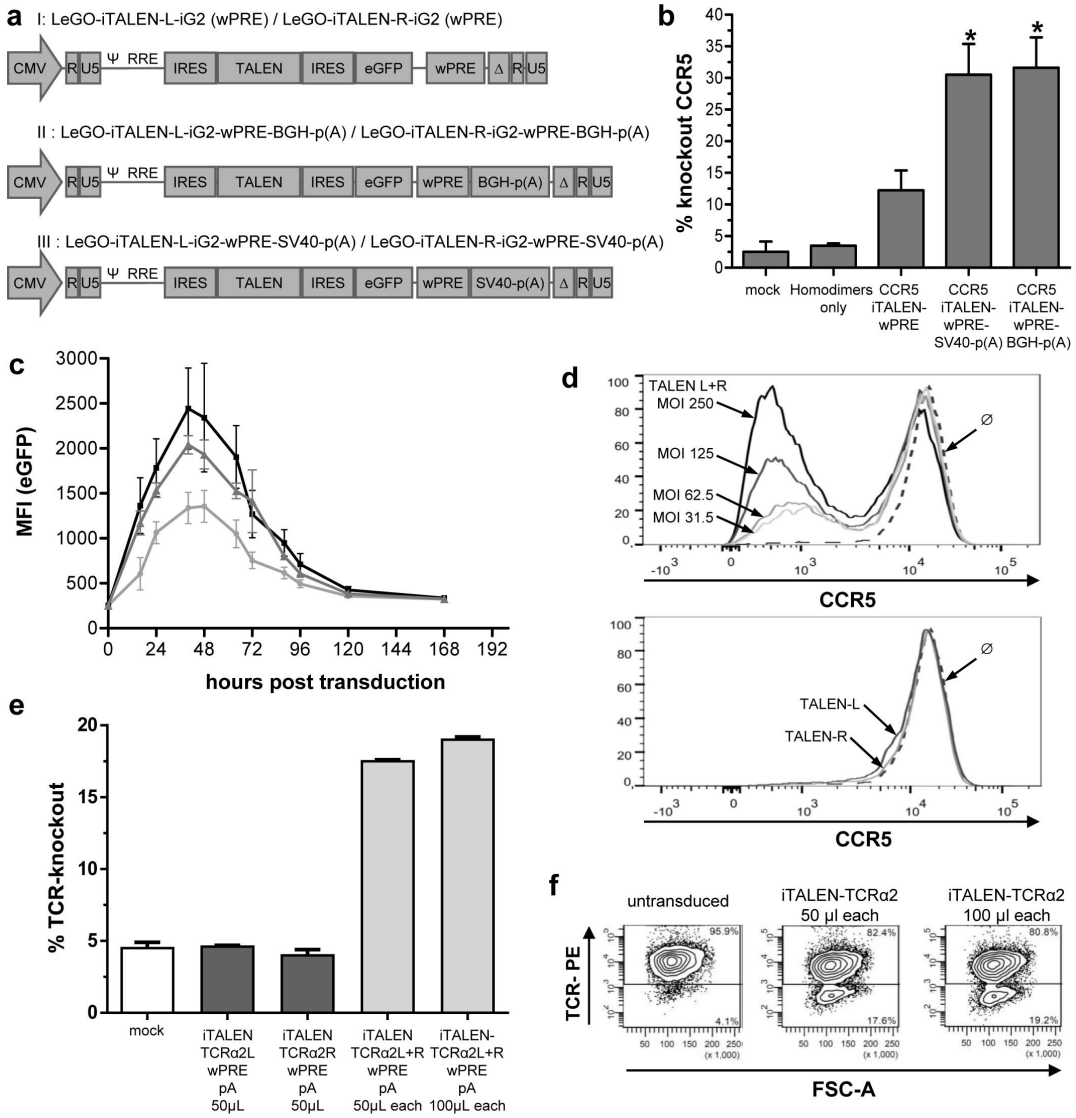

Figure 3 | Functional analysis of NRTLV-delivered TALEN constructs. (a) Schematic vector design of I: LeGO-iTALEN-iG2 (wPRE), II: LeGO-iTALEN-iG2-wPRE-BGH-p(A) and III: LeGO-iTALEN-iG2-wPRE-SV40-p(A) 3rd generation LVV derived from LeGO-system[11]. CMV = CMV-ie promoter; Δ (ΔU3), R, U5 = elements of SIN-LTR, self-inactivating long terminal repeat; Ψ = *Psi*, packaging signal; RRE = Rev response element; SFFV = promoter of spleen focus-forming virus; wPRE = Woodchuck hepatitis virus posttranscriptional regulatory element; IRES = internal ribosome entry site; eGFP = enhanced green fluorescent protein; p(A) = polyadenylation signal; BGH = bovine growth hormone; SV40 = simian virus 40. (b) Knockout of CCR5 in reporter cell line CCR5+/293T-cell clones were co-transduced with non-concentrated NRTLVs delivering different iTALEN-constructs with either no internal polyadenylation (p(A)) signal (iTALEN-wPRE), or internal BGH-p(A) (iTALEN-wPRE-BGH-p(A)) or SV40-p(A) (iTALEN-wPRE-SV40-p(A)) signals downstream of the wPRE-element, respectively. Mock-transduction and transduction of left or right TALEN-arms, only, served as negative controls (homodimers only). Measured for 3 independently produced vector preparations, each time in duplicates, *$p<0.0005$. (c) Kinetics of eGFP-expression for constructs containing a wPRE followed by different p(A)-signals. Transduction of 293T cells was performed with NRTLV encoding eGFP followed by endogenous HIV-1-p(A)-Signal (iG2 (light grey)) or internal, exogenous BGH-p(A) (iG2-wPRE-BGH (black)) or SV40-p(A) (iG2-wPRE-SV40 (dark grey)), respectively. eGFP expression was measured as mean fluorescent intensity (MFI) at different time points post transduction by flow cytometry. Mean values for 3 independently produced vector preparations are depicted, error bars indicate SEM. (d) Representative histograms of identical CCR5+/293T cells co-transduced with concentrated preparations of the respective NRTLV-TALEN-expressing constructs (LeGO-iTALEN-iG2-wPRE-BGH-p(A) – Fig. 3b: II). Top: Transduction with increasing MOIs of both arms. Bottom: Transduction of only one arm at an MOI of app. 125. Mock-transduction (dashed line) served as negative control. For quantification of knockout, see Suppl. Fig. 5. (e – f) Knockout of T- cell receptor (TCR) with NRTLV-delivered TCRα-specific TAL effector nucleases in Jurkat T cells. (e) Quantification of knockout and (f) representative dot plots of two independent experiments (means ± SD). As controls either no arm (white) or only one of the respective TALEN-arm (dark grey) were applied. Measured at d4 post transduction.

upper panel). On the contrary, we did not detect successful CCR5 knockout – neither on the protein (Suppl. Fig. 6, lower panel) nor on the DNA level by next generation sequencing (data not shown). This is in line with the assumption that targeted genome editing requires threshold levels of the designer nuclease, which were not achieved in primary T lymphocytes with the given configuration. Thus, additional improvements seem to be necessary to efficiently edit the genome of difficult targets such as primary T cells.

To verify applicability of the novel TALEN-NRTLVs we additionally tested a second TALEN-pair, namely the TCR-α2-TALEN already established in our lab[36]. The respective TALEN-arms were cloned into the optimised NRTLV-backbones containing the IRES,

wPRE and the BGH-pA-signal (Fig. 3a, construct II), and lentiviral particles were used to transduce the Jurkat T-cell line as well as primary T lymphocytes. Using this approach, we obtained successful knockout of up to 15% of the natural TCR-locus in Jurkat cells (Fig 3e and f), but not in the primary cells (not shown). Based on this data it is safe to conclude that the NRTLV delivery system not only facilitates editing of reporter constructs, but also of endogenous gene loci. In addition, this data is also in agreement with the assumption that threshold TALEN protein levels need to be reached to mediate gene editing in primary T cells.

Discussion

Targeted genome editing with designer nucleases is a promising strategy that has already entered clinical trials[37]. Based on their characteristics (relatively easy design combined with very high specificity), TAL effector nucleases seem to be particularly attractive for clinical applications. To this end, sufficiently high TALEN expression needs to be ensured in the target cells, which at the same time should be temporarily restricted ("hit and run") to minimise the risk of off-target cutting. However, until now efficient and ideally specific TALEN delivery to the respective target cells has remained a major obstacle[8,9] resulting in the exploration of different vector types. Extrachromosomal DNA vectors, such as plasmids, adenoviral, or adeno-associated vectors have been suggested as suitable candidates, since they mediate high, but transient transgene expression. However, in non-dividing cells even non-integrating vectors can remain relatively stable over time, and occasional genome integration may represent an additional risk[15,38,39]. To avoid this hazard delivery of TALEN by cell-penetrating peptides has been proposed[40].

Lentiviral vectors represent an alternative, popular vector system with an already established clinical track record[41] and high potential for targeted cell entry[42]. However, standard lentiviral vectors were not suitable for TALEN delivery for two reasons. First, integration into the host genome facilitating permanent transgene expression is an obligatory step in their life cycle – a problem easily resolved by the use of non-integrating lentiviral vectors[29]. The second obstacle is more difficult - it was previously shown that in a very high proportion of lentivirally transduced cells TALEN genes were not functional due to genetic rearrangements within the integrated provirus[12].

Here we demonstrated that the observed genetic rearrangements led to exact in-frame elimination of complete repeat modules, suggesting template switch-mediated recombination during reverse transcription as the underlying mechanism[17,22,23]. In line with this assumption, we also demonstrated that the majority of lentiviral particles contained full-length vector genomes. We therefore reasoned that inhibition of recombination should allow transfer of intact lentiviral mRNA genomes. To address this question, we genetically derived a novel, functionally incompetent mutant of the lentiviral reverse transcriptase. Incorporation of this inactivated reverse transcriptase (RT) into vector particles indeed allowed mRNA transfer into transduced cells. Based on their specific characteristics we named the novel gene transfer vehicles non-reverse transcribable lentiviral vectors (NRTLV).

In proof-of-principle experiments we used NRTLVs for the transient transfer of previously described functional CCR5- and TCR-TALENs[16,36]. Importantly, NRTLVs not only solve the recombination issue, but at the same time also the potential problems associated with vector integration, prolonged nuclease expression and insertional mutagenesis[43,44]. Moreover, the low probability of unwanted genome insertion of lentiviral mRNA can be further reduced by combining NRTLV with additional modifications to prevent integration, such as mutated primer-binding sites and/or defective integrases[28,45].

We here used the new vector system for TALENs cloned with the very common cloning kit from the Zhang-Lab[46], but it should also work with any other TALEN-cloning strategy. Moreover, the NRTLV principle can be easily adapted for the short-term expression of other "difficult" proteins that, for instance, are toxic upon long-term expression or contain repetitive sequences.

The use of lentiviral vectors as mRNA transfer vehicles required several optimization steps. Indeed, although lentiviral genomic RNA structurally resembles cellular mRNA, it is not intended to be directly translated. In line with this, we observed very weak cap-dependent translation initiation from standard lentiviral vector genomes. However, translation could be strongly improved by the inclusion of an internal ribosomal entry site (IRES) derived from encephalomyocarditis virus[47]. In addition, we showed that translation efficiency can be further enhanced by the use of strong polyadenylation signals. Taken together these vector modifications allowed us to achieve expression levels high enough to facilitate efficient genome editing of an artificial CCR5 locus in a 293T-cell reporter cell line and the natural TCR locus in Jurkat cells by NRTLV transduction. In contrast, translation levels were not yet high enough to facilitate TALEN-mediated genome editing in primary T lymphocytes, even though transient transduction was detectable based on eGFP-marker gene expression. Further genome modifications may help to achieve the threshold levels of TALENs obviously required for successful genome editing in primary T cells. Also, in future studies it might be interesting to characterise permissivity to NRTLVs in association with alternative TALENs in other primary target cell lineages.

Even in the light of the necessity of further improvement, the opportunity to use lentiviral vectors for transient TALEN-transfer provides several advantages. As compared to frequently used plasmid transfection, lentiviral transduction is much more gentle and efficient for many cell types. Lentiviral vectors can easily be produced at relatively high titres that can be further increased by concentration. Moreover, availability of alternative envelope proteins improving transduction of certain cell types[48] and the opportunity to generate receptor-targeted hybrid env proteins has increased applicability of lentiviral vectors[42]. Finally, lentiviral-vector technology is established in numerous laboratories and thus readily available to many researchers. In this regard, it is notable that production of NRTLV follows standard protocols where just the gag-pol plasmid needs to be replaced. In addition, vectors themselves need to be modified to ensure efficient translation as outlined above.

In conclusion, by introducing a non-functional reverse transcriptase we report a novel lentiviral vector-based TALEN transfer system. We suggest that the novel non-reverse transcribable lentiviral vectors will also be exploited for the transfer of other effector proteins that require definite short-term expression.

Methods

Vectors. All vectors were generated using standard cloning methods and confirmed by sequencing. Sequences of oligonucleotides used for cloning can be found in Supplementary Table 1.

dRT-pMDLg/pRRE was generated by megaprimer-PCR on previously described pMDLg/pRRE (ref. 49) using primer u44, u45 and u46 to exchange the amino acids VV at position 249/250 against DD. The respective CCR5-specific LeGO-TALEN-iG2 constructs were cloned by SacII/ApaI excision and blunting of TALEN-sequences of the previously described pAC CMV TALE RM1 long FokI 1317 and RM2 long Fok1 1318 (ref. 16), respectively, prior to ligation into StuI site of LeGO-iG2 (ref. 11). Insertion of an internal ribosomal entry site (IRES) upstream of the TALEN-sequences to create the respective LeGO-iTALEN-iG2 was performed by ligation of the PstI/NcoI IRES-fragment of LeGO-iG2 with an NcoI-NheI-linker (u47/u48) and into the respective LeGO-TALEN-iG2 vectors. LeGO-iTALEN-iG2 construct with an polyadenylation (p(A))-signal from either the bovine growth hormone (BGH-p(A)) or the Simian virus 40 (SV40-p(A)) either upstream or downstream of the wPRE were generated by PCR based cloning using either blunt (downstream of wPRE) or BsrGI-flanked PCR fragments (upstream of wPRE) of the respective p(A)-signal. The previously described pAC CMV TALE RM1 long FokI 1317 (ref. 16) served as a template for either BGH-p(A) (u58, u59) or SV40-p(A) (u60, u61). The respective iTALEN-TCRα2-wPRE-pA constructs were cloned by ligation of the lentiviral-backbone (NotI/PstI-fragment of iTALEN-wPRE-BGH-p(A)) to the IRES (PstI/XbaI-fragment of iTALEN-wPRE-BGH-p(A)) and the respective, previously described TCRα2-TALEN-arms[36] (PCR-product u62/u63, digested with NotI/PstI). PCRs on TALEN were performed by the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies).

Cells. The CCR5$^+$-GHOST cell line was kindly provided by Dr Littman, Dr V KewalRamni (NIBSC, UK) and the Programme EVA Centre for AIDS Reagents (EVA CFAR). CCR5$^+$-293T cells were generated by lentiviral transduction of 293T cells with the LeGO-CCR5-iB2-puro+ encoding for human CCR5, mTagBFP and a puromycin resistance. Cell clones were obtained by FACS-based single cell cloning. Primary T cells were isolated from peripheral blood mononuclear cells (PBMCs) by Ficoll gradient centrifugation from blood (buffy coats) donated by healthy individuals after informed consent. T lymphocytes were activated for 72 h by Dynabeads Human T-Activator CD3/CD28 (Life Technologies) according to the manufacturer's instructions and kept in X-VIVO 10, supplemented with 16% autologous plasma and 200 U/ml IL-2. Where not otherwise stated cell culture was performed under standard conditions (37°C, 5% CO2).

Lentiviral transduction. Third-generation lentiviral particles (ILV and NRTLV) were produced and cells in accordance with our standard protocols[11,50,51]. If indicated concentration of viral particles was performed for 16 h at 7,000 g. Second-generation lentiviral particles (NILV) were produced as described previously by transient transfection of 293T cells with vector plasmid, VSV-G envelope plasmid (pMDG) and integrase-deficient gag/pol plasmid (pCMVΔ8.74D64V)[45]. Viral supernatant was harvested 48 h later, filtered and concentrated by ultracentrifugation (2 h, 50,000 g). Titration of NILV/NRTLV vector particles was performed using the p24-based ELISA (Zeptometrix). In order to compare obtained values with functional (gene-transducing) titres, an integrating LeGO-G2 vector with a known eGFP-transducing titre measured by FACS was titrated in parallel by the p24 ELISA.

Cells transduced with ILV/NILV were cultivated at 37°C, cells treated with NRTLV were cultivated at 32°C for 48 h followed by cultivation at 37°C unless otherwise indicated.

Inhibition of mRNA translation. For translation inhibition, 10^5 293T cells in 500 µl medium were plated in each well of a 24-well plate. After the cells attached, we added cycloheximide (CHX) at 5 µg/ml or 50 µg/ml or DMSO alone (controls) to individual wells. Two hours later NRTLV-iG2-containing supernatant and polybrene (8 µg/ml) were added in triplicates, and cells were spinoculated for 1 h at 1000 × g at 25°C before they were incubated at 32°C and 5% CO_2 in a humidified cell culture incubator. FACS analysis was performed 24 hours after transduction.

Recombination. Proviral DNA of the integrated, respective TALEN-iG2 constructs was amplified either from a bulk-culture or after FACS-based single cell sorting of transduced 293T-cells with TALEN-specific primers in a nested PCR (1st PCR: u40/u41; 2nd PCR: u42/u43). Fragments were analysed by gel electrophoresis, extracted from gel and subcloned into pJET1.2/blunt using the CloneJetPCR Cloning Kit (Fermentas) according to the manufactures instructions. Sequencing was performed using pJet 1.2 forward and reverse primer (Fermentas).

Viral RNA of lentiviral particles was isolated using the NucleoSpin RNA Virus-Kit (Macherey-Nagel). Reverse Transcription and amplification was performed using the RevertAid Minus H First Strand cDNA Synthesis Kit (Fermentas) and SFFV/wPRE-specific primer (p74/u37). Sequencing was performed using primer u28, u29, u30 and u31.

For sequences of oligonucleotides see Supplementary Table 1.

Flow Cytometry. Flow cytometry and FACS-based cell sorting was performed on LSRFortessa (405, 488, 561, 640 nm laser, BD Bioscience) or CantoII (407, 488, 633 nm laser, BD Bioscience) and on the FACSAria Illu (407, 488, 561, 633 nm laser, BD Bioscience), respectively. The data were analysed using BD FACSDiva (BD Bioscience) and Diva-Fit[52]. Surface staining was performed by anti-human CD195-APC-Cy7 (BD, 557755); anti-human CD195-PerCP-Cy5.5 (Biolegends, 313716); anti-human CD3-PE (eBioscience, 12-0038-73) and anti-human TCR-PE (Miltenyi, 130-091-236).

Statistical Analysis. Data sets shown as bar graphs represent the average of at least three independent experiments with error bars indicating standard deviation (SD), if not specified otherwise. Statistical significance was determined using a two-tailed, homoscedastic Student's t-test.

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Author contributions

U.M. Conception and design of the study, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing. K.R. Conception of vector parts, Collection and/or assembly of data, Data analysis and interpretation, Final approval of manuscript. B.B. Collection and/or assembly of data for TCR-part, Data analysis and interpretation, final approval of manuscript. W.Q. Conception and Design, Data analysis and interpretation, Final approval of manuscript. E.C. Cloning of NILV-ZFN constructs, Final approval of manuscript. T.C. TALEN development, Final approval of manuscript. B.F. Conception and design, Data analysis and interpretation, Manuscript writing.

Additional information

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