Retroviral Particle-Mediated mRNA Transfer

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I. Kurzzusammenfassung

Die ektope und reversible Expression von Proteinen kann das Schicksal von Zellen beeinflussen. Da derzeitige transiente Expressionsmethoden (z.B. physikochemische oder adenovirale Systeme) eine geringe Effizienz, eine zu hohe Toxizität oder unerwünschte, residuale Integrationen aufweisen können, ist die Entwicklung neuer und verbesserter Alternativen von großem Interesse. In dieser Arbeit wurde untersucht, ob sich modifizierte, auf dem Mausleukämievirus basierende Vektoren für die gezielte und transiente Zellmodifikation eignen. Wir konstruierten drei Vektormutanten, die entweder in reverser Transkription (aPBS, ΔPBS) oder Integration (ΔU5) defizient waren, und testeten sie in ihrer Fähigkeit zur transienten Proteinexpression. Alle drei Vektormutanten vermittelten einen effizienten und transienten Transfer der Rekombinase Cre in humane und murine Fibroblasten. Da hierbei die aPBS den anderen Mutanten überlegen war, wurde sie bevorzugt in dieser Arbeit verwendet. Diese Art des Cre Transfers war Rezeptor mediiert und erforderte die Formation von Partikeln sowie die Präsenz des retroviralen Verpackungssignals. Wir bezeichneten daher diese Technik als retroviralen Partikel mediierten mRNA Transfer (RMT).

Die biochemische Charakterisierung von Wildtyp- und RMT-Partikeln zeigte keine signifikanten Unterschiede. Im Vergleich zu episomalen, lentiviralen Vektoren vermittelten RMT-Partikel eine geringere und kürzeranhaltende Proteinexpression, wiesen jedoch keinerlei residuale Integrationen auf. In einem weiteren Schritt untersuchten wir die Sensitivität dieser Partikel gegenüber zellulären Restriktionsfaktoren. Hierbei stellte sich heraus, dass sowohl huTRIM5α als auch in einem geringeren Maße Fv1 in der Lage waren, RMT zu inhibieren, und dass die huTRIM5α vermittelte Restriktion zu einem beschleunigten Abbau retroviraler RNA-Genome führte. Die Expression von shRNAs in Zielzellen, die gegen das eindringende RNA-Genom gerichtet waren, hemmte RMT, deutlich zeigend, dass das retrovirale RNA-Genom die verantwortliche Komponente für RMT ist. Dies suggeriert ein Modell, in welchem das retrovirale RNA-Genom als Translationsmatrize dienen kann, wenn es nicht revers transkribiert wird. Die Anwendung von RMT ist daher ausichtsreich für Applikationen, in denen eine geringe und transiente Expression von Proteinen zu deutlichen biologischen Effekten führt. Desweiteren könnte RMT der Analyse von Retrovirus-Wirt-Wechselwirkungen dienen.

II. Abstract

Short-term, reversible expression of foreign proteins could be useful to modify cell fate. Since current transient expression methods (e.g. physicochemical or adenoviral systems) may suffer from low efficiency, high cytotoxicity or unwanted residual integration events, the development of new and improved alternatives is of great interest. The present study addressed the question whether modified murine leukemia virus-based vectors can be exploited for targeted and transient cell modification. Thus, three different gammaretroviral vector mutants, being either disabled in reverse transcription (aPBS, ΔPBS) and/or integration (ΔU5), have been generated and tested for their capability of transient expression of recombinant proteins in target cells. As a paradigm, we could show that all three vector mutants were able to mediate transient, efficient and non-toxic delivery of Cre recombinase into human and mouse fibroblasts. Interestingly, the reverse transcription deficient aPBS mutant was superior to ΔPBS and ΔU5 mutants and therefore primarily used in this study. This type of Cre delivery was receptor-mediated, required particle assembly and depended on the presence of the packaging signal within the retroviral vector genome. For that reason we named this technique retroviral particle-mediated mRNA transfer (RMT).

Biochemical characterization of RMT vector particles did not reveal any significant differences when compared to the wild-type counterpart. The comparison with non-integrating lentiviral vectors revealed that RMT expressed foreign proteins shorter and at lower levels, but without the risk of residual integration events. To understand the mechanism of RMT, we explored the sensitivity of RMT particles to cellular restriction factors, targeting the incoming retroviral capsid. Both huTRIM5α and to a lesser extent the Friend virus susceptibility factor 1 were capable of effectively restricting RMT. Interestingly, huTRIM5α-mediated restriction of RMT resulted in reduced levels of retroviral mRNA genomes. Furthermore, cells expressing short hairpin RNAs targeting the retroviral genome inhibited RMT, clearly showing that the genomic mRNA is responsible for functional RMT. This suggests a model, in which – after entry - the retroviral mRNA becomes accessible to ribosomes and serves as a translation template if it is not undergoing reverse transcription. In conclusion, RMT is promising for applications in which low and transient expression of proteins achieves striking biological effects, and might be a useful tool to analyze retrovirus-host interactions early after entry before proviral DNA synthesis.

Schlagworte:

Mausleukämievirus

Retroviraler Lebenszyklus

Gentherapie

Keywords:

Murine leukemia virus

Retroviral life cycle

Gene therapy

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V. List of abbreviations

aPBS	Artificial primer binding site
APOBEC	Apolipoprotein B mRNA-editing enzyme catalytic protein
ASV	Avian sarcoma virus
att	Integrase attachment site
AZT	Azidodeoxythymidine
BAF-1	Barrier to auto-integration factor-1
bp	Base pairs
CA	Capsid protein, retroviral protein
cDNA	Complementary DNA, complementary to mRNA
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
Cre	Site-specific recombinase Cre
C-terminal	Carboxy-terminal
ECL	Enhanced chemiluminescence
Eco	Ecotropic, MLV glycoprotein
EGFP	Enhanced green fluorescence protein
eIF	Eukaryotic initiation factor
eRF1	Eukaryotic release factor 1
Env	Envelope, retroviral glycoproteins
FACS	Fluorescence activated cell sorter / sorting
FSC	Forward scatter
FCS	Fetal calf serum
Fv1	Friend virus susceptibility factor 1
Gag	Group specific antigen, retroviral structural proteins
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
huTRIM5α	Human tripartite motif 5α
IN	Integrase, retroviral protein
IRES	Internal ribosomal entry site
kb	Kilo bases
kDa	Kilo Dalton
LAP2α	Lamina-associated polypeptide 2α
LTR	Long terminal repeat
MA	Matrix, retroviral protein
MESV	Murine embryonic stem cell virus
MG132	Proteasomal inhibitor
MHC	Major histocompatibility complex
miRNA	Micro RNA
MOI	Multiplicity of infection
MoMLV	Moloney murine leukemia virus
MPSV	Myeloproliferative sarcoma virus
mRNA	Messenger RNA
MLV	Murine leukemia virus
MVB	Multivesicular body
NC	Nucleocapsid, retroviral protein
nlsCre	Cre recombinase containing a nucear localization signal
nt	Nucleotides
N-terminal	Amino-terminal
OH-group	Hydroxyl-group
ORF	Open reading frame
PABP	PolyA binding protein
PBS	Primer binding site

PCR	Polymerase chain reaction
PIC	Preintegration complex
Pol	Polymerase, retroviral enzymatics proteins
PPT	Polypurine tract
PR	Protease
RBCC motif	RING-Bbox-coiled-coil motif
RCR	Replication competent retrovirus
RD114	Envelope protein of feline endogenous retrovirus
rER	Rough endoplasmic reticulum
rhTRIM5α	Rhesus macaque tripartite motif 5α
RING	Really interesting new gene, N-terminal domain of TRIM5 proteins
RMT	Retroviral particle-mediated mRNA transfer
RNA	Ribonucleic acid
RNase H	Ribonuclease H, subunit of the reverse transcriptase
rpm	Rounds per minute
R-region	Redundant region
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	Reverse transcription complex
SA	Splice acceptor
SD	Splice donor
SFFV	Spleen focus-forming virus
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SSC	Side scatter
SU	Surface subunit
TM	Transmembrane subunit
TRIM	Tripartite motif
tRNA	Transfer ribonucleic acid
U	Units
UTR	Untranslated region
Vif	Viral infectivity factor, HIV-1 protein
VSV-G	Glycoprotein of vesicular stomatitis virus
WHV	Woodchuck hepatitis virus
wPRE	Woodchuck posttranscriptional regulatory element
Wt	Wild-type
Ψ	Retroviral packaging signal
ZAP	Zinc finger antiviral protein

AMINO ACIDS

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

A. Introduction

Targeted and controlled manipulation of cells is widely used in order to better understand the role and functional relevance of cellular proteins in cellular processes. The ectopic expression or down-regulation (by RNA interference, including short hairpin RNAs (shRNAs) or micro RNAs (miRNAs)) of selected proteins helps to elucidate the complex biochemical processes in a given cell. Currently, there are several techniques available for the specific manipulation of cells. A general distinction is made between the transfer of nucleic acids (DNA or RNA transfection, and viral gene transfer) and the transfer of proteins (e.g. transduction of recombinantly produced proteins) into target cells. Since protein transduction is a relatively young technology and still has strong limitations, the transfer of nucleic acids by appropriate vector systems is preferably used. Vectors consist of nucleic acid molecules which encode the genetic information to be transferred and contain all necessary regulatory elements for successful gene expression (e.g. promoters and polyadenylation signal). Such vector systems serve for the transfer of genetic material into cells and have different efficiencies in regard to expression level and duration (Thomas et al. 2003).

A major distinction is made between transient and stable expression vector systems. Transient expression vectors temporarily transfer RNA- or DNA-based vectors into target cells, from which the desired proteins are expressed. The transfer of these vectors can be performed using physicochemical methods, such as electroporation (Neumann et al. 1982), calcium phosphate-mediated transfection (Graham and van der Eb 1973; Wigler et al. 1978; Jordan et al. 1996) or lipofection (Felgner et al. 1987; Felgner et al. 1997). Disadvantages of physicochemical transfer methods are target cell type specific and include limited efficacy, high cytotoxicity or spontaneous, residual, unwanted integration events into the host cell chromatin (Hsiung et al. 1980; Robins et al. 1981; Chen and Okayama 1987; Murnane et al. 1990; Kjer and Fallon 1991; Baum et al. 1994; O'Mahoney and Adams 1994; Nguyen et al. 2007).

Transient protein expression can also be achieved through use of viral vector systems, which are naturally adapted to cellular pathways. Utilization of viral features, such as packaging and protection of genetic information in viral particles, which interact with cellular host factors (Brass et al. 2008), facilitates the entry into the cytoplasm and/or nucleus of a given cell. Examples are the adenoviral vector systems, which are used in many gene therapy trials (overview in http://www.wiley.co.uk/genetherapy/clinical), and the integrase-deficient lentiviral

vector systems (Yanez-Munoz et al. 2006; Philpott and Thrasher 2007). Both types of viral gene transfer establish nuclear episomal DNA structures, which are lost over time in dividing cell populations. Infrequently, spontaneous, residual integrations are observed, supported by the the cellular DNA recombination machinery.

For applications in which a stable ectopic expression is desirable, it is preferred to use retroviral (based on gammaretroviral or lentiviral technology) vector systems, which incorporate their genetic information into the host genome. The ability of retroviruses to infect a variety of target cells coupled with the relatively well understood retroviral life cycle (see below), makes them promising candidates for human gene therapy.

This work explores retroviral technology as a tool for the transient delivery of proteins, which presents with advantageous features including low cytotoxicitiy, specific cell targeting and prevention of residual integration events.

1. Retroviruses and their replication

a. Overview

Retroviruses are plus-stranded, lipid-enveloped RNA viruses, approximately 100 nm in size, with either icosahedral (e.g. MoMuLV) or conical (e.g. HIV-1) capsids. The name “retrovirus” is based on the ability to retrograde the flow of information from RNA to DNA via a specific enzyme, the reverse transcriptase (RT). The family of the Retroviridae consists of the following 7 genera, whereof most were assigned to be exogenous retroviruses (Modrow et al. 2003).

1) α-retrovirus (e.g. Rous sarcoma virus, RSV)
2) β-retrovirus (e.g. Murine mammary tumour virus, MMTV)
3) γ-retrovirus (e.g. Murine leukemia virus, MLV)
4) δ-retrovirus (e.g. Human T-cell leukemia virus type 1, HTLV-1)
5) ε-retrovirus (Fish retroviruses, e.g. Walleye dermal sarcoma virus, WDSV)
6) Lentivirus (e.g. Human immunodeficiency virus type 1, HIV-1)
7) Spumavirus (e.g. Human foamy virus, HFV)

In respect to the variety of proteins encoded by the retroviral genome, the retrovirus family can be further classified as simple or complex organized retroviruses. The genome of simple retroviruses (alpha-, beta- and gammaretroviruses) exclusively

encodes for the three viral proteins Gag (group-specific antigen, structural proteins), Pol (polymerase, enzymatic proteins) and Env (envelope protein). This set of genes is common to all retroviruses. In contrast, the genomes of complex retroviruses (delta-, lenti- and spumaviruses) harbor additional open reading-frames (ORF) for regulatory and accessory proteins.

Retroviruses enter their target cells in a receptor-mediated manner, reverse transcribe their genomic RNA into double stranded DNA and integrate as a so called “provirus” into the host genome. All essential components for the generation of new virus progeny are then produced by taking advantage of the host transcriptional, translational and other cellular machineries. After assembly, the nascent virions are released at the cell surface by a so called “budding” process. The budding retroviral particles are initially immature and through subsequently processing of viral proteins within virions by the encoded viral protease, mature particles capable of infecting new target cells are produced. A more extensive description of the retroviral life cycle is provided below. Since the present work is based on the utilization of retroviral vectors derived from the gammaretroviral murine leukemia virus (MLV), the following paragraphs will mainly discuss the aspects of retroviral replication and retrovirus-host interactions in the light of simple structured gammaretroviruses.

b. The gammaretroviral genome

The genome of retroviruses (Fig. 1) consists of two copies of a linear, plus- and single-stranded RNA molecule of 7-12 kb. Similar to cellular messenger RNAs (mRNA), retroviral genomic RNA molecules are equipped with a 5´ Cap and a 3´ PolyA-tail (consisting of approximately 200 adenosine residues). Additionally, each genome carries a cellular tRNA molecule, which serves as the primer for the initiation of reverse transcription upon infection.

The viral coding regions are flanked by regulatory sequences, which are essential for reverse transcription and the integration of the provirus into the host genome. The 5´ and 3´ end of the retroviral genome contains a redundant region (R-region) which entails the transcription start point and a polyadenylation signal. The 5´ R-region is followed by the U5 region (U=unique) which harbors one of the two integrase attachment sites (*att*). The adjacent primer binding site (PBS) binds via hydrogen-bonds to the 3´ end of a tRNA-molecule, whose free 3´ hydroxyl group serves as initiation point for reverse transcription (Leis et al. 1993). In addition to the splice

donor site (SD), the sequence located between the PBS and the beginning of *gag* contains the highly structured packaging signal (Ψ) (D'Souza and Summers 2005), which is involved in dimerization as well as encapsidation of the retroviral genomes. This 5´ untranslated region (UTR), which comprises the sequences located between the Cap and *gag*, is termed the leader region. The following coding region consists of the three successive genes *gag*, *pol* and *env*, whereof expression of env is enabled through a splice acceptor (SA) in *pol*. The PPT (polypurine tract) is located downstream of the coding region and contains a series of at least 9 adenosine and guanosine residues important for the initiation of plus strand DNA synthesis during reverse transcription. Subsequent to the PPT and adjacent to the 3` R-region follows the U3 region. The U3 region is unique to the 3´ end of the retroviral genome and provides enhancer and promoter sequences for the regulation of retroviral gene expression as well as the second integrase attachment site.

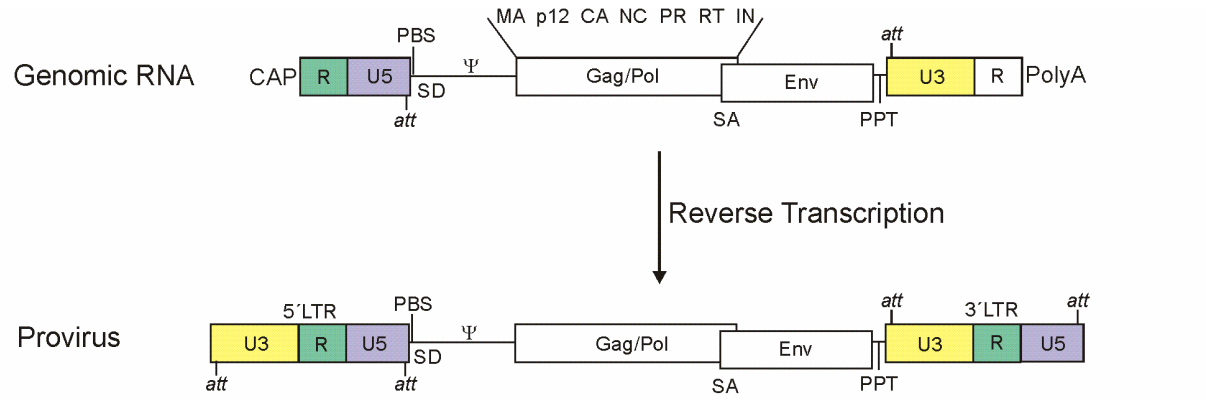

Fig.1: The gammaretroviral RNA genome and its conversion into proviral DNA. The figure schematically illustrates the genomic RNA (top) and the proviral DNA formed during reverse transcription (bottom) of MLV. The retroviral RNA genome harbors a 5' CAP site and a 3' PolyA tail. During reverse transcription, the 5´ R (green) and U5 (purple) regions are copied to the 3´ end, whereas the 3 ´U3 region (yellow) is copied to the 5´ terminus. The resulting long terminal repeats (LTR, consisting of U3, R, U5 and integrase attachment site (*att)*) flank internal regulatory motifs, which include PBS (primer binding site), splice donor (SD), packaging signal ψ, splice acceptor (SA) and the polypurine tract (PPT), as well as the open reading frames for Gag/Pol and Env. The single components of Gag and Pol are given above: MA (matrix), p12, CA (capsid), NC (nucleocapsid), PR (protease), RT (reverse transcriptase), IN (integrase).

During infection, the retroviral RNA genome is reverse transcribed into double-stranded DNA and integrated as a provirus into the host genome. In the process of reverse transcription, the 5´ R and U5 regions and the 3´ U3 region of the retroviral genome are each copied to the opposite termini. This results in a provirus flanked by long terminal repeats (LTR) consisting each of the U3, R and U5 sequences (Fig. 1).

c. The expression of gammaretroviral proteins

The simply organized genome of gammaretroviruses encodes for the retroviral proteins Gag, Pol and Env. Whereas the structural Gag and enzymatic Pol proteins are expressed from the same unspliced (genomic) viral mRNA transcript, the Env glycoproteins originate from a spliced variant (Fig. 2).

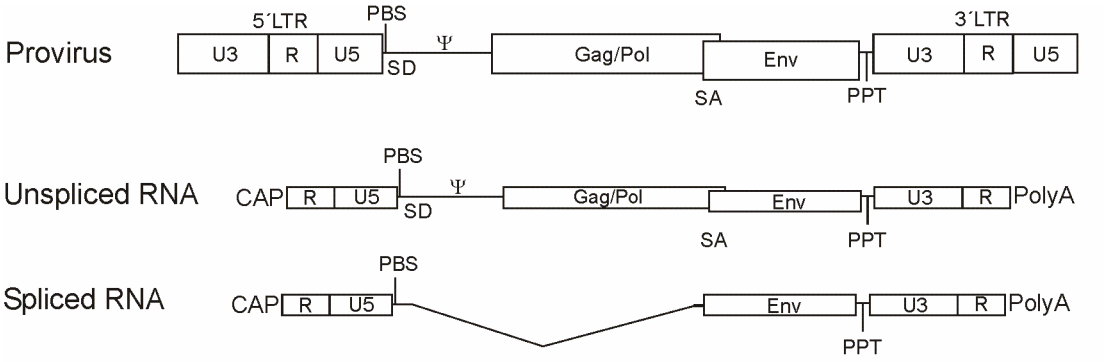

Fig. 2: The gammaretroviral transcripts. After integration, the proviral DNA serves as template for the generation of two gammaretroviral transcripts. Both RNAs are equipped with a 5´ Cap structure and a 3´ PolyA-tail. Whereas Gag and Pol proteins are expressed from the unspliced, genomic RNA transcript, the Env protein originates from a spliced message. The spliced RNA is not packaged into retroviral particles, because it lacks the ψ region.

Since *gag* and *pol* form one large open reading frame that is separated by a stop codon, retroviruses had to evolve translational strategies allowing the expression of both proteins from a single unspliced mRNA transcript. Retroviruses solved this problem by the synthesis of a Gag/Pol polyprotein precursor, which is further proteolytically processed by the viral protease after budding (see below). In Moloney MLV (MoMLV) the reading frame of *gag* is terminated by an amber stop codon (UAG). This amber stop codon is occasionally misread by the tRNA for glutamine, thus allowing continued translation through *pol* (Yoshinaka et al. 1985). This process of “translational suppression of termination” is a well-known mechanism in bacteria, but is not known so far to occur in normal eukaryotic gene expression. In the case of MoMLV, the efficiency of translational suppression is about 4% to 10% and involves a purine-rich sequence 3´ of the amber stop codon, as well as an RNA pseudoknot structure further downstream. It has been suggested that the pseudoknot causes the ribosome to pause and, together with the eight-nucleotide, purine-rich segment of the suppression signal, allows the suppressor tRNA (glutamine) to compete with translational termination/release factors at the suppression site. Interestingly, the Pol protein of MLV regulates its own synthesis by modulating translational read-through.

The RT protein of the Gag/Pol precursor binds and inhibits the mammalian translational release factor eRF1, thereby increasing the misreading frequency of the stop codon by the glutamine tRNA (Orlova et al. 2003). The resulting increased level of Gag/Pol production is suggested to be required for production of infectious particles.

After synthesis, the Gag and Gag/Pol precursor molecules are myristoylated at their N-terminus, packaged into viral particles and following budding subsequently processed into their single components by the viral protease. Whereas the Gag precursor of MLV is cleaved into the components matrix (MA, p15), p12, capsid (CA, p30), and nucleocapsid (NC, p10), Pol is processed into protease (PR, p14), reverse transcriptase (RT, p80) and integrase (p46) (see also Fig. 1).

The glycoprotein (Env) of retroviruses is embedded in the lipid envelope of the virion and consists of two subunits, which are initially expressed as a precursor molecule from the spliced subgenomic retroviral *env* RNA. The N-terminal glycoprotein subunit is designated the surface (SU) subunit and is responsible for binding to the ecotropic receptor on target cells. The carboxy-terminal subunit, which anchors the virus protein in the viral envelope, is termed the transmembrane (TM) subunit. Unlike the translation of Gag and Gag/Pol precursors, which takes place at free polyribosomes, the retroviral Env precursor ($gp85^{env}$) of MLV is synthesized, like other cell surface proteins, at polyribosomes associated with the rough endoplasmic reticulum (rER). Following translation into the lumen of the rER, the nascent Env polypeptides trimerize and undergo a glycolysation process (addition of N-linked oligosaccharides, mostly mannose sugars). Subsequently, the nascent Env glycoproteins are transported to the Golgi apparatus, where the N-linked oligosaccharides are further modified (trimming of the high mannose core, addition of other carbohydrates to the branches, addition of terminal sialic acid residues) and a cellular protease of the furin family cleaves the Env precursor molecule into the hydrophilic SU ($gp70^{env}$) and the hydrophobic TM ($p15^{env}$) moieties (Pinter et al. 1978; Henderson et al. 1984; Kamps et al. 1991). During virus budding, the viral protease cleaves TM to generate $p12^{env}$ and $p2^{env}$ molecules (Van Zaane et al. 1976), thereby activating the Env protein for fusion (Rein et al. 1994).

d. The gammaretroviral particle

Nascent retroviruses bud from the host membrane in a rather immature state. These immature particles are enveloped and consist each of an internal viral core that is formed by Gag and Gag/Pol precursor proteins embedding two copies of the plus-stranded retroviral RNA genome. Subsequently, processing of the Gag and Gag/Pol proteins into their subunit components by the viral protease (see above) during and/or shortly after the budding process leads to structural reorganization of the inner protein core, thereby generating mature and infectious virions. A schematical illustration of a mature gammaretroviral particle is given in figure 3. The mature gammaretroviral particle consists of an internal core, whose shell is formed by the viral CA proteins giving the core its icosahedral structure. The inner part of the core houses the retroviral genome complexed with NC proteins, the viral enzymes PR, RT and IN as well as cellular factors like the primer tRNA essential for replication. The core is covered with a layer of the p12 protein, whose exact function remains to be elucidated. The MA protein is found adjacent to p12 and in closest association with the viral envelope. The modification of the amino termini of the MA proteins by the addition of fatty, myristic acid groups during the translation of Gag allows MA direct interactions with the cell membrane derived viral lipid envelope. The viral core, p12 and MA proteins are surrounded by the envelope, in which the processed Env proteins are deposited (Levy 1993).

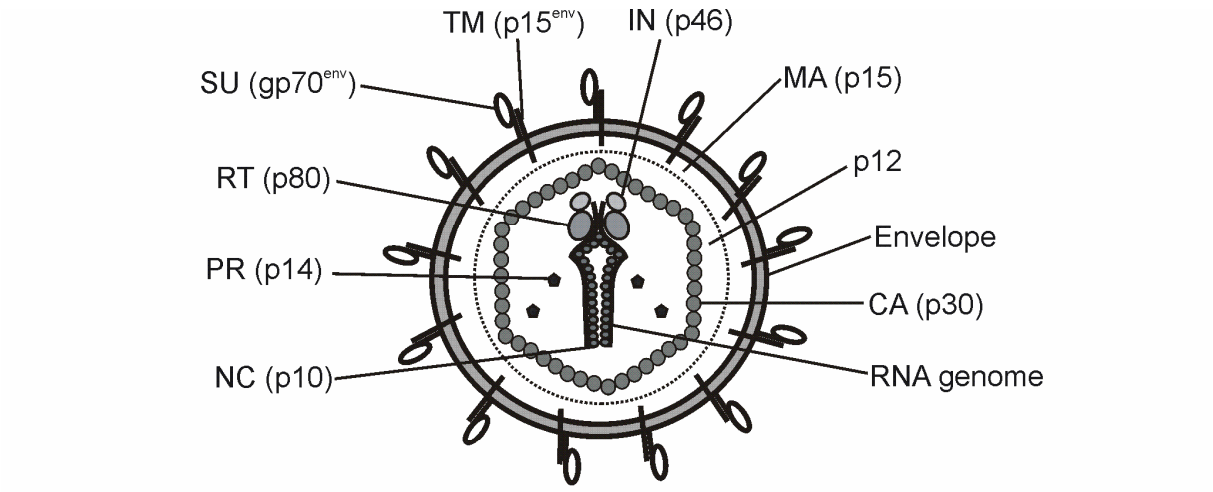

Fig. 3: The gammaretroviral virion. The figure shows a highly schematic illustration of a mature MLV particle. All retroviral components and their relative locations within the particle are indicated. IN: integrase, MA: matrix, CA: capsid, NC: nucleocapsid, PR: protease, RT: reverse transcriptase, SU: surface subunit, TM: transmembrane subunit.

e. The retroviral life cycle

The infectious and mature retroviral particle (Fig. 3) contains two plus-stranded RNA genomes embedded within the viral protein core. The latter is surrounded by a cell membrane-derived envelope bearing the glycoproteins (encoded by *Env*) necessary for the initiation of infection. The retroviral life cycle (Fig. 4) can be divided into early and late phases of replication, as described below.

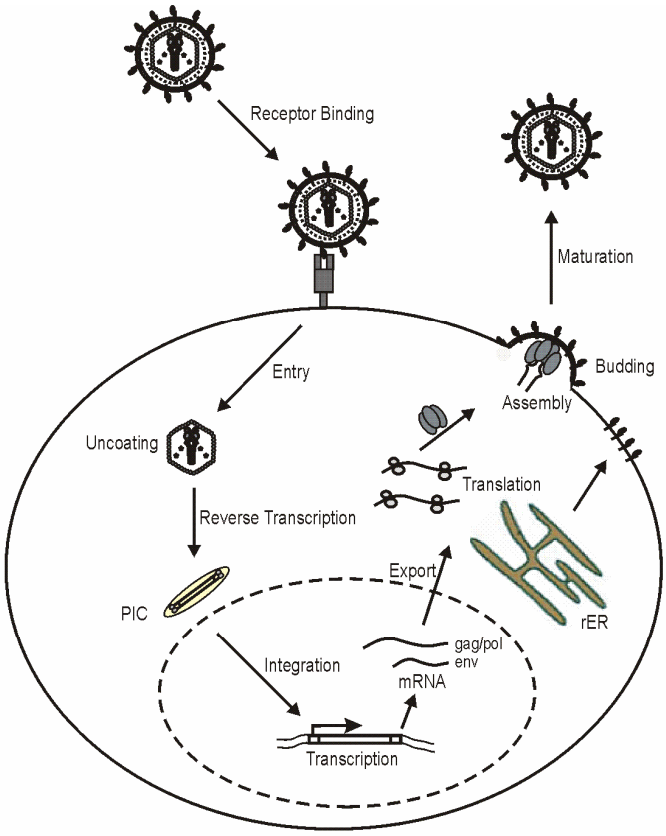

Fig. 4: The gammaretroviral life cycle. After receptor binding, the retroviral virion enters the cytoplasm by fusing its envelope with the host membrane, either at the cell surface or after internalization into endosomes (entry). Subsequently, the retroviral virion undergoes an uncoating process and reverse transcription is initiated. The resulting preintegration complex (PIC) gets access to the host´s chromatin during mitosis and the reverse transcribed, linear, double-stranded DNA is integrated into the genome via the viral integrase. By taking advantage of the cellular Polymerase II, the retroviral genes are transcribed, processed and exported into the cytoplasm for translation. The Gag and Gag/Pol precursor proteins are produced at free cytoplasmic polyribosomes, myristoylated and transported to the cell membrane. In contrast, the viral glycoproteins (Env) are synthesized at ribosomes of the rough endoplasmic reticulum (rER), undergo glycosylation and are transported to the cell membrane via the Golgi apparatus. After assembly of the retroviral components, the budding process is initiated at the cell membrane. During or shortly after budding, the nascent virions are converted into infectious and mature retroviral particles (maturation).

Early phases of replication

In the first step of infection, the virion envelope glycoprotein binds to specific and reciprocal cell surface receptor/s (Fig. 4). Subsequently, the viral envelope membrane fuses with the host membrane, either at the cell surface or after

internalization into endosomes, leading to the release of the internal virion core into the cytoplasm. Soon after viral penetration into the cell cytoplasm and a poorly understood uncoating step, the reverse transcription of the retroviral genome into double-stranded proviral DNA is initiated.

As already mentioned, the retroviral enzyme responsible for retrograding the viral RNA genome into DNA is the reverse transcriptase (RT) (Baltimore 1992). RT combines two enzymatic properties, DNA polymerase activity and ribonuclease H (RNase H) activity, within one molecule. It uses both RNA and DNA as templates for DNA synthesis (Hurwitz and Leis 1972; Leis and Hurwitz 1972) and cleaves RNA within an RNA/DNA hybrid (RNase H activity) (Molling et al. 1971; Hansen et al. 1987; Starnes and Cheng 1989). Because RTs lack proofreading activity and have been shown to be error-prone *in vitro*, they are presumed to contribute to the high mutation rate seen in retroviruses (Preston et al. 1988; Goodenow et al. 1989).

As shown in figure 5, MLV reverse transcription is initiated by RT-catalyzed synthesis of the minus strand DNA using the free 3´ OH residue of the host derived $tRNA^{Pro}$ primer (Peters et al. 1977). Since $tRNA^{Pro}$ complementarily binds the MLV PBS, which is located at the 5´ end of the retroviral RNA genome, the earliest product during minus strand DNA synthesis is a copy of the short region consisting of R and U5 lying between the PBS and the 5´ end of the RNA genome (Coffin and Haseltine 1977). This first reverse transcription intermediate is called minus strand strong stop DNA. Once minus strand strong stop DNA is generated, the newly made strand is translocated to the 3´ end of the retroviral RNA genome in order to complete synthesis of full-length minus strand DNA (Telesnitsky and Goff 1993). This process is described as minus strand transfer and is dependent on RT polymerase and RNase H activity as well as template homology. During minus strand transfer, the R region at the 3´ end of the strong stop DNA anneals to the 3´ R region of the genomic RNA, thereby allowing continued full-length minus strand synthesis. Concomitant with synthesis of the full-length minus DNA strand, the RNase H cleaves the newly copied RNA genome into short oligonucleotide fragments from either the 5´ or 3´ end (Mizrahi 1989). While many of these oligonucleotides can dissociate from the nascent minus DNA strand, the PPT sequence located upstream of the 3´ U3 region is resistant to RNase H cleavage. Therefore, the PPT sequence remains associated with the nascent minus DNA strand and serves as primer for plus strand DNA synthesis (Charneau et al. 1992). Plus strand synthesis initiates at the 3´ end of the PPT sequence and continues to the 5´ end of the minus strand DNA, thereby using

the first 18 nucleotides of the tRNA primer as a template for the generation of the PBS (Gilboa et al. 1979). Subsequently, the tRNA primer is removed by RNase H activity and a second strand transfer occurs, whereby the newly generated plus strand strong stop DNA is transferred to the 3´ PBS sequence of the minus strand DNA (Ben-Artzi et al. 1996). Finally, minus and plus strand DNA synthesis resumes, with each strand using the other as a template until the double stranded DNA containing LTR termini is fully synthesized. Interestingly, it has been shown that minus strand strong stop DNA is transferred at similar frequencies to the 3´ end of the same or the other co-packaged retroviral RNA genome, whereas plus strand transfer seems to predominantly occur intramolecularly (van Wamel and Berkhout 1998; Yu et al. 1998).

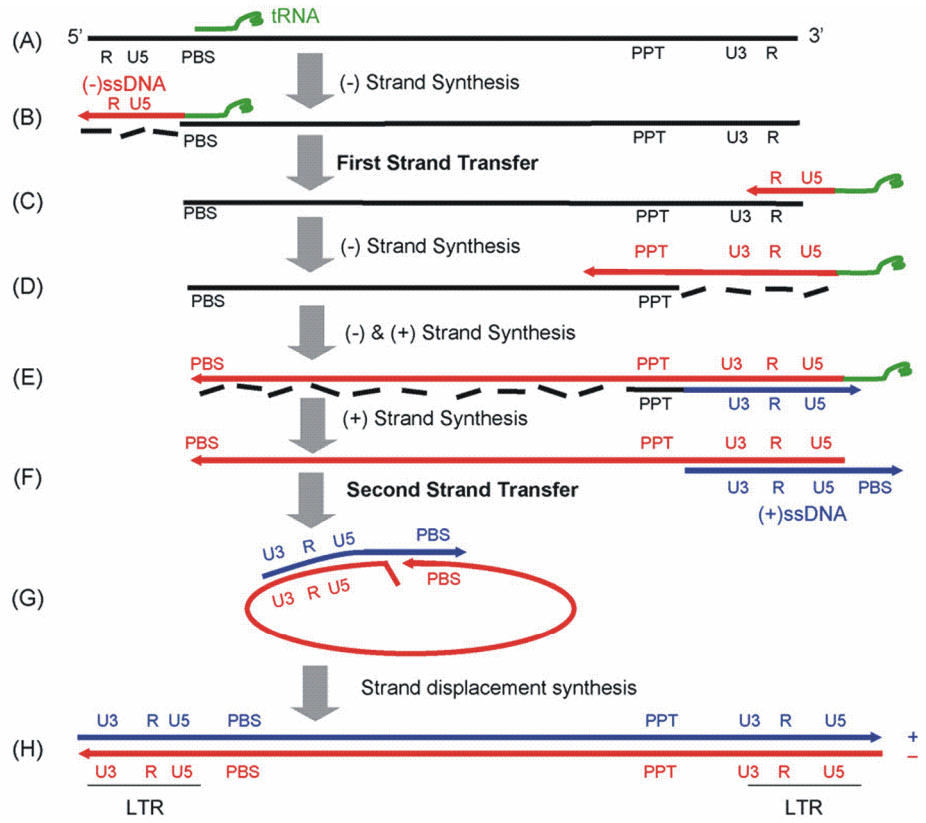

Fig. 5: The process of reverse transcription. (A) Reverse transcription is initiated from the 3´-end of a primer tRNA (green) partially annealed to the PBS region of the retroviral genomic RNA (black). (B) The primer extends through the 5' end and generates the minus-stranded strong stop DNA [(-)ssDNA, in red]. The 5' end of the retroviral RNA is degraded by RNase H. (C) Strand transfer of the (-)ssDNA to the 3' R region of the retroviral genome. (D) Continued minus strand DNA synthesis occurs and the DNA within the DNA/RNA hybrid is susceptible to RNase H degradation (except the PPT sequence). (E) The PPT serves as a primer for plus strand DNA synthesis [(+)ssDNA, blue]. Thereby, the first 18 nucleotides of the tRNA primer are used for the generation of the PBS DNA sequence. (F) Subsequent degradation of the tRNA primer by RNase H. (G) The newly synthesized plus strand strong stop DNA is transferred to the 5' end of the minus DNA strand. Base pairing of plus and minus DNA PBS sequences leads to a loop-like structure. (H) DNA synthesis resumes after second strand transfer with each strand using the other as a template. *The figure is adapted from Basu et al., Virus Research, 2008.*

The viral complex in which reverse transcription occurs is called the reverse transcription complex or RTC. So far, little is known about the structure, the composition and the properties of the RTC, particularly during the early steps after virus internalization. In 1999, Goff and colleagues demonstrated that the RTC of MoMLV contains integrase (IN) and capsid (CA) proteins in addition to the viral RNA genomes and the reverse transcriptase (RT) proteins (Fassati and Goff 1999). The process of reverse transcription, which is generally completed within 8 to 12 h, leads to the formation of the cytoplasmic preintegration complex (PIC). The cytoplasmic PIC of MLV has a relatively large size, sedimenting at 160S. The PIC contains the synthesized full-length, linear viral DNA, retains components of the virion core (including MA, CA, NC, RT and IN), and is, if isolated, competent to integrate the DNA *in vitro* (Bowerman et al. 1989). Recently, at least two host proteins have been found to be important for proper PIC structure and function. One is BAF-1 (barrier to auto-integration factor-1), a small DNA-binding protein identified as a component of the MLV and HIV-1 PIC (Chen and Engelman 1998; Suzuki and Craigie 2002; Lin and Engelman 2003; Mansharamani et al. 2003). BAF-1 is suggested to bridge the viral DNA in a discrete, higher-order nucleoprotein complex and thereby avoids a suicidal autointegration of the viral termini into internal sites on the viral DNA (Umland et al. 2000; Zheng et al. 2000). The second host component of the MLV PIC, which has been identified, is the lamina-associated polypeptide 2α (LAP2α), a component of the nuclear envelope which is required for infection by MLV (and by HIV-1 entering the cell using the HIV envelope protein) (Jacque and Stevenson 2006). LAP2α was shown to bind BAF-1 (Shumaker et al. 2001) and promote productive PIC integration (Suzuki et al. 2004).

How does the retroviral PIC reach the nucleus? The mechanism is poorly understood, but it seems rather unlikely that the relatively large PIC passes through the viscous cytoplasm which is filled with cytoskeleton networks, organelles and cellular vesicles by simple diffusion (Goff 2007). For HIV-1, it was demonstrated that the PIC traffics to the nucleus by latching onto dynein motor proteins and moving along the microtubules (McDonald et al. 2002; Goff 2007). This might also be true for MLV, although treatment of cells with nocodazole, which depolymerizes micro-tubules, affects MLV infection in some but not all cell-types (Kizhatil and Albritton 1997).

When the PIC has reached the nucleus, it must cross the nuclear membrane to integrate its reverse transcribed genome into the host genome (Suzuki and Craigie

2007). In contrast to HIV-1, which is able to infect dividing as well as nondividing cells, MLV depends upon mitosis to gain access to the host´s chromosomal DNA. It was shown that MLV derived PICs solve the nuclear entry problem by waiting for the nuclear envelope to disperse during mitosis (Harel et al. 1981; Miller et al. 1990; Roe et al. 1993; Lewis and Emerman 1994; Hatziioannou and Goff 2001). When the cell cycle is arrested at the G1-S transition by serum starvation or chemical treatment (e.g. aphidicolin), MoMLV PICs containing full-length viral DNA are present in the cytoplasm, but integration is blocked (Roe et al. 1993). Interestingly, when allowing the cell cycle to progress to metaphase, during which the nuclear envelope breaks down, the replication block is released and integration occurs (Roe et al. 1993).

After gaining access to the host´s chromatin, the reverse transcribed linear DNA is integrated into the genome via the viral integrase. During the step-wise integration reaction, the viral integrase binds to the integrase attachment sites, which are located at the ends of the 5' and 3' LTRs after reverse transcription. Initially, the integrase removes two bases from the 3' strand (3' end processing), adjacent to a highly conserved CA dinucleotide, leaving a highly nucleophilic free 3' OH-group. When brought together with the target cell genome, a nucleophilic attack by the 3' OH-group on a phosphate residue of the exposed 3' end of the cell genome is initiated. This is follwed by a strand transfer and joining reaction. The remaining gap is then filled by the cellular DNA repair machinery (Engelman et al. 1991; Vink et al. 1991; Hindmarsh and Leis 1999; Lewinski and Bushman 2005).

The position at which a retroviral genome integrates into the host cell genome is not random. Different integration preferences have been identified for individual retroviruses. Whereas MLV has a tendency to integrate into promoter-dense regions (near the transcriptional start site), HIV-1 prefers gene-dense regions and favorably integrates into transcription units (Schroder et al. 2002; Wu et al. 2003; Hematti et al. 2004). Interestingly, the integrase itself has been been identified to be a major determinant of integration site selection (Lewinski et al. 2006; Derse et al. 2007). Of note, a cellular tethering factor (lens epithelium derived growth factor) has been identified for HIV-1, but not (yet) for MLV.

Late phases of replication

After integration, the retroviral gene expression follows cellular rules. By taking advantage of the cellular Polymerase II, the retroviral genes are transcribed, spliced

if necessary (see *env* transcript), polyadenylated and exported into the cytoplasm either for translation of the retroviral precursor proteins or, in case of the unspliced genomic RNA transcript, also for encapsidation into newly forming virions. As already mentioned, Env proteins are synthesized at the rER and subsequently transported to the plasma membrane via the secretory pathway. In contrast, the retroviral Gag and Gag/Pol precursors are cytosolic proteins that are synthesized at free polyribosomes and are subsequently myristoylated at their N-termini. However, production of infectious retroviral particles requires the co-localization and assembly of retroviral cytosolic and transmembrane proteins as well as the retroviral RNA genomes at the membrane budding site. Initially, the budding site has been postulated to be localized at the plasma membrane. However, recent reports suggest that budding also occurs in multivesicular bodies (MVBs) (Nguyen and Hildreth 2000; Pelchen-Matthews et al. 2003; Sherer et al. 2003; Houzet et al. 2006). MVB are late endosomal compartments which accumulate internal vesicles produced from intracisternal invagination of the endosomal membrane. These internal vesicles are released either into lysosomes to allow associated protein and lipid degradation or into the extracellular space as exosomes for intercellular communication (Thery et al. 2002).

Three major domains within the Gag precursor are important for assembly and release of virus progeny. These domains are referred to as membrane targeting (M), interacting (I) and late (L) domains. The M-domain is located at the N-terminus of the matrix protein. Myristoylation of the N-terminal glycine of the matrix protein mediates the association of Gag and Gag/Pol precursors with cellular membranes (Hill et al. 1996; Suomalainen et al. 1996; Ono and Freed 1999). In addition, matrix contains a stretch of basic residues that are also believed to stabilize its association with the plasma membrane (Freed 1998; Garoff et al. 1998). The I-domain promotes Gag-Gag multimerization that drives the assembly process and is located within the nucleocapsid protein (Sandefur et al. 2000; Derdowski et al. 2004). When the virus is successfully assembled, the viral membrane must separate (bud) from the cellular membrane. The budding and pinching off of retroviral particles is mediated by the L-domain, which is a tetrapeptide PPPY sequence in the p12 protein or PTAP sequence in p6 for MLV and HIV, respectively (Huang et al. 1995; Yuan et al. 1999). Interestingly, late domain sequences were found to direct the interaction between the Gag protein and some cellular host factors involved in the protein sorting process and vesicle formation during the MVB biogenesis (Garrus et al. 2001; VerPlank et al. 2001; Wang et al. 2003; Demirov and Freed 2004). It seems that retroviruses have

evolved mechanisms to hijack complexes of the MVB machinery (e. g. endosomal sorting complexes required for transport, ESCRTs), thereby promoting their release from the cell.

The encapsidation of retroviral RNA genomes within nascent virions is a further prerequisite for retroviral infectivity. Once delivered into the cytoplasm, unspliced, genomic viral mRNA serves as a protein synthesis template and as a viral genome when packaged. For HIV-1, it was demonstrated that the RNA genome, which is packaged into a retroviral particle, can be the same as that used for the translation of the *gag/pol* gene. (Butsch and Boris-Lawrie 2002; Poon et al. 2002; Anderson and Lever 2006). In contrast, the genome of MLV appears to be mainly segregated into two distinct populations that function independently as genomic RNA for packaging into progeny virions or as an mRNA template for protein synthesis (Levin et al. 1974) (Levin and Rosenak 1976; Dorman and Lever 2000).

How is encapsidation of the two retroviral RNA genomes regulated? Although the exact mechanism is still under investigation, it has been shown that packaging of retroviral RNA genomes in nascent virions is mediated by specific interactions between the retroviral NC domain of Gag and the highly structured packaging signal Ψ of the viral genome. The NC domains of all retroviruses, except the spuma-retroviruses, contain one or two conserved Cys-X_2-Cys-X_4-His-X_4-Cys (X = variable amino acid) zinc finger motifs that bind specifically to sequences within Ψ (Henderson et al. 1981; D'Souza and Summers 2004). After dimerization of two RNA genomes, possibly promoted by the nucleic acid chaperone activity of NC (Darlix et al. 1995), high-affinity binding sites within Ψ are exposed to mediate packaging of these two genomic RNA molecules into the viral particle. Since the spliced *env* transcripts and cellular RNAs lack Ψ sequences, preferentially the full-length RNA genomes are incorporated into new viral progeny. Of note, cellular RNAs (e.g. mRNAs and tRNAs) have been also detected in purified retroviruses, an event which is assumed to occur randomly and at low rates (Muriaux et al. 2001; Rulli et al. 2007).

Less is known about the site where Gag captures the RNA genomes. However, it has been shown for HIV-1 that Gag interacts with the RNA genomes at or adjacent to the centriole, near the nuclear membrane (Poole et al. 2005). In addition, it was shown that MLV RNA genomes traffic in association with Gag along recycling endosomal vesicles (Basyuk et al. 2003).

Once assembly is completed, the viral particles are released and extracellular conversion creates a fully infectious particle (as described above).

2. The retroviral vector system

Retroviruses are well adapted to their hosts and infect a variety of cells. They enter the cytoplasm in a receptor mediated manner, and stably integrate their viral genes into the host genome after reverse transcription. Due to these abilities, retroviruses represent a useful tool for the delivery of genetic information into target cells. By inserting the gene of interest (transgene) within the retroviral genome and taking advantage of all retroviral proteins necessary for successful infection, retroviral particles serve as well evolved and specialized “gene ferries” that permit the transfer of genetic information into the nucleus of a target cell. To avoid the generation of replication competent retrovirus (RCR), it is necessary to separate genes encoding for structural and enzymatic proteins (Gag/Pol) as well as the gene encoding envelope proteins (Env) from the retroviral genome (i.e. the so-called split packaging design) (Fig. 6). The result is a so-called “retroviral vector”, which still contains the packaging signal (Ψ), the primer binding site (PBS) and the long terminal repeats (LTR), but harbors the transgene instead of genes encoding for structural and enzymatic retroviral proteins. The viral structural proteins (Gag) and replication enzymes (Pol) as well as the glycoproteins (Env) are encoded on separate helper expression plasmids, which lack all other retroviral components including the retroviral packaging signal. To lower the probability of recombination events, which could recreate a wild-type retrovirus, the genetic information for *gag*/*pol* and e*nv* are usually located on separate expression plasmids. For the production of gammaretroviral vector particles, both Gag/Pol and Env proteins as well as the retroviral vector construct are either transiently or stably co-expressed in so-called “packaging cell lines” (e.g. human embryonic kidney derived 293T cells) (Fig. 6). Since *gag*/*pol* and *env* expression constructs lack the packaging signal, viral structural proteins only recognize the Ψ-containing retroviral vector construct leading to a preferential packaging of retroviral vector genomes into infectious particles. After entry of the particle into the target cell (transduction), only the nucleic acid of the retroviral vector construct is reverse transcribed and stably integrated into the host genome. Since *gag*/*pol* and *env* are only transferred in the form of proteins (and not

as nucleic acid) the generation of replication competent retroviral vector progeny is prevented.

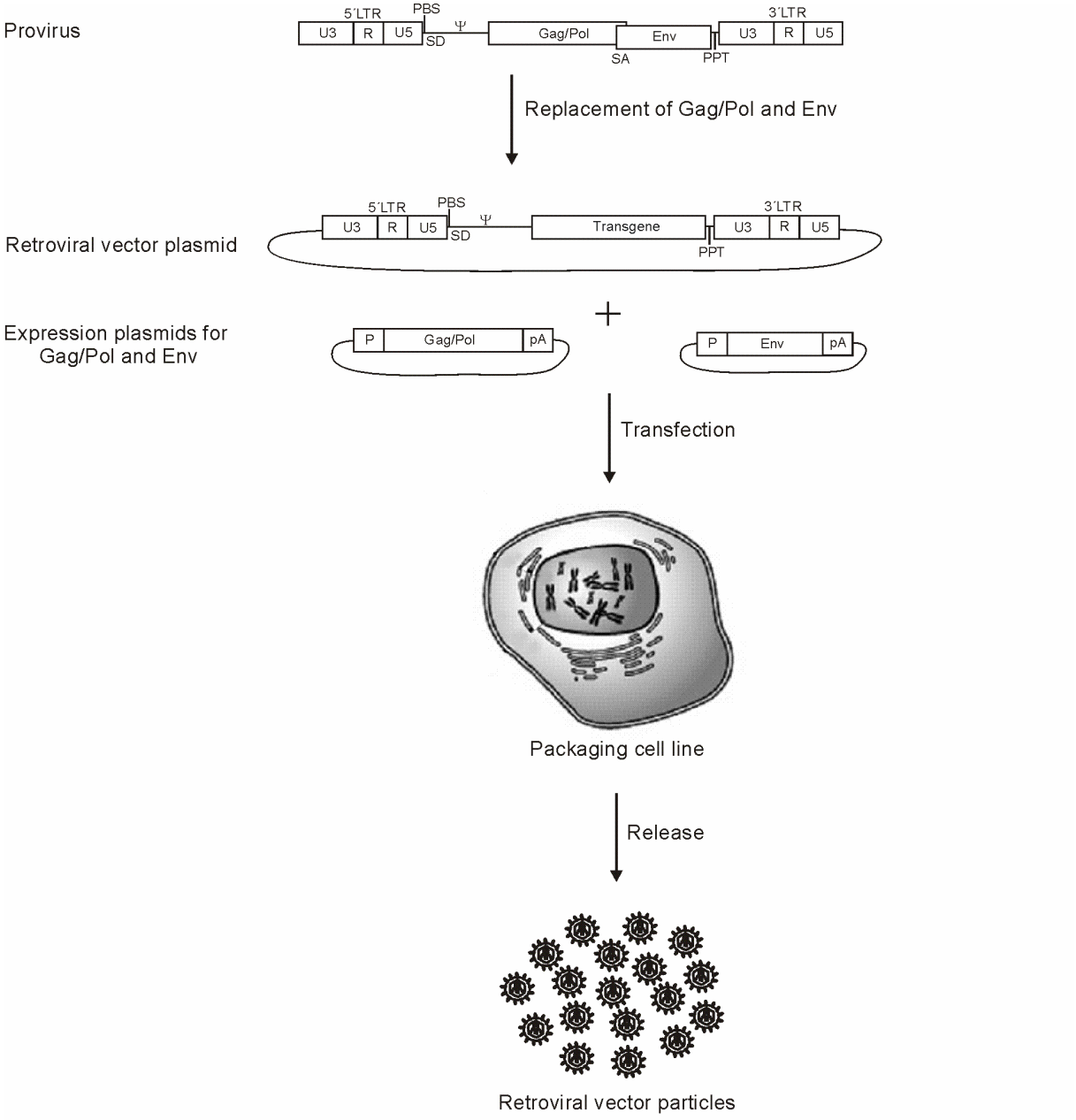

Fig. 6: From replication competent gammaretroviruses to gammaretroviral vector systems. For the generation of replication-deficient gammaretroviral vectors, the gag/pol and env reading frames are separated from the proviral genome and replaced by a transgene expression cassette. The retroviral vector is co-transfected into a packaging cell line together with gag/pol and env expression plasmids (P: promoter; pA: PolyA signal). The retroviral vector particles are released into the cell supernatant following assembly within the packaging cell line. *The image of the cell is derived from www.medizin.uni-tuebingen.de/webim2/moldiag/moldiag.htm and modified.*

a. The gammaretroviral vector used in this study

Retroviral vectors, derived from gammaretroviruses (Miller and Rosman 1989) or lentiviruses (Naldini et al. 1996), are most frequently used for gene therapy

applications. The gammaretroviral vector constructs used in this study (Fig. 7) are derived from Moloney MLV and were optimized stepwise for high transgene expression levels in early hematopoietic cells (Baum et al. 1995; Hildinger et al. 1999; Schambach et al. 2000). Substitution of the enhancer/promoter region (3´ U3) of MoMLV within the retroviral genome with enhancer/promoter sequences from the polycythemic strain of mouse spleen focus-forming virus (SFFV) leads to higher expression levels in both multipotent and lineage-committed hematopoietic cells (Baum et al. 1995). The PBS sequence of MoMLV (complementary to the 3´ end of the $tRNA^{Pro}$) negatively controls viral gene expression by transcriptional silencing in embryonic carcinoma (EC) and embryonic stem cells (Kempler et al. 1993;Teich et al. 1977; Barklis et al. 1986). This repression has been overcome in the gammaretroviral vector used here by introducing the PBS sequence (complementary to the 3´end of the $tRNA^{Glu}$) of murine embryonic stem cell virus (MESV) (Grez et al. 1990). Recently, the factor which is responsible for the repression has been identified to be the tripartite motif 28 (TRIM28) (Wolf and Goff 2007), a nuclear protein that is known as a transcriptional corepressor (Le Douarin et al. 1996; Schultz et al. 2001; Schultz et al. 2002). The removal of all potential and aberrant ATG start codons within the 5´ UTR leader, the introduction of a minimal splice acceptor site, thereby generating a functional intron including the packaging signal, or the addition of the woodchuck posttranscriptional regulatory element (wPRE, derived from woodchuck hepatitis virus) further improved transgene expression from gammaretroviral vector constructs (Hildinger et al. 1999; Zufferey et al. 1999; Schambach et al. 2000).

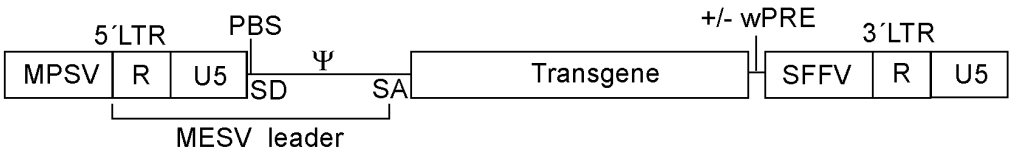

Fig. 7: The basic gammaretroviral vector plasmid used in this study. The plasmid´s 5´ enhancer/promoter region originates from myeloproliferative sarcoma virus (MPSV), whereas the 3´ U3 region comes from a polycythemic strain of mouse spleen focus-forming virus (SFFV). The retroviral leader region, including 5´ R, 5´ U5, PBS and SD sequences, is derived from murine embryonic stem cell virus (MESV). The introduction of a splice acceptor site (SA) upstream of the transgene creates a functional intron that improves transgene expression. Optionally, the woodchuck posttranscriptional regulatory element (wPRE) further improves gene expression.

b. Non-integrating retroviral vectors

The infection of target cells with functional retroviral vector particles usually results in stable ectopic transgene expression. However, integration of foreign DNA into the

genome can influence the expression of neighboring alleles (Hayward et al. 1981; Kung et al. 1991; Barker et al. 1992; Fan 1994). Replication-deficient vectors can lead to “insertional mutagenesis” causing dysregulation of neighboring genes, e.g. proto-oncogenes with subsequent induction of leukemia (Li et al. 2002; Hacein-Bey-Abina et al. 2003b). Non-integrating vector tools might be of interest for some gene therapeutic or other applications in biotechnology or basic research in order to express the selected transgene in a non-permanent manner. Since conventional transient expression methods (e.g. transfection of nucleic acids) are in general limited in terms of gene transfer and can lead to toxicity (particularly in primary cells), the development of new and improved transient expression methods is of great interest.

An alternative and novel transient expression method for the ectopic expression of proteins is the use of integrase-deficient lentiviral vector particles. The disruption of normal retroviral integrase function by introducing specific point mutations within the integrase sequence allows the molecular separation of cellular and nuclear entry from the intrinsic integration process. As shown in figure 8, the lentiviral integrase protein consists of an N-terminal zinc finger domain followed by a catalytic core domain and a C-terminal DNA-binding domain (Engelman et al. 1993). The catalytic domain comprises a core DDE amino acid sequence motif that is absolutely required for successful integration and is common with all retroviral integrase proteins as well as many cellular and bacterial transposases (Engelman and Craigie 1992; Kulkosky et al. 1992) (Johnson et al. 1986; Baker and Luo 1994; Radstrom et al. 1994). The DDE motif of the HIV integrase is located at positions D64, D116 and E152. Point mutations which result in amino acid changes at these positions specifically inhibit the integration of the lentiviral DNA into the host genome and result in an episomal intermediate (linear DNA, 1LTR and 2LTR circles) (Leavitt et al. 1993; Ansari-Lari et al. 1995; Wiskerchen and Muesing 1995; Leavitt et al. 1996). However, despite of the destruction of the catalytic core domain of the retroviral integrase (within the DDE motif), residual integration events are still observed. The quantification of residual integration events revealed that integrases with D116I mutations integrated 3 log units less efficiently compared to wild-type integrases; and integrases with D64V or E152G mutations are 4 log units less efficient than wild-type integration events (Leavitt et al. 1996). Combination of the D64V with *att* site mutations did not further decrease residual integration events and subsequent integration site analysis suggested that residual integration events of the D64V mutant was not mediated by

integrase but by background integration events (Nightingale et al. 2006). Thus, the development of new techniques for the transient expression of proteins for cell manipulation would be of great interest and is addressed in this study.

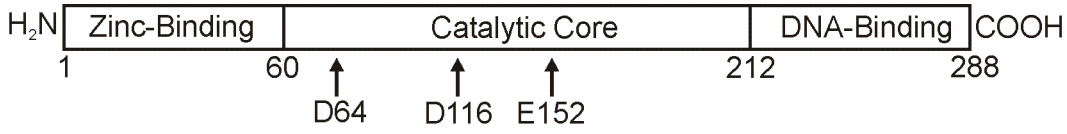

Fig. 8: Scheme of the HIV-1 integrase. The amino-terminal zinc-binding domain is followed by the catalytic core domain and the C-terminal DNA-binding domain. The DDE motif within the catalytic triad is indicated. The numbers represent the positions of the amino acid residues.

3. Retroviral restriction factors

Retroviruses are obligatory intracellular parasites that have coevolved with their hosts over millions of years. They cause infections which are responsible for significant diseases in mammals, including a variety of pathologies such as immune deficiency, malignancies, and neurological and immunological symptoms. Therefore, it is not surprising that mammals evolved antiviral defense mechanisms to protect themselves against these pathogens. Besides type I (α and β) interferons, which are historically considered to be the first line of defense against viral infection (Isaacs and Burke 1958), mammals have evolved additional proteins to counteract viruses. One well-known example of a retroviral restriction factor is the family of apolipoprotein B mRNA-editing enzyme catalytic proteins (APOBEC), which are cytidine deaminases capable of removing the amino group from cytosine to form uracil. Human APOBEC3G and APOBEC3F were shown to have potent inhibitory activity against HIV-1 (Sheehy et al. 2002; Wiegand et al. 2004; Zheng et al. 2004) by hypermutating the incoming retroviral genome during reverse transcription. However, recent data indicate that APOBEC proteins act against the virus by blocking accumulation of complete reverse transcription products rather than through their enzymatic cytidine deaminase activity (Bishop et al. 2006; Holmes et al. 2007a; Holmes et al. 2007b). The restriction factor is packaged into virions in producer cells, but does not display its negative influence on viral replication until the viral RNA is reverse transcribed in the target cell. Interestingly, HIV-1 has coevolved a strategy to prevent the deleterious actions of APOBEC3G. The HIV-1 Vif protein apparently binds to APOBEC3G in the producer cell and prevents its packaging into the virus by

targeting it to the proteasomal degradation pathway (Mariani et al. 2003; Yu et al. 2003).

Several other viral restriction factors have been identified and cloned so far. Among these is the zinc finger antiviral protein (ZAP). ZAP posttranscriptionally inhibits the replication of MoMLV and several alphaviruses by preventing the accumulation of *de novo* synthesized viral RNA in the cytoplasm (Gao et al. 2002) (Bick et al. 2003). It has been shown that the N-terminal located CCCH-type zinc finger motifs of ZAP directly bind defined sequences within the viral genome and presumably target it to an exoribonuclease complex for degradation (Guo et al. 2004).

Other retroviral restriction factors, such as the Friend virus susceptibility factor 1 (Fv1) and the cytoplasmic body component TRIM5α (a member of the tripartite motif family of proteins) target the incoming retroviral capsid instead of the retroviral nucleic acid. Since part of this study is based on the interaction of gammaretroviral particles with Fv1 or TRIM5α, these two restriction factors are explained below in more detail.

a. The restriction factor Fv1

The first gene described to display retroviral restriction properties in mammals is the friend virus susceptibility factor 1 (*Fv1*). *Fv1* was found to confer resistance of inbred strains of mice to leukemia caused by MLV (Lilly 1967). Two main alleles of *Fv1* have been described, $Fv1^{n}$ from NIH mice and $Fv1^{b}$ from BALB/c mice. The specific restriction ability also enables the division of MLVs into two subgroups depending on their tropism for these two mouse strains (Steeves and Lilly 1977). Thus, N-tropic MLV (N-MLV) strains are able to cause leukemia in NIH mice ($Fv1^{n/n}$) but do not infect BALB/c mice ($Fv1^{b/b}$). Conversely, B-tropic MLV strains (B-MLV) infect BALB/c ($Fv1^{b/b}$) but not NIH mice ($Fv1^{n/n}$). Since the *Fv1* gene is inherited co-dominantly, crossing NIH mice with BALB/c mice generated a heterozygous phenotype, which restricted both N-MLV and B-MLV (Rowe and Hartley 1972). NB-tropic MLVs, such as the common lab strain MoMLV (Moloney 1960), are able to replicate well in both BALB/c and NIH cells and do not show any apparent sensitivity to Fv1. However, recent studies involving over-expression of $Fv1^{b}$ indicate that its gene product can interact to a certain degree with both N-tropic and NB-tropic virus (Bock et al. 2000). The main viral determinant conferring susceptibility to either $Fv1^{n}$ or $Fv1^{b}$ is the amino acid residue at position 110 within the retroviral capsid. Whereas N-tropic MLV

harbors an arginine residue at that position, which determines the sensitivity to $Fv1^b$, the glutamate residue within the capsid of B-MLV is responsible for the susceptibility to restriction by $Fv1^n$ (Kozak and Chakraborti 1996). Interestingly, substitution of arginine 110 of N-MLV with glutamate creates a B-tropic-like particle which is sensitive to $Fv1^n$. The opposite (i.e. conversion of glutamate 110 of B-MLV into arginine) renders B-MLV sensitive to $Fv1^b$. On the other hand, conversion of B- or N-tropic MLV into NB-tropic particles is more complex and requires a number of additional amino acid changes (Kozak 1985; Stevens et al. 2004; Lassaux et al. 2005).

Fv1 encodes a Gag-like protein, which exhibits sequence similarities to the *gag* gene (approximately 60%) of endogenous retroviral elements (ERV-L, endogenous retrovirus with leucine t-RNA primer) in mice and humans (Best et al. 1996; Benit et al. 1997). Hypothesizing a gammaretrovirus-like Gag processing of ancient ERV-L, the homology of Fv1 to Gag covers a region extending from matrix through capsid and into the first part of the nucleocapsid. The most likely reason for the survival of the *Fv1* ORF and the loss of the surrounding retroviral sequences might be a selective pressure provided by pathogenic MLV infection.

It has been shown that Fv1 blocks MLV in a saturable manner and that capsid processing is essential for recognition by Fv1 (Dodding et al. 2005). It is suggested that early after cellular entry, Fv1 binds the capsid of the incoming particle, still allowing reverse transcription of the retroviral genome, but blocking the formation of circular viral DNA. Since the latter is thought to be indicative for nuclear entry, the observation of reduced circular viral DNA levels in a restrictive cell implies that Fv1 blocks infectivity before nuclear entry (Jolicoeur and Rassart 1980; Yang et al. 1980). However, the exact mechanism by which Fv1 restricts MLV is currently unknown and still needs to be elucidated.

b. The tripartite motif TRIM5α

Members of the TRIM protein family are involved in various cellular processes, including cell proliferation, differentiation, development, oncogenesis and apoptosis. Some TRIM family members possess antiviral properties, which target retroviruses in particular. One of the TRIM family members displaying antiviral features is the cytoplasmic protein TRIM5α, the largest isoform of at least three splice variants of the *TRIM5* gene (α, γ and δ). So far, a variety of *TRIM5α* cDNAs from a large number of

primates and bovines have been cloned and tested for antiviral activity against retroviruses (Song et al. 2005; Ohkura et al. 2006; Si et al. 2006; Ylinen et al. 2006). Interestingly, the virus specificity of TRIM5α proteins is species-dependent. The TRIM5α protein expressed in rhesus macaque cells (rhTRIM5α) potently restricts HIV-1, whereas the human variant of TRIM5α (huTRIM5α) only modestly inhibits HIV-1 but clearly affects the infection of N-tropic (N-MLV), but not B-tropic murine leukemia virus (B-MLV).

Although the exact mechanism of TRIM5α restriction is still under investigation, it was demonstrated that TRIM5α, like Fv1, interacts with the capsid of the retroviral particle at an early postentry step (Himathongkham and Luciw 1996; Towers et al. 2000; Besnier et al. 2003; Passerini et al. 2006; Towers 2007). However, in contrast to Fv1, TRIM5α usually does not allow the reverse transcription of the retroviral genome (Shibata et al. 1995; Himathongkham and Luciw 1996; Towers et al. 2000; Besnier et al. 2002; Cowan et al. 2002; Munk et al. 2002). Interestingly, squirrel monkey TRIM5α, which restricts SIV_{mac}, does not block SIV_{mac} DNA synthesis (Ylinen et al. 2005), rather resembling the Fv1-like restriction mechanism.

Similar to Fv1, the susceptibility of gammaretroviral particles to huTRIM5α is mainly controlled by the amino acid residue at position 110 within the retroviral capsid. The arginine 110 of N-MLV confers sensitivity to huTRIM5α, whereas the corresponding glutamic acid residue within the B-MLV capsid renders the particle insensitive to restriction (Towers et al. 2000; Perron et al. 2004). Replacement of glutamic acid 110 of the B-MLV capsid with arginine generates a virus that is susceptible to huTRIM5α restriction. Conversely, replacing the arginine residue of the N-MLV capsid with the corresponding glutamic acid residue from B-MLV generates a virus that can only partially overcome huTRIM5α restriction (Perron et al. 2007), arguing for the involvement of additional amino acid residues in this specific setting.

The fact that restriction of retroviral particles by TRIM5α usually inhibits the accumulation of reverse transcription products may implicate degradation of retroviral nucleic acids as part of the TRIM5α restriction process. However, to date there is no evidence that TRIM5α directly or indirectly (by an unknown factor X) targets the retroviral RNA for degradation, thereby preventing reverse transcription (Chatterji et al. 2006).

A common feature of all TRIM proteins, including TRIM5α, is the RBCC motif, which comprises a RING (“really interesting new gene”) domain, a Bbox-2 and a predicted coiled-coil region. In addition to the RBCC motif, each TRIM contains a specific

carboxyl-terminal (C-terminal) domain. In the case of TRIM5α, the specific C-terminal is the B30.2 or PRYSPRY domain (Fig. 9), a motif also found in members of the immunoglobulin superfamily.

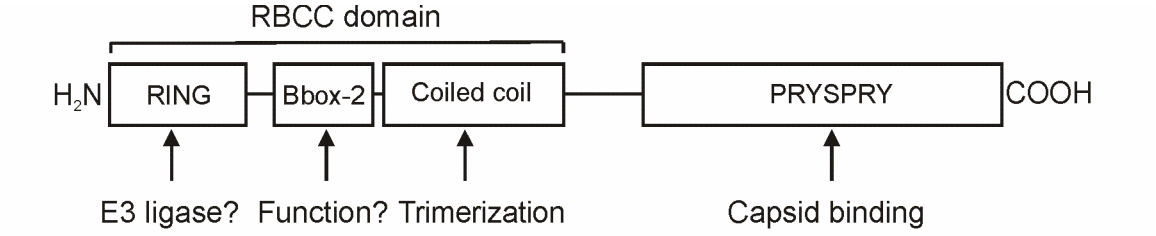

Fig. 9: Schematical illustration of the TRIM5α protein. TRIM5α consists of an N-terminal RBCC (RING, Bbox-2 and the Coiled-Coil domain, respectively) and a C-terminal PRYSPRY domain. The putative function of each domain is shown below.

The C-terminal PRYSPRY domain forms one compact 13-stranded β-sandwich, containing a hydrophobic core and a putative ligand-binding pocket. Although all attempts of conventional co-immunoprecipitations have failed so far, the PRYSPRY domain of TRIM5α has been proven to be the specificity determinant for retroviral restriction (Stremlau et al. 2004; Sawyer et al. 2005; Sebastian and Luban 2005; Yap et al. 2005). It is suggested that PRYSPRY interacts directly with the incoming retroviral capsid and perturbs the continuation of the infectious cycle. Since virus-like particles (not containing nucleic acid) saturate TRIM5α restriction activity, but the expression of capsid monomers in a restrictive cell does not (Dodding et al. 2005), TRIM5α seems to recognize rather a higher order structure of capsid multimers (Mortuza et al. 2004). Deletion of the PRYSPRY domain completely abrogates the efficacy of TRIM5α to restrict HIV-1 or N-MLV (Stremlau et al. 2004; Perez-Caballero et al. 2005a). Mutational analyses of TRIM5α showed that the amino acid composition between approximately residues 320 and 345 within the PRYSPRY domain confers the specificity of retroviral restriction among hosts (Nakayama et al. 2005; Perez-Caballero et al. 2005a; Sawyer et al. 2005; Stremlau et al. 2005; Yap et al. 2005). Interestingly, altering the arginine at position 332 of huTRIM5α to the proline residue found in the PRYSPRY domain of rhTRIM5α results in a protein that can potently restrict HIV-1 and, surprisingly, SIV_{mac} infection (Stremlau et al. 2005; Yap et al. 2005). A further study revealed that even the removal of this positively charged arginine residue 332 within the PRYSPRY domain of huTRIM5α is sufficient to allow huTRIM5α to bind HIV-1 capsids and to restrict infection (Li et al. 2006).

The N-terminal RING domain is a specialized zinc finger, which binds two zinc atoms via two cysteine residues, forming a “cross-brace” motif. The role of the RING domain during TRIM5α-mediated restriction is currently unknown. It has been shown that RING finger mutants (mutations of the Zn-coordinating cysteine residues) of TRIM5α retain partial restriction activity (Perron et al. 2004; Stremlau et al. 2004; Javanbakht et al. 2005; Perez-Caballero et al. 2005b), indicating that the RING domain is not absolutely required but also not completely dispensable for function during retroviral restriction. Since an ubiquitin auto-E3 ligase activity was found in the RING domain of the TRIM5δ isoform (Xu et al. 2003), the RING finger domain of TRIM5α might also confer similar activity. It was shown that the rapid turnover of the TRIM5α protein itself is likely mediated via auto-polyubiquitination of the RING domain followed by proteasomal degradation (Diaz-Griffero et al. 2006). To what extent (if at all) ubiquitin-conjugating activity is necessary for retroviral restriction still needs to be elucidated. However, two reports suggest that proteasomal degradation plays an important role during TRIM5α restriction. The authors of these papers showed that proteasomal inhibition preserves the overall restriction activity of rhTRIM5α or huTRIM5α but uncouples the reverse transcription block, leading to formation of functional, but still restricted preintegration complex intermediates (Anderson et al. 2006; Wu et al. 2006).

The RING finger following, the Bbox-2 domain, a motif exclusively found in the TRIM family of proteins, forms an additional zinc finger by binding one zinc atom via conserved cysteine and histidine residues. In general, the function of B-boxes remains unknown. However, in contrast to mutations within the RING domain, deletion of both RING and Bbox-2 as well as mutation of the zinc-coordinating residues in the B-box-2 motif caused a complete loss of antiviral activity (Perez-Caballero et al. 2005a; Javanbakht et al. 2005), indicating an essential role during restriction.

The adjacent coiled-coil motif is a typical hyper-secondary structure (formed by intertwining of multiple α-helices) and is thought to mediate homo- and hetero-interactions between TRIM molecules (Reymond et al. 2001). Cross-linking studies revealed that TRIM5α exists as a trimer (Mische et al. 2005), a conformation which is suggested to be necessary for retroviral capsid binding (Javanbakht et al. 2006). Deletion of the coiled-coil motif completely abrogates the restriction activity of TRIM5α.

B. General aim of the study

Novel and improved transient expression methods for many approaches in gene therapy, biotechnology or basic science are of great interest. Retroviruses are evolutionary optimized and adapted to the host´s cellular machinery, which makes them interesting candidates for the transient and therefore reversible expression of transgenes. Since conventional retroviral gene delivery results in stable transgene expression, the generation of vector particles which are defective for either reverse transcription or integration are necessary. Introduction of specific mutations within the retroviral vector genome or the Gag/Pol expression construct may allow the inhibition of specific steps (depending on the type of the introduced mutation) within early phases of the retroviral life cycle. An already existing transient retroviral expression method is the use of integrase-deficient lentiviral vector particles, where specifically introduced point mutations within the catalytic core domain of the lentiviral integrase (e.g. D64V point mutation within the DDE motif) result in the formation of non-integrating episomal lentiviral DNA molecules. However, one undesirable side effect is the potential of residual integration events of these episomal DNA molecules (Nightingale et al. 2006).

Infectious virions of retroviruses contain two copies of their plus-stranded RNA genome. Retroviral RNA genomes are equipped with a 5´ Cap structure and a 3´ PolyA-tail, and therefore highly resemble cellular messenger RNAs. Thus, when reverse transcription is disabled, retroviral particles might serve as an interesting mRNA delivery tool for a receptor-mediated, transient, and ectopic expression of proteins in target cells. Thus, one could generate reverse transcription-deficient vector particles that - after receptor-mediated uptake and the retroviral uncoating process – release their genomic vector mRNA (encoding for the transgene of interest) into the cytoplasm for translation. The contents of the following publications are based on this hypothesis.

The aim of publication 1 was the generation and comparison of MLV-based vector mutants that are deficient in reverse transcription and/or integration. As a paradigm, these mutants were tested for their capability to mediate transient transfer of the site-specific recombinase Cre into human and mouse fibroblasts.

The second publication focussed on mechanisms underlying “retroviral particle-mediated mRNA transfer” (RMT) and how it might be limited by cellular restriction factors. The sensitivity of RMT to shRNAs and retroviral restriction factors (huTRIM5α and Fv1) were explored in this work.

Self-inactivating (SIN) retroviral vectors were designed to lack U3 enhancer/promoter sequences of their LTRs after proviral integration, and therefore transcription of the transgene is initiated by an internal promoter (Yu et al. 1986). However, in contrast to corresponding lentiviral packaging systems, packaging of gammaretroviral SIN vectors suffers from suboptimal titers (Yu et al. 1986; Ailles and Naldini 2002). Thus, the topic of publication 3 was to understand the mechanism underlying titer reduction and to improve gammaretroviral SIN vector packaging by introducing stronger 5' enhancer/promoter sequences within the retroviral vector plasmid. In line with this study, we investigated whether the improved 5' enhancer/promoter sequences could also enhance RMT (supplementary figure 1).

These three studies show that the retroviral genomic RNA of gammaretroviral vector particles may serve as a translation template when reverse transcription is disabled and that this type of modified vector particles may be a potential tool for targeted and transient cell modification.

C. Publication 1

Retroviral Pseudotransduction for Targeted Cell Manipulation

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Author's contribution statement:
Melanie Galla constructed the Cre encoding gammaretroviral vectors, acquired and analyzed the data for Figs. 1-4, Suppl. Fig. 1C and Suppl. Fig. 3. Furthermore, MG aquired an additional data set for Suppl. Fig. 2 (except for adenoviral Cre vector obtained data; data not shown), participated in designing the figures and writing the manuscript. Elke Will supervised all experiments, acquired and analyzed the data for Suppl. Fig. 1B and Suppl. Fig. 2, participated in designing the figures and writing the manuscript. Janine Kraunus designed, cloned and verified the EGFP encoding gammaretroviral vector mutants (Fig. 1). Lei Chen provided the adenoviral Cre vector supernatant (Suppl. Fig. 2A). The Suppl. Fig. 4 was designed by Shawn Wheeler as mentioned in the acknowledgments. Christopher Baum is the corresponding author, designed the whole study and developed the concept. In addition, Christopher Baum critically evaluated and analyzed the data, and participated in writing the paper.

Retroviral Pseudotransduction for Targeted Cell Manipulation

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a. Abstract

The present study addressed whether retroviral vectors could be modified to achieve receptor-mediated, dose-controlled, and transient delivery of proteins or nucleic acids into targeted cells. As a paradigm, we generated mouse leukemia virus-based vectors encoding the site-specific recombinase Cre. The vectors were disabled in primer binding site function, blocking reverse transcription of the virion mRNA. While reducing transgene insertion more than 1000-fold and abolishing toxic effects of constitutive Cre expression, transient Cre delivery was still highly efficient, receptor-restricted, and insensitive to pharmacologic inhibition of reverse transcription. This form of Cre transfer required the retroviral packaging signal, cap-proximal positioning of the translation unit, as well as *gag* and *env* expression in producer cells, revealing retroviral mRNA transfer as the underlying mechanism. Thus, retrovirally delivered mRNA may serve as an immediate translation template if not being reverse transcribed. This approach allows multiple modifications for targeted and reversible cell manipulation with nucleic acids.

b. Introduction

Numerous applications of advanced cell biology and cellular therapy would profit from the development of new methods for targeted and reversible delivery of protein or mRNA. Retroviruses infect cells through receptor-mediated uptake, reversely transcribe plus-stranded genomic mRNA into double-stranded proviral DNA, and integrate the proviral copy into the host genome. Thereafter, spliced and unspliced proviral mRNA is transcribed, the latter being specifically packaged into novel retroviral particles. Retroviral pseudotransduction has been described as a process that may lead to the expression of foreign proteins, without delivering integrating proviral DNA (Haas et al., 2000; Nash and Lever, 2004). Previously, this phenomenon has not been exploited to exert specific biological functions. Here we show that retroviral particles can be manipulated for targeted delivery of mRNA, resulting in efficient transient cell manipulation. Proof of principle is established with transfer of Cre recombinase.

The bacteriophage endonuclease Cre excises double-stranded DNA flanked by *loxP* recognition sites from the genome of prokaryotic or eukaryotic cells. Cre may thus reverse a specific transgene insertion, or also trigger site-specific insertion of a transgene into a single *loxP* site. Cre represents a member of a larger family of site-specific endonucleases that are of great interest for experimental and therapeutic cell manipulation (Ghosh and Van_Duyne, 2002; Gorman and Bullock, 2000). Constitutive expression of Cre is genotoxic, and therefore, reversible delivery of Cre as a recombinant protein (“protein transduction”) or from self-excising retroviral vectors has been developed (Jo et al., 2001; Loonstra et al., 2001; Peitz et al., 2002; Pfeifer et al., 2001; Silver and Livingston, 2001; Will et al., 2002). However, transduction of recombinant Cre protein does not allow targeting to a specific cell type in a mixed population, and retroviral self-excision may still disrupt cellular genes or trigger translocations with *loxP* sites present in the targeted allele or in another integrated provirus copy.

c. Results

Design of retroviral vector mutants

To investigate the potential of retroviral pseudotransduction for delivery of Cre, we inserted a nuclear localizing variant of Cre (nlsCre) into mouse leukemia virus (MLV)

based retroviral vectors that mediated stable integration into the target cell genome (SF91-nlsCre, Figure 1A). To inhibit stable gene transfer, we designed several mutants. Vector dPBS lacks the retroviral primer binding site (PBS) to disable reverse transcription (RT) into proviral DNA. Vector aPBS contains an artificial PBS that cannot prime RT unless being complemented by a recombinant tRNA (Lund et al., 1997). Vector dU5 contains all elements required for RT into double-stranded DNA but lacks the *att* recognition motif of the retroviral integrase (Basu and Varmus, 1990), along with flanking sequences of the U5 region (Figure 1A).

Mutations strongly inhibit stable retroviral gene delivery

Experiments with retroviral vectors expressing enhanced green fluorescent protein (EGFP) revealed that all three mutants transiently expressed high levels of EGFP after physicochemical transfection into retroviral packaging cells. Ecotropic particles of the intact retroviral vector SF91-EGFP mediated stable gene transfer with a titer of ~10^6 infectious particles per milliliter of unconcentrated supernatant. Using concentrated supernatants of SF91-EGFP on NIH3T3, 50% of cells were stably transduced (Figure 1B). Under identical experimental conditions, supernatants of packaging cells transfected with mutants dU5-EGFP, aPBS-EGFP and dPBS-EGFP were at least 1000-fold attenuated in their capacity of stable gene transfer (Figure 1B).

Retroviral pseudotransduction occurs immediately after particle exposure

The process of retroviral receptor binding, particle uptake, nuclear transport, integration, and *de novo* transcription of mRNA requires several hours. By flow cytometric detection of EGFP, we investigated the kinetics of retroviral pseudotransduction (Figure 1C). Starting 5 hr until 13 hr after exposure, SF91-EGFP and aPBS-EGFP mediated an equal, weak but significant increase of EGFP expression when compared with control cells. Culminating between 13 and 55 hr, SF91-EGFP mediated a >60-fold higher fluorescence intensity than the peak level observed with aPBS-EGFP, which started to decline 19 hr after exposure. These data indicate that the early phase after exposure (5-13 hr) was dominated by pseudotransduction activity, irrespective of the vector's capacity to undergo RT and integration. If, however, stable delivery of retroviral DNA occurs, *de novo* transcription results in a much higher expression of the encoded gene, depending on the strength

of the vector. The persistence of EGFP fluorescence until 36h after exposure to aPBS particles is best explained by the long half-life of the encoded protein.

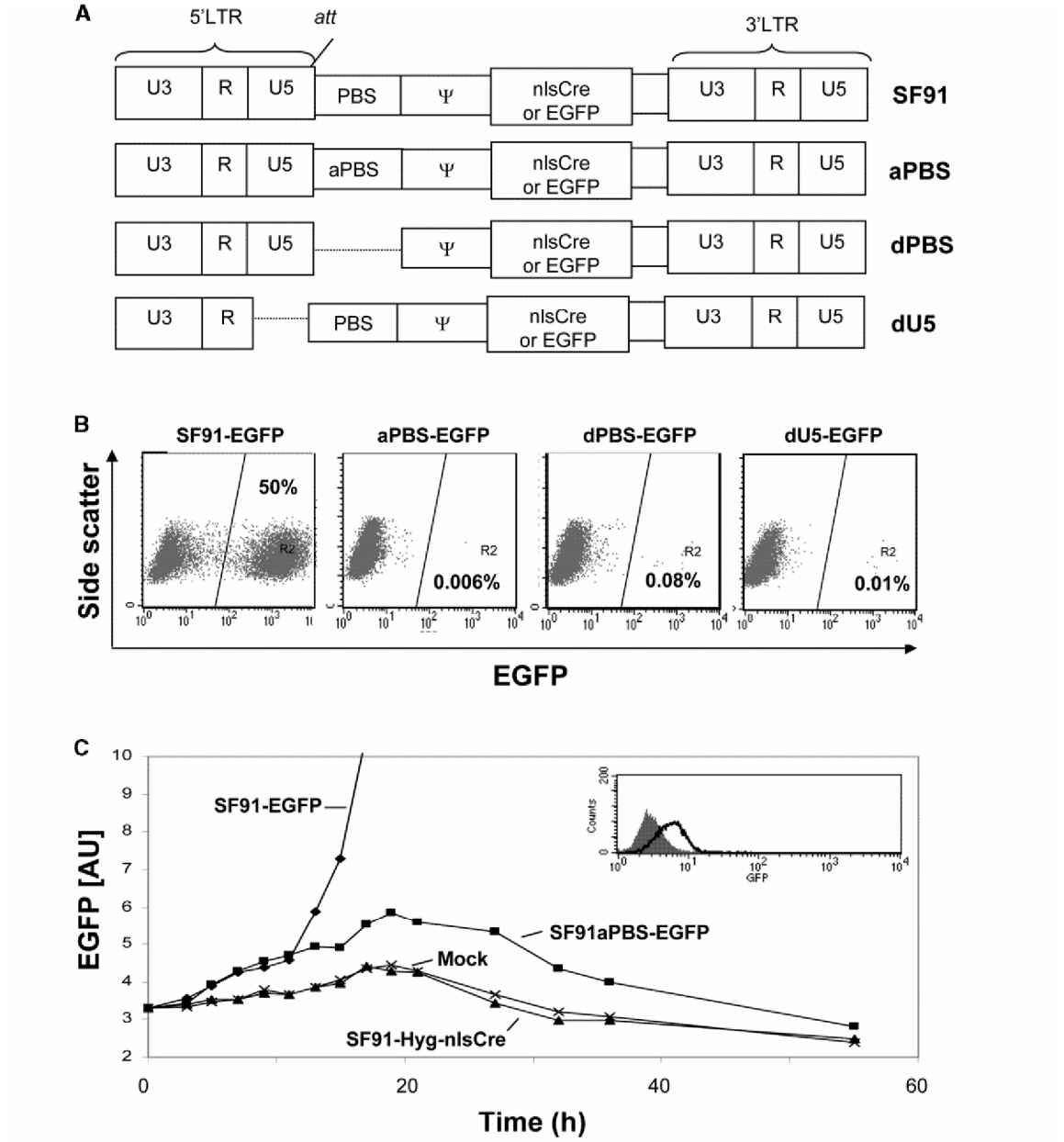

Fig. 1: Retroviral vector mutants used for pseudotransduction experiments, expressing either nlsCre or EGFP. (A) SF91 contains all *cis* elements required for RT and integration. Mutant aPBS contains a defective PBS, dPBS lacks the PBS required for RT, and dU5 lacks the *att* signal required for integration. (B) Compared with SF91-EGFP, mutants aPBS, dPBS, and dU5 show a greatly reduced incidence of stable EGFP transduction. NIH3T3 cells were analyzed 5 days after exposure to particles. (C) Pseudotransduction generates slightly increased EGFP expression between 5 and 13 hr after exposure of cells to retroviral particles, both with aPBS-EGFP and SF91-EGFP. Subsequently, EGFP expression (AU, arbitrary units) rises only when using SF91-EGFP, reflecting *de novo* synthesis of mRNA after provirus integration. In contrast, cells exposed to aPBS-EGFP return to baseline levels after ~40 hr. SF91-Hyg-nlsCre was used as a non-EGFP expressing vector control. The inset shows histograms of mock-transduced cells (gray) and cells exposed to aPBS-EGFP (black line), 19 hr after treatment.

Retroviral mutants deliver Cre activity with high efficiency

To detect the ability of retroviral particles to transfer nlsCre into target cells, we used mouse and human fibroblasts as indicator cells. These contained a reporter allele SFr-2 in which *loxP*-flanked coding sequences of red fluorescent protein (DsRed2) is deleted and transcription of EGFP is initiated only following Cre exposure (See Supplemental Figure 1 at http://www.molecule.org/cgi/content/full/16/2/309/DC1) (Will et al., 2002).

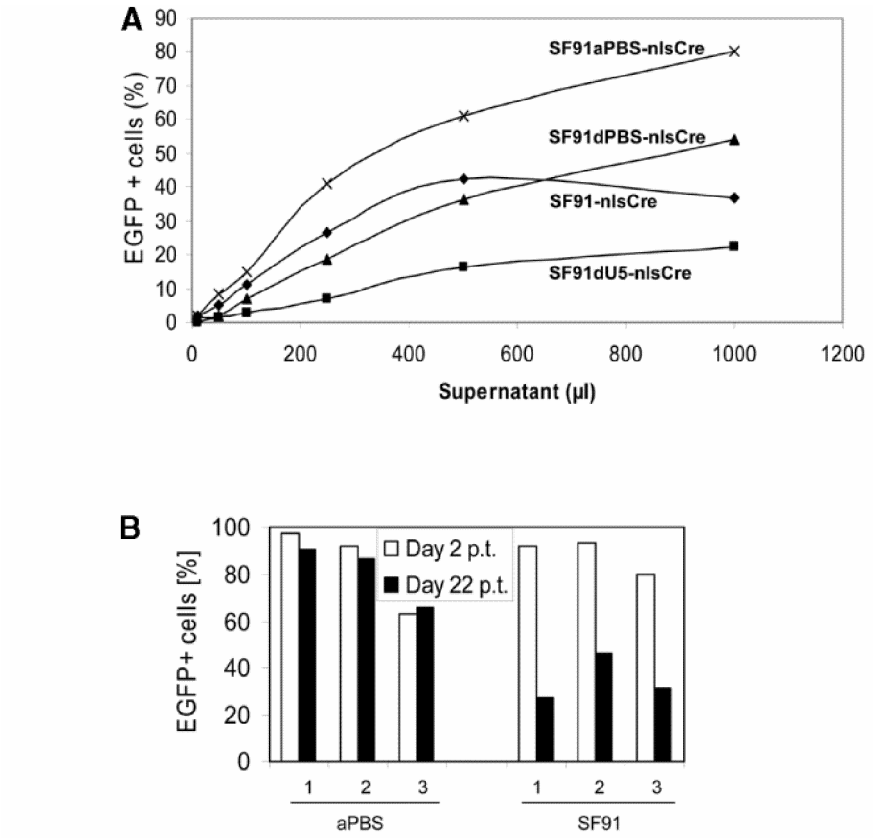

Fig. 2: Cre expression mediated by pseudotransduction is dose-dependent and avoids toxic side effects. (A) Treatment of reporter cells with supernatants containing either the integrating vector SF91-nlsCre or the mutants aPBS-nlsCre, dPBS-nlsCre or dU5-nlsCre resulted in highly efficient, dose-dependent recombination. (B) Permanent expression of nlsCre by the integrating vector SF91-nlsCre leads to a competitive growth disadvantage of EGFP+ Cre+ cells during 20 days of culture in three independent experiments. No such effect is seen with mutant aPBS-nlsCre.

As judged by the conversion to the EGFP+ phenotype all retroviral mutants mediated efficient and dose-dependent Cre activity in target cells (Figure 2A). Cre-mediated induction of EGFP expression was most efficient when using as vehicle retroviral particles of mutants aPBS-nlsCre. The potency of the cellular supernatants showed some variability possibly due to differences in the transfection efficiency of the producer cells. Therefore, comparative experiments were performed with

supernatants from virus productions performed in parallel under identical conditions. Using the most potent preparations, almost complete conversion of the target cell population could be achieved with a single treatment, even with unconcentrated supernatants (Figure 2B).

Cre delivery by retroviral mutants is transient and not toxic

Importantly, prolonged analysis of uncloned target cells after exposure to retroviral Cre supernatants revealed that mutant aPBS-nlsCre did not induce any overt target cell toxicity, whereas cells harboring integrating retroviral vectors encoding Cre were counterselected, likely due to genotoxic side effects of persistent Cre expression (Loonstra et al., 2001; Pfeifer et al., 2001; Silver and Livingston, 2001). To demonstrate this important advantage of retroviral pseudotransduction, we started with populations of target cells where EGFP expression was achieved with 80%-96% efficiency using the integrating retroviral vector SF91-nlsCre. The frequency of EGFP+ cells decreased by 45% within 22 days. In contrast, using aPBS-nlsCre the frequency of EGFP+ cells remained constant, independent of the initial Cre load (Figure 2B). Detection of Cre by Western blot and of Cre encoding DNA by PCR revealed counterselection of cells with persistent Cre expression following use of SF91-nlsCre or dU5-nlsCre, but not aPBS-nlsCre (Figures 3A and 3B).

Physicochemical transfection and adenoviral vectors may be considered as alternative procedures for transient Cre delivery. With either method, a high efficiency of Cre transfer was achieved. Comparing similar populations with 50%-70% of reporter cells in which Cre had been active, we found that adenovirally transduced cells were counterselected with similar kinetics as cells transduced with the integrating retroviral vector SF91-nlsCre (Supplemental Figure 2A). This could be explained by persisting nlsCre expression from episomal adenoviral transgenes. Cells physicochemically transfected with a Cre plasmid were not significantly counterselected but the overall toxicity was very high, probably due to the transfection reagent (Supplemental Figure 2B). Under identical conditions, aPBS-nlsCre neither caused counterselection nor general toxicity, leading to an 8-10x higher recovery of cells 2 days after exposure.

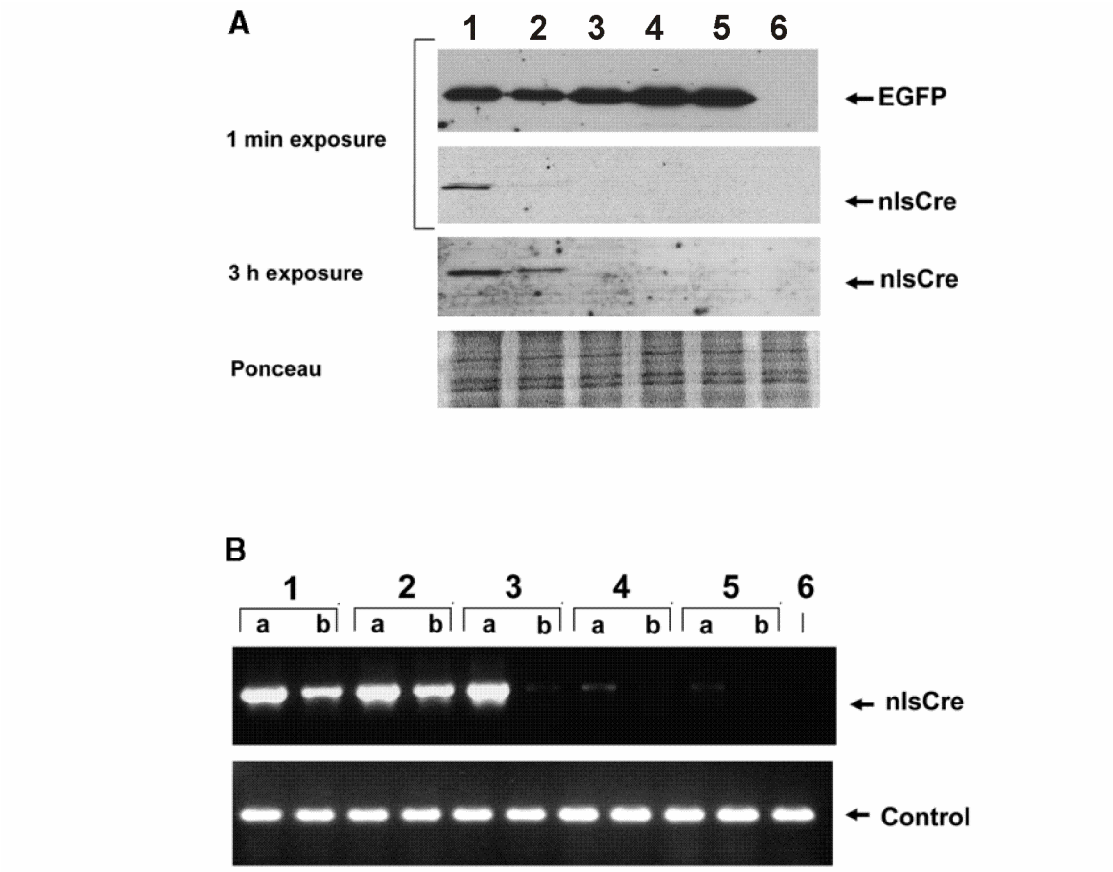

Fig. 3: Counterselection of cells constitutively expressing nlsCre. (A) Western blot of protein extracts harvested from target cells 9 days after particle exposure reveals persisting Cre expression from SF91-nlsCre (lane1). Prolonged exposure (3 hr instead of 1 min) revealed a low level of persisting Cre after use of mutant dU5-nlsCre (lane 2), but not with mutants dPBS-nlsCre (lane 3) and aPBS-nlsCre (lanes 4 and 5). EGFP expression from the converted Cre reporter allele is even stronger in cell populations treated with dPBS and aPBS. Lane 6 shows mock-transduced cells. The Ponceau stain (lower panel) shows equal protein load. (B) Semi-quantitative PCR to detect integrated nlsCre DNA. Genomic DNA from Sc-1 reporter cell populations was prepared 9 (a) or 22 days (b) after transduction and a PCR using nlsCre-specific primers was performed. Integrated nlsCre was detected in SF91-nlsCre (1), dU5-nlsCre (2) and dPBS-nlsCre (3) treated cells. A much weaker signal was seen in aPBS-nlsCre-treated cells (4 and 5). This is consistent with the residual leakiness of these mutants (Fig. 1B). A control PCR amplifying the EGFP sequence in the SFr-2 reporter allele indicated that equal amounts of genomic DNA were used (lower panel).

Pseudotransduction depends on retroviral particle assembly and mRNA packaging

Controls addressed which retroviral components were required for transfer of Cre activity by constructs aPBS-nlsCre and dPBS-nlsCre. No evidence of Cre delivery was observed when omitting either of the three key components of the retroviral packaging process (Gorelick et al., 1988): the *gag-pol* expression plasmid, the *env* expression plasmid, or the retroviral packaging signal (Ψ) of the Cre plasmid. The latter construct encoded large amounts of Cre mRNA and protein in transfected 293T

cells (data not shown). However, uptake of a cellular mRNA lacking Ψ into retroviral particles is expected to be very inefficient (Gorelick et al., 1988). The latter control also excluded passive protein transfer and contamination of retroviral particles by transfected plasmid DNA as the underlying mechanisms of Cre transfer by dPBS or aPBS (Chen et al., 2001; Will et al., 2002). Thus, retroviral pseudotransduction requires retroviral particle formation with incorporation of Ψ+mRNA and an active retroviral infection process triggered by Env. The retroviral mRNA must be able to serve as an immediate translation template if not undergoing RT. In line with this hypothesis, pseudotransduction with mutant dPBS was impossible when expressing Cre from an internal promoter located 3' of Ψ on the retroviral mRNA (data not shown). In this case, cap-dependent ribosomal scanning could only occur after *de novo* synthesis of mRNA in transduced cells.

Further evidence for the role of Ψ+mRNA in retroviral pseudotransduction was obtained when inhibiting RT in target cells with 3´-Azido-3´-deoxythymidine (AZT) (Strair et al., 1991). This drug inhibited stable EGFP transfer by the intact vector SF91-EGFP in a highly efficient manner (Supplemental Figure 3). As expected, residual pseudotransfer leading to a weak shift of cellular fluorescence was still observed. However, while nlsCre delivery by the vector aPBS-nlsCre was completely insensitive to AZT, Cre delivery by the intact vector SF91-nlsCre was reduced by up to 50% (Supplemental Figure 3). This suggested that pseudotransduction of intact retroviral vectors may not be as efficient as that of RT deficient mutants.

Retroviral pseudotransduction allows targeting of specific cells

Finally, to address whether transfer of Cre activity by aPBS-nlsCre was receptor-mediated, we mixed human HT1080 and murine Sc-1 cells carrying the same indicator allele SFr-2. EGFP conversion was restricted to murine cells when using ecotropic supernatants. In contrast, use of the RD114 Env largely restricted Cre transfer to human cells (Figure 4). Even when human cells represented a minor population (<5%), specific targeting with RD114 enveloped particles containing aPBS-nlsCre was possible (data not shown). The data are consistent with the known species restriction of these pseudotypes (RD114 may confer residual infectivity in mouse cells [F.L. Cosset, personal communication]) (Hanawa et al., 2002). As expected, the tropism was independent of the type of vector used (SF91 or PBS or dPBS).

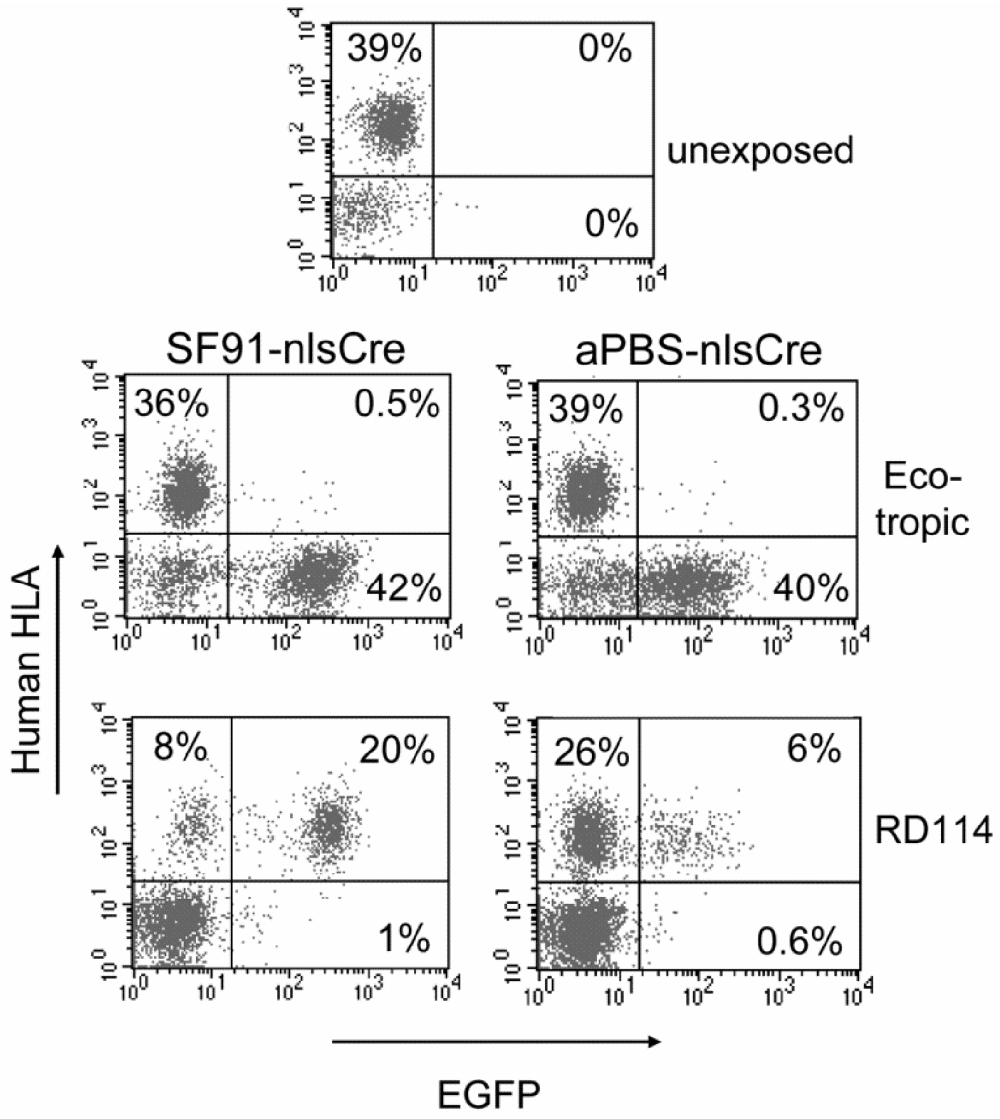

Fig. 4: Pseudotransduction is receptor mediated and allows targeting of distinct cells in a mixed population. Human HT1080 and murine Sc-1 cells were mixed and transduced using either ecotropic (middle panel) or RD114 pseudotyped particles (lower panel) containing either the integrating SF91-nlsCre or mutant aPBS-nlsCre vectors. Flow cytometry was performed 4 days after exposure to particles. The mixed cell population was stained with anti human HLA(A,B,C) antibody to identify the HT1080 subpopulation. The unstained Sc-1 cells are mostly located in the first channel detecting green fluorescence. EGFP expression induced by Cre activity is strictly dependent on the tropism of the envelope protein, and targeting is independent of the type of expression vector used (SF91-nlsCre, aPBS-nlsCre, or dPBS-nlsCre, data not shown). The different efficiencies reflect variations in vector preparations.

d. Discussion

By delivering Cre recombinase, we demonstrate here that retroviral pseudotransduction can be exploited for highly efficient, dose-controlled, transient, and targeted manipulation of specific cells in a mixed population. Introducing mutations in the PBS or U5 region of the retroviral mRNA resulted in an up to 10,000-fold reduction of stable gene transfer efficiency while still mediating sufficient Cre delivery for site-specific recombination in up to 95% of exposed cells. Our study demonstrates

that receptor-mediated uptake of retroviral particles serves the key mechanism of retroviral pseudotransduction, strongly arguing against a major role of cellular microvesicles or transfected plasmid DNA which may contaminate producer cell supernatants. After receptor-mediated entry, it is formally possible that retroviral pseudotransduction depends on transient delivery of RNA, protein and/or episomal DNA.

Retroviral pseudotransduction after blockade of integrase function

When using the dU5 mutant, in which crucial *cis*-regulatory elements required for RT are preserved (PBS, R region and polypurine tract), double-stranded DNA may still be formed and transported to the nucleus. As the deletion of the U5-located *att* recognition motif inhibits the function of the retroviral integrase (Basu and Varmus, 1990), a strong reduction of stable transgene insertion by this mutant was expected. A deletion of the second *att* motif in the U3 region and structural or pharmaceutical blockade of the retroviral integrase may allow even more stringent retroviral delivery of unintegrated DNA. A recent report suggests that such a version of retroviral pseudotransduction may allow transient or semipermanent *de novo* RNA synthesis (Vargas et al., 2004). However, this form of pseudotransduction bares a residual risk of stable transgene insertion and unpredictable duration of transgene expression.

Retroviral pseudotransduction after blockade of RT

When using mutants aPBS or dPBS, in which the initiation of RT is severely disabled (Lund et al., 1997), retroviral pseudotransduction must be dependent on “early translation” of the retroviral mRNA, and/or on passive transfer of proteins in retroviral particles (Supplemental Figure 4). However, retroviral particles are not expected to uptake major non-retroviral protein cargo except that specific domains are present which direct an interaction with *gag* proteins or proviral RNA. Such a process may result in uptake of inhibitory cellular proteins such as APOBECG3 or accessory viral proteins such as Vif (KewalRamani and Coffin, 2003). Using highly concentrated supernatants of lentiviral vector preparations pseudotyped with the cytotoxic glycoprotein of vesicular stomatitis virus, Nash and Lever (2004) recently reported transient EGFP protein transfer into target cells. However, in this study microvesicels and cellular debris may also have contributed to the EGFP transfer, raising concerns regarding the specificity of the method for practical use.

In the present study, several controls indicated that passive delivery of (nuclear localizing) Cre protein does not explain pseudotransduction by any of the mutants tested here. Pseudotransduction was only possible when the Cre-encoding RNA contained the retroviral Ψ signal and when the Cre translation unit was accessible by a cap-dependent scanning process. mRNAs in which Cre was encoded from an internal promoter located downstream of Ψ were inefficient in this process. Finally, experiments with AZT showed that the characteristic RT step of retroviruses is not necessary for this form of pseudotransduction, and therefore, the term "*retro*viral" may even be somewhat misleading. In a broader sense, we suggest this phenomenon be addressed as retroviral particle-mediated mRNA transfer (RMT), leading to "early translation" of the encoded proteins after particle disassembly. This process may also be exploited when using other viruses with a genomic plus-stranded mRNA (such as picornaviridae) (Wilson, 1985).

Our study revealed that RMT occurred in the first hours after exposure of cells to retroviral particles. As these contain just two strands of mRNA, it seems plausible that the efficiency of RMT depends on the substantial excess of infectious particles over integration events (McDonald et al., 2002). Our data suggest that following uptake in the cytoplasm, many retroviral particles will not undergo RT but rather disassemble to release their genomic RNA for subsequent translation (Supplemental Figure 4). While this process is likely to represent a dead end of retroviral replication, it is not expected to be hindered by postentry defense mechanisms targeting downstream steps of RT or nuclear translocation (Besnier et al., 2003; KewalRamani and Coffin, 2003; Towers and Goff, 2003) and, therefore, should be cell-cycle independent even when using gammaretroviral particles.

Developing pseudotransduction as a method for cell manipulation

Following these considerations, it may be possible to adapt gammaretroviral RMT to applications with increased potency requirements by supernatant concentration, particle preloading, or repetitive exposure. It may also be possible to manipulate the disassembly process to increase the efficiency. Additional mutations of the transferred mRNA or the retroviral *pol* functions are expected to completely avoid residual RT and DNA integration. The major advantage of the method is the potential to avoid DNA transfer while still introducing specific mRNA, in a receptor-mediated manner. Of note, envelope modifications are not only useful for retargeting of

retroviral particles to defined subsets of cells, such as human T lymphocytes or cancer cells (Schnierle et al., 1997; Chowdhury et al., 2004); they might also allow a combination of RMT with delivery of growth factor signals (Verhoeyen et al., 2003). In general, we expect RMT to be particularly useful for applications where relatively low and transient expression of proteins may lead to striking biological effects: examples are the expression of receptors involved in homing of circulating cells, transcription factors, or cellular proteins regulating cell expansion and differentiation, and, as exemplified by Cre, recombinases or integrases for targeted genetic interventions.

e. Experimental Procedures

Cre reporter cell lines

Murine and human fibroblast lines containing a Cre reporter allele were generated by transducing Sc-1 (ATCC CRL-1404) and HT1080 (ATCC CCL-121) with the retroviral vector SFr-2, containing DsRed2 cDNA flanked by loxP sites. Clones expressing DsRed2 were obtained by single cell sorting. SFr-2 is a derivate of SFr (Will et al., 2002) encoding DsRed2 instead of DsRed1 and the woodchuck hepatitis B virus posttranscriptional regulatory element (Schambach et al., 2000).

Retroviral vectors and plasmids

Retroviral vector SF91-nlsCre was derived from SF91-EGFP (Schambach et al., 2000) by replacing an EGFP NcoI-NheI fragment with a NcoI-NheI fragment of pGEX-nlsCre (Will et al., 2002). Mutant vectors lacking the U5 region of the 5'LTR located 70-145 bp downstream of the CAP site (SF91dU5-EGFP) or lacking the PBS located 146-163 bp downstream of the CAP site (SF91dPBS-EGFP) were derived from SF91-EGFP by overlapping PCR, resulting in precise deletions. Corresponding vectors SF91dU5-nlsCre and SF91dPBS-nlsCre were obtained by replacing the EGFP NcoI-NheI fragment with a NcoI-NheI fragment of pGEX-nlsCre (Will et al., 2002). In SF91aPBS-EGFP, sequences 149-160 bp downstream of CAP were replaced by TCAGCTGCAGGG using site-directed mutagenesis, according to Lund et al. (1997). Correct deletions or nucleotide replacements were confirmed by sequencing. The eukaryotic Cre expression plasmid pCMVnlsCre lacking Ψ was generated by replacing the EGFP cDNA in pEGFP-C1 (Clontech, Heidelberg, Germany).

Production of retroviral particles

Packaging of SFr-2, SF91-nlsCre, SF91dU5-nlsCre, SF91dPBS-nlsCre and SF91aPBS-nlsCre in retroviral particles was performed by cotransfection of the retroviral plasmid with expression plasmids for MLV *gag-pol* and either ecotropic (Morita et al., 2000) or RD114 envelopes (Cosset et al., 1995) into Phoenix GP (G. Nolan, Stanford University, Palo Alto, CA) or 293T cells. Transfection, harvest and concentration of virus-containing supernatants was performed as described previously (Beyer et al., 2002).

Cell culture and transduction

293T, Phoenix-GP, NIH3T3, Sc-1, and HT1080 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. The day before transduction 5 x 10^4 cells were plated. 1 ml undiluted or serial dilutions of retroviral supernatants were applied to the cells. Transduction was assisted by adding 4 µg/ml protamine sulfate and centrifugation for 60 min at 400 x g and 25ºC-32℃. After 2 days the percentage of EGFP+ cells was analyzed by FACS. For specific detection of human cells, mixed populations were stained with anti human HLA(A,B,C)-APC conjugate (BD Pharmingen, San Diego, CA).

Lipofection and adenoviral gene transfer

10^5 reporter cells were seeded 12 hr before treatment. Transfection with pCMVnlsCre was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer´s instructions. Cells were washed with PBS and incubated with a mixture of 1 µg pCMV-nlsCre with either 1, 2 or 3 µl Lipofectamine in a total volume of 200 or 400 µl Opti-MEM I (Gibco, Grand Island, NY) containing no or 5% FCS for 5 or 12 hr. Standard culture conditions were used until FACS analysis.

AdCreM2 supernatants (Microbix, Toronto, Canada) were produced according to the manufacturer's instructions. Cells were incubated for 1 hr at 37℃ with 0.5 to 40 µl of AdCreM2 supernatant in a total volume of 200 µl PBS/4 % FCS. Cells were washed twice with PBS, and cultivated as above.

The percentage of dead cells and cells targeted by Cre (EGFP+) was determined by FACS analysis after staining with 7AAD (BD Pharmingen). 7AAD+ events and events

with low forward scatter were considered to be dead cells (verified by Trypan blue staining).

Western blot

Cell lysates were obtained after 15 min incubation with 50µl RIPA buffer containing proteinase inhibitors (Complete, Roche, Indianapolis, IN). Samples were separated by SDS/PAGE(12.5%), transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and probed with anti-Cre (Novagen, Madison, WI) 1:7,000 or anti-GFP (Santa-Cruz, Santa Cruz, CA) antiserum 1:500 in TBST/3% dry milk. The secondary antibody anti-rabbit-HRP (Santa Cruz) was used at a 1:10,000 dilution in TBST/3% dry milk. Detection was carried out by chemiluminescence (ECL, Pierce, Rockford, IL).

Semiquantitative PCR

Genomic DNA was isolated 9 or 22 days post transduction with QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) after the manufacturer's protocol. 500 ng of DNA was used for PCR amplification of Cre DNA sequence using oligonucleotides GGTGAACGTGCAAAACAGGCTCTA and GCTTGCATGATCTCCGGTATTGAAA. PCR was performed using Taq polymerase (New England Biolabs, Beverly, MA), 2 min 94℃, followed by 41 cycles of 30 s at 94℃, 30 s at 57℃, and 40 s at 72 ℃. For the control amplification of EGFP-wPRE oligonucleotides ACGAGAAGCGCGATCAC ATGGTCCTG and CCAAATCAAGAAAAACAGAACAAATA were used under identical conditions.

Acknowledgements

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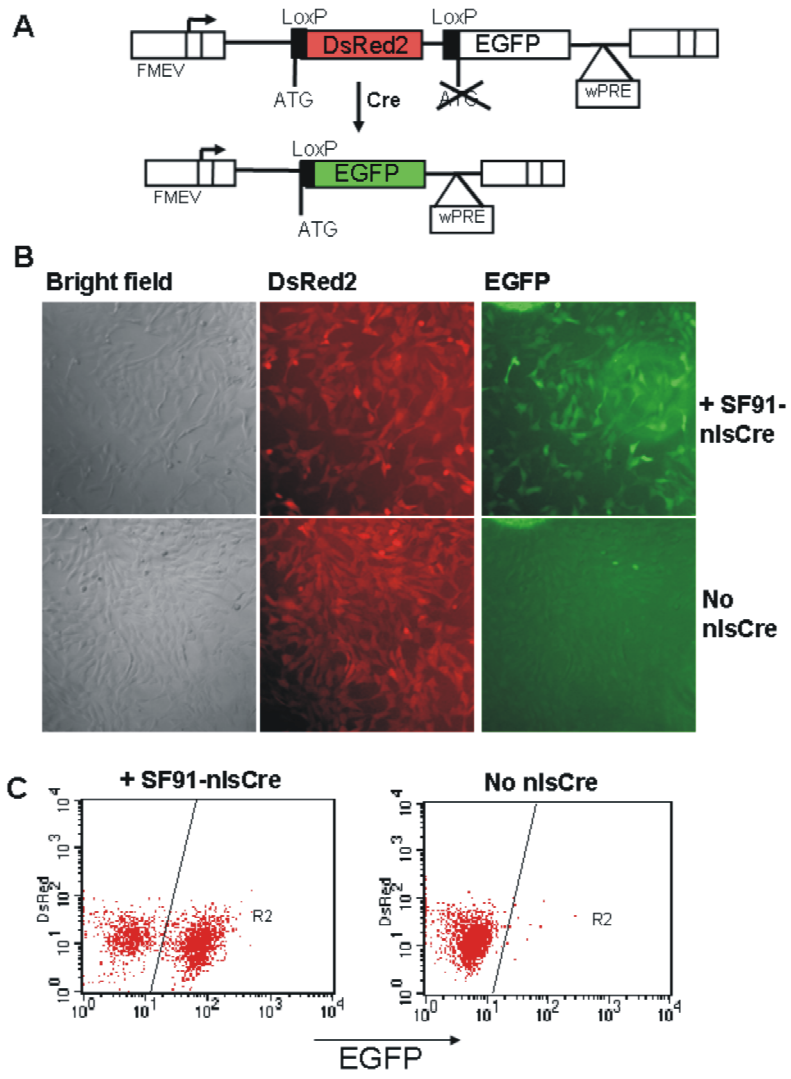
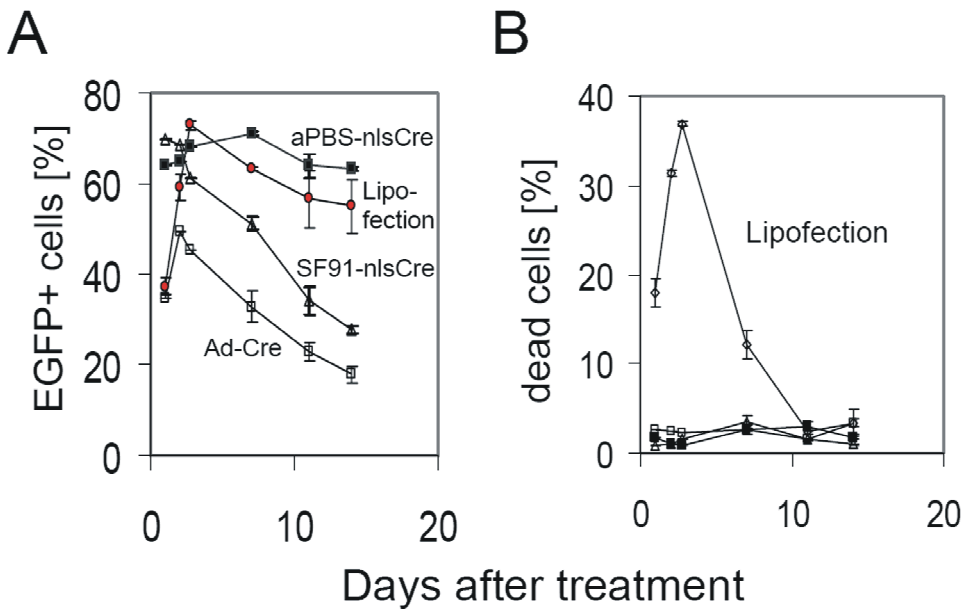
g. Supplementary Figures

Supplementary figure 1



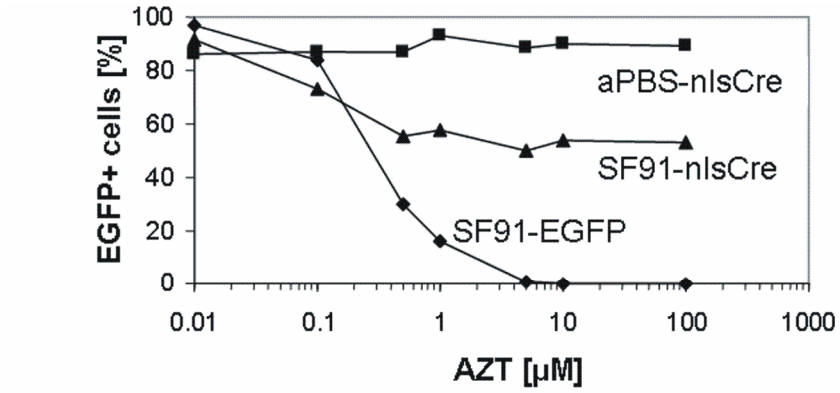
Suppl. Fig. 1: Cre activity mediates a switch from red to green fluorescence in Sc-1 cells containing the Cre reporter allele SFr-2 (A). Two days after treatment, EGFP expression can be detected only after exposure to Cre recombinase (B). The percentage of reporter cells modified by Cre-mediated recombination can be clearly defined by flow cytometry (C). Controls revealed that EGFP expression leads to a shift of fluorescence in the DsRed channel, such that the loss of DsRed expression after Cre-mediated gene excision cannot be monitored. Excision of DsRed was documented by PCR (Will et al., 2002).

Supplementary figure 2



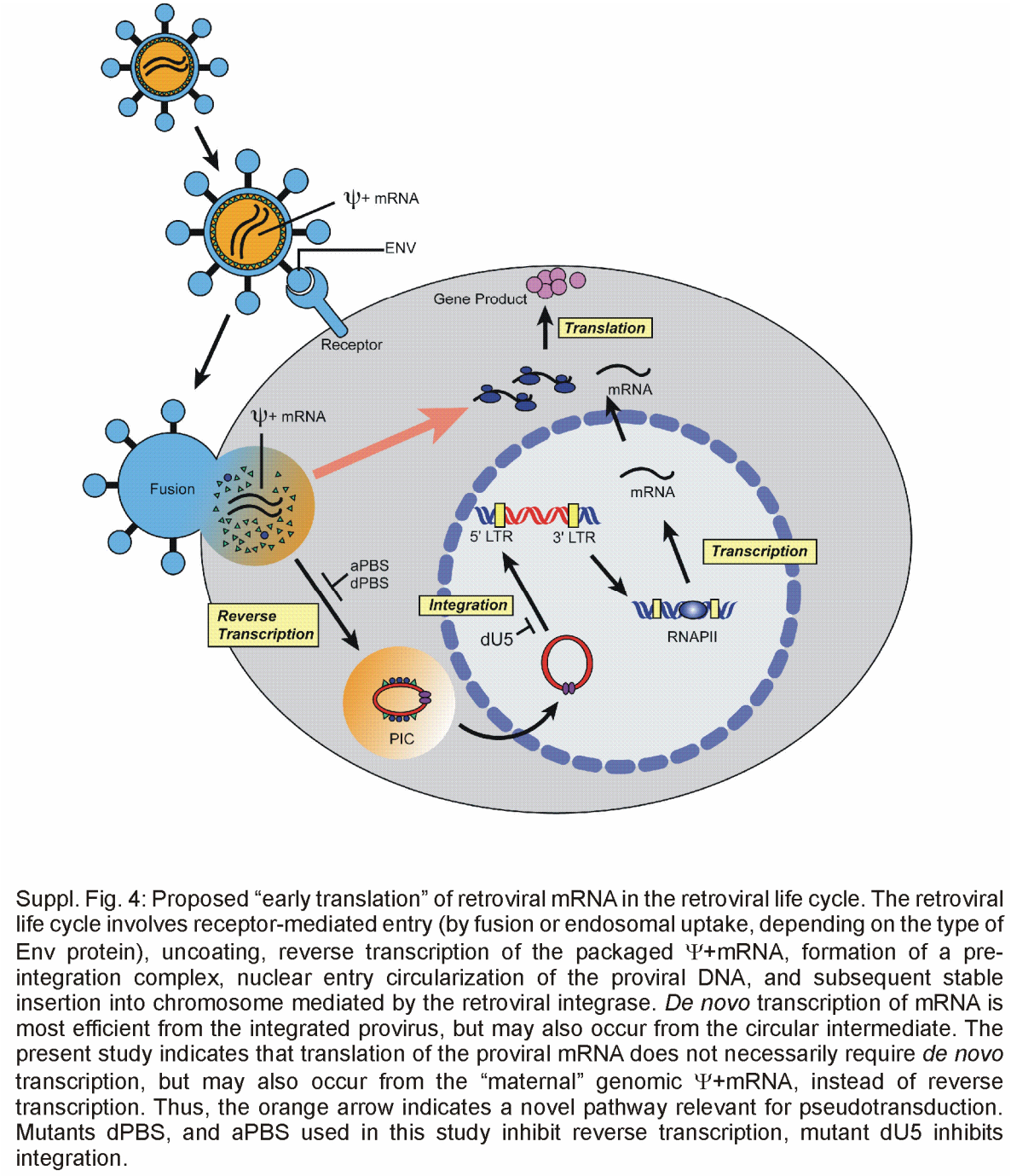
Suppl. Fig. 2: Pseudotransduction of Cre is superior to adenoviral or physicochemical DNA delivery. Sc-1 reporter cells were treated side by side with different concentrations of retrovirus particles containing SF91-nlsCre (triangle), aPBS-nlsCre (black square), Adenovirus encoding Cre (white sqare) or a Lipofectamine/pCMVnlsCre complex (red circle). Treated cells were analyzed over a period of 14 days with respect to (A) efficacy (%EGFP+ cells) and (B) toxicity (% dead cells). Shown are representative results of at least two independent experiments with initially 50%–70% EGFP+ cells. After 14 days, the yield of Cre-manipulated (EGFP+) cells was highest after pseudotransduction with aPBS-nlsCre, whereas stable retroviral and transient adenoviral transduction of Cre led to counterselection of manipulated cells (A). In all experiments, toxicity of Lipofectamine was very high, even when transfection was performed in the presence of serum, or a non-Cre plasmid was used (B and data not shown). Thus, total cell yield was also highest (comparable to untreated cells) when using pseudotransduction. Error bars represent variations between duplicates in one representative experiment.

Supplementary figure 3



Suppl. Fig. 3: The nucleoside analog 3´-Azido-3´-deoxythymidine (AZT) does not inhibit retroviral pseudotransfer, while strongly inhibiting stable retroviral gene transfer. Sc-1 cells were transduced with wild-type or reverse transcription deficient retroviral particles in the presence or absence of AZT (Zidovudine, Sigma). 5 x 10^4 Sc-1 reporter cells were plated per well in a 24-well-plate the night before transduction. Retroviral supernatants were diluted with DMEM/10%FCS/20mM HEPES containing different concentrations of AZT as indicated and applied to the cells. (Pseudo)transduction was assisted by adding 4 µg/ml protamine sulfate and centrifugation for 60 min at 400 x g and 25°C–32°C. After an incubation time of 5–12 hours retroviral supernatants were replaced by DMEM/10%FCS supplemented with AZT. The percentage of EGFP+ cells was analyzed by flow cytometry 3 days posttransduction. As AZT does not inhibit Cre transfer by SF91-nlsCre in more than 50% of target cells, pseudotransfer must also occur with otherwise intact retroviral vectors. The gate detecting EGFP expression mediated by SF91-EGFP was set such that pseudotransduction of EGFP was excluded.

Supplementary figure 4



Suppl. Fig. 4: Proposed "early translation" of retroviral mRNA in the retroviral life cycle. The retroviral life cycle involves receptor-mediated entry (by fusion or endosomal uptake, depending on the type of Env protein), uncoating, reverse transcription of the packaged Ψ+mRNA, formation of a pre-integration complex, nuclear entry circularization of the proviral DNA, and subsequent stable insertion into chromosome mediated by the retroviral integrase. *De novo* transcription of mRNA is most efficient from the integrated provirus, but may also occur from the circular intermediate. The present study indicates that translation of the proviral mRNA does not necessarily require *de novo* transcription, but may also occur from the "maternal" genomic Ψ+mRNA, instead of reverse transcription. Thus, the orange arrow indicates a novel pathway relevant for pseudotransduction. Mutants dPBS, and aPBS used in this study inhibit reverse transcription, mutant dU5 inhibits integration.

D. Publication 2

Cellular Restriction of Retrovirus Particle-Mediated mRNA Transfer

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Author's contribution statement:
Melanie Galla acquired and analyzed the data for Figs. 1-6 and partially for Table 1. Furthermore, MG constructed the SF91aPBS.GFP.pre vector (Fig. 1), introduced the Zeocin resistance gene cassette in the $Fv1^n$ encoding gammaretroviral vector provided by G. Towers, generated the $Fv1^n$ expressing human Cre indicator cell line (Figs. 5 and 6C) and shRNAs expressing SC-1 cells (Fig. 3), designed the figures and participated in writing the paper. Axel Schambach designed RRL.PPT.SF.GFP.pre (Fig. 1) and lentiviral constructs encoding shControl or shGFP (Fig. 3). In addition, AS participated in generating the data of Table 1, helped in formatting the figures and in writing the paper. Greg Towers provided the N- and B-tropic *gag/pol* expression constructs, donated the gammaretroviral vector encoding the $Fv1^n$ gene, critically evaluated the data and participated in writing the paper. Christopher Baum is the corresponding author, developed the overall concept of the study, supervised all experiments and participated in writing the paper.

Cellular Restriction of Retrovirus Particle-Mediated mRNA Transfer

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a. Abstract

Analyzing cellular restriction mechanisms provides insight into viral replication strategies, identifies targets for antiviral drug design, and is crucial for the development of novel tools for experimental or therapeutic delivery of genetic information. We have previously shown that retroviral vector mutants that are unable to initiate reverse transcription mediate a transient expression of any sequence which replaces the *gag-pol* transcription unit, a process we call retrovirus particle-mediated mRNA transfer (RMT). Here, we further examined the mechanism of RMT by testing its sensitivity to cellular restriction factors and shRNAs. We found that both human TRIM5α and, to a lesser extent *Fv1*, effectively restrict RMT if the RNA is delivered by a restriction-sensitive capsid. While TRIM5α restriction of RMT led to reduced levels of retroviral mRNA in target cells, restriction by *Fv1* did not. Treatment with the proteasome inhibitor MG132 partially relieved TRIM5α-mediated restriction of RMT but not TRIM5α-mediated restriction of wild-type integrating vectors. Finally, cells expressing shRNAs specifically targeting the retroviral mRNA, inhibited RMT particles, but not reverse-transcribing particles. Retroviral mRNA may thus serve as a translation template if not used as a template for reverse transcription. Our data imply that retroviral nucleic acids become accessible to host factors, including ribosomes, as a result of particle remodeling during cytoplasmic trafficking.

b. Introduction

Retroviruses enter cells in a receptor-mediated manner, following which a reverse transcription (RT) complex is formed in the cytoplasm to reverse transcribe the genomic mRNA into double-stranded DNA. During the completion of RT, a hybrid virus-cellular nucleoprotein structure known as the preintegration complex (PIC) is formed. Eventually, active transport of the PIC into the nucleus or dissolution of the nuclear membrane during mitosis allows the viral integrase to integrate the viral double-stranded DNA into chromosomal cellular DNA (35). We have previously shown that retroviral vector mutants that are unable to initiate RT of their capped, plus-stranded mRNA genomes mediate a transient expression of the sequences cloned into the *gag-pol* equivalent position of the vector genome (8). Particles conferring this activity required the presence of the retroviral mRNA packaging signal within the vector sequence as well as the expression of both Gag and Env in the vector packaging cell, but not reverse transcriptase. We refer to this previously unexplored aspect of the retroviral life cycle as retrovirus particle-mediated mRNA transfer (RMT). Using replication-defective retroviral vectors in which the gene of interest is cloned in the position of the *gag* reading frame, RMT can be exploited as a novel approach for the transient expression of a gene of interest (8).

The analysis of cellular restriction factors that belong to the innate immune response against retroviruses may provide further insights into the mechanisms of RMT. The cellular restriction factor *Fv1* (Friend virus susceptibility factor 1) has been shown to impact on the sensitivity of mice to murine leukemia viruses (MLV) (17). Further studies identified two major alleles of *Fv1*. Whereas the $Fv1^n$ allele confers resistance to B-tropic MLV (B-MLV), but not N-tropic (N-MLV), infection in NIH mice, $Fv1^b$ renders BALB/c mice resistant to N-MLV but susceptible to B-MLV infection (3, 9). The differences in N- and B-MLV infectivity are due to a single amino acid residue at position 110 (arginine and glutamic acid, respectively) in the retroviral capsid. The exact mechanism of action of *Fv1* remains to be elucidated.

Another retroviral restriction factor is the cytoplasmic body component TRIM5α, a member of the tripartite motif family of proteins (TRIM) (10, 14, 24, 33, 40). As a defining feature of TRIM proteins, TRIM5α harbors an RBCC motif, which consists of a RING (“Really Interesting New Gene”) domain at the N-terminus followed by a B-box-2 domain and a coiled-coil domain (25). The C-terminal domain of TRIM5α is a B30.2 or PRY/SPRY domain, whose amino acid sequence confers the specificity of

retroviral restriction (28, 32, 34, 41). Whereas rhesus monkey TRIM5α has the ability to restrict human immunodeficiency virus type 1, human TRIM5α (huTRIM5α) restricts N-MLV, but not B-MLV infection (40). Interestingly, as for *Fv1*, the same amino acid residue at position 110 within the retroviral capsid controls susceptibility to TRIM5α restriction (24, 36). However, although *Fv1* and huTRIM5α seem to interact with the retroviral capsid at an early postentry step, the mechanism of restriction appears to differ. huTRIM5α usually acts before RT, whereas *Fv1* allows RT, but blocks subsequent steps including integration into the host genome (2, 7, 12, 21, 36, 37).

In the present study, we examined the sensitivity of RMT to cellular restriction factors and short hairpin RNA (shRNA). We found that RMT is sensitive to restriction by both huTRIM5α and *Fv1*. The restriction of RMT by huTRIM5α could be partially relieved by the proteasome inhibitor MG132. Interestingly, the restriction of RMT by huTRIM5α, but not by *Fv1*, correlated with the degradation of the retroviral genomic RNA in the cytoplasm of infected cells. shRNAs specifically targeting the retroviral genomic RNA inhibited RMT but did not interfere with reverse-transcribing particles. These observations shed new light on the cytoplasmic fate of nucleic acids contained in retroviral particles.

c. Results

Kinetics of RMT vectors in comparison with those of non-integrating lentiviral episomes

As a first step to elucidate the mechanisms underlying RMT, we compared the kinetics of gene expression after RMT, the delivery of episomal lentiviral (eLV) DNA or transduction with integrating gammaretroviral (iRV) or integrating lentiviral (iLV) vectors encoding enhanced green fluorescent protein (EGFP). All vectors contained the woodchuck hepatits virus post-transcriptional regulatory element (wPRE) for the optimization of titers and RNA processing (42). The RMT vector SF91aPBS.GFP.pre encodes EGFP downstream of the splice acceptor sequence (Fig. 1A). RT was blocked by the presence of an artificial primer binding site (aPBS) that does not correspond to any cellular tRNA (18). RT is therefore possible only if the corresponding tRNA is cotransfected into packaging cells (8, 18). In the present study, we used a vector with a PBS for the $tRNA^{Gln}$ as the integration-competent control (SF91.GFP.pre). We also produced a third-generation self-inactivating

lentiviral vector (RRL.PPT.SF.GFP.pre) expressing EGFP under the control of the strong enhancer-promoter derived from the long terminal repeat of the MLV spleen focus-forming virus (SFFV). Additionally, integration-defective lentiviral particles, competent to form episomal DNA by using an integrase-deficient variant of the lentiviral *gag-pol* plasmid (integrase D64V) were produced (20, 26).

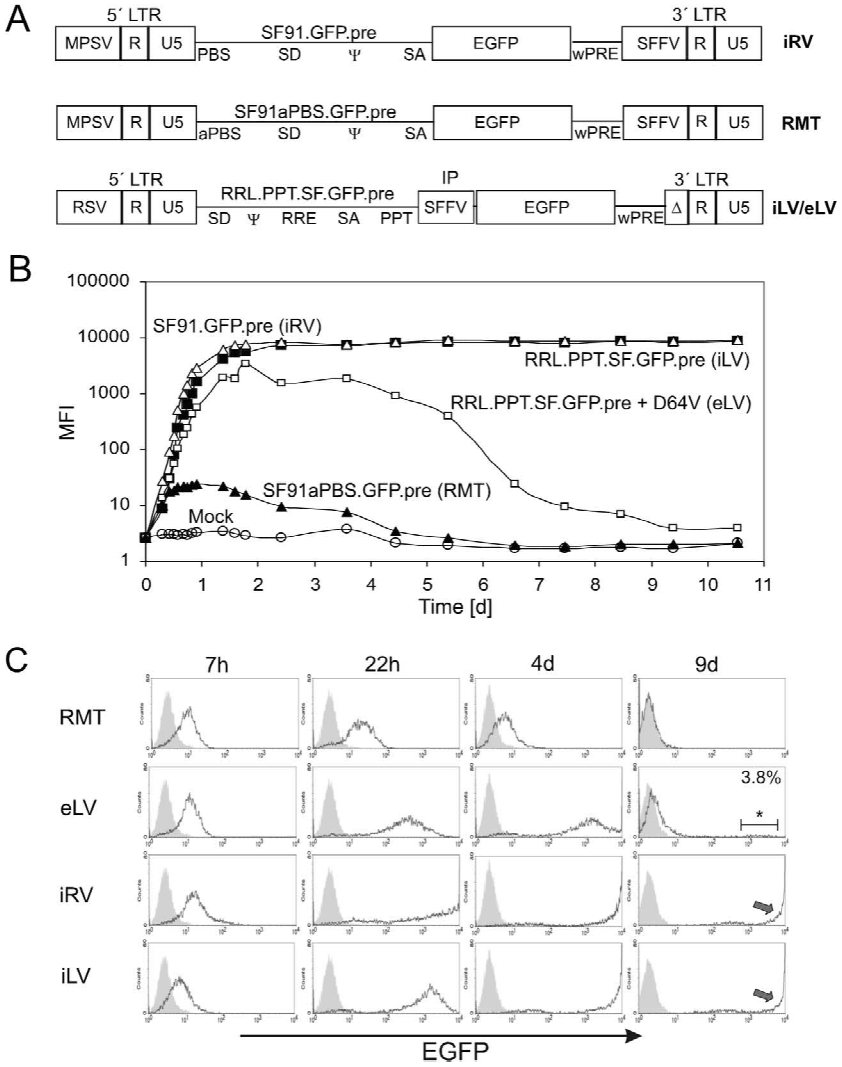

Fig. 1: Comparison of RMT using integrase-deficient lentiviral particles (eLV) and integrating gammaretroviral (iRV) and lentiviral particles (iLV). (A) Gammaretroviral vectors SF91.GFP.pre and SF91aPBS.GFP.pre with long terminal repeats (U3, R, U5), a functional PBS or an aPBS, splice donor (SD), packaging signal (Ψ), splice acceptor (SA), EGFP, and the post-transcriptional regulatory element of woodchuck hepatitis virus (wPRE). The plasmid´s 5´ enhancer-promoter is from the myeloproliferative sarcoma virus (MPSV), and the 3' U3 region from spleen focus-forming virus (SFFV). The lentiviral self-inactivating vector RRL.PPT.SF.GFP.pre contains the Rev responsible element (RRE), the central polypurine tract (PPT), and SFFV as the internal promoter (IP). (B) Vectors were packaged into integration-competent (SF91.GFP.pre and RRL.PPT.SF.GFP.pre) or RT-deficient (SF91aPBS.GFP), as well as integrase-deficient (RRL.PPT.SF.GFP.pre+D64V) VSVg-pseudotyped particles. The mean fluorescence intensities (MFI) of transduced SC-1 fibroblasts were monitored by FACS, starting 7 h and terminating 10.5 days posttransduction. Mock-transduced cells were negative controls. (C) Histogram plot overlays of the results for the transduced cells described for panel B (dark gray lines). Transduction efficiencies are displayed in comparison to those of mock-transduced cells (light gray) at different time points (7 h, 22 h, 4 days, 9 days). Arrows point to cell populations transduced by integration-competent particles (iRV, iLV). The asterisk indicates putative residual integrations caused by eLV vectors.

In this set of experiments, all vector particles were pseudotyped with the glycoprotein from vesicular stomatitis virus (VSVg). Using a high multiplicity of infection (MOI), we transduced SC-1 fibroblasts with these four different vector preparations: SF91aPBS.GFP.pre for RMT, RRL.PPT.SF.GFP.pre+D64V for delivery of episomal lentiviral DNA, RRL.PPT.SF.GFP.pre packaged with intact lentiviral *gag-pol* for delivery of integrating lentiviral vectors, and SF91.GFP.pre for delivery of integrating gammaretroviral vectors. All integrating vectors were used at an MOI of 10. EGFP expression was monitored by flow cytometry at regular intervals, starting 7 h after transduction and ending after 10.5 days. Mock-transduced cells served as negative controls.

This side-by-side comparison of the kinetics of the expression of EGFP revealed that RMT particles, which are RT-deficient retrovirus mutants containing EGFP vector RNA (18), express EGFP for a relatively short duration and to a low level (Fig. 1B). The peak of EGFP expression was 1 order of magnitude above background fluorescence and occurred 24 h after transduction. The continuous decay to background levels until day 6.5 is consistent with the half-life of EGFP (6). There was no evidence for residual integration events following the use of aPBS vectors, consistent with earlier reports (8, 18). After transduction with the episomal or integration-competent vectors, the peak of expression occurred later (day 2) and reached much higher levels: 3 orders of magnitude above background with the integration-defective vector and saturating levels with the integrating vectors. While expression remained stable over the observation period with both integrating vectors, EGFP expressed from the integration-defective lentiviral vectors decayed within 8 days but did not return to background levels. Continued expression in more than 1% of the target cell population was suggestive of residual integration events, as previously described (Fig. 1C) (20). The residual integration of the D64V mutant may be circumvented by using a double or triple mutant at the DDE catalytic site.

These data show that two important features distinguish RMT from other forms of retroviral delivery of genetic information (episomal or integrated DNA): the relatively low levels of expression and the complete reversion to background levels.

Characterization of RMT particles and wild-type viral particles

The aPBS within RMT vectors was designed not to match any naturally occurring tRNA molecule (18); therefore, the retroviral genomic RNA is packaged without the

primer for initiation of RT. To address the possibility that these modifications could affect the biochemistry of this type of viral particle, we compared viral RNA content, reverse transcriptase activity, capsid (p30) load and biological titers of RMT versus wild-type (iRV) retroviral supernatants (Fig. 2; Table 1). For this analysis, we packaged SF91.nlsCre (iRV) and SF91aPBS.nlsCre vectors (RMT), which are similar to the GFP vectors described above (Fig. 1A) but harbor the nlsCre cDNA instead and do not contain the wPRE. For each vector type (iRV and RMT vector), we analyzed four different retroviral preparations (B- and N-tropic, VSVg, and ecotropic pseudotypes). After harvest, the supernatants were concentrated via ultracentrifugation and the obtained pellets resuspended in phosphate buffered saline for further analysis. The retroviral genomic RNA contents of the supernatants were determined via real-time PCR (Table 1). In all samples, DNA contamination were excluded. Table 1 shows a clear correlation between the biological titer of a retroviral supernatant and its retroviral genomic RNA content (R^2 = 0.99 for RMT supernatant and R^2 = 0.89 for iRV supernatant). In the case of RMT vector, the strict correlation of retroviral genomic RNA content and titer suggests that the biological activity is entirely mediated by packaged RNA, whereas in the case of iRV, subsequent steps of RT, integration and *de novo* transcription contribute to the biological activity.

Vector	type of vector	gag/pol	envelope	RNA content [fmol/mL]	RT activity [U/mL]	biological titer [10^7 t.U/mL]
SF91.nlsCre	iRV	B	VSVg	1.2	9.2	0.8
SF91.nlsCre	iRV	N	VSVg	1.0	7.8	0.9
SF91aPBS.nlsCre	RMT	B	VSVg	6.1	8.6	4.5
SF91aPBS.nlsCre	RMT	N	VSVg	8.3	9.8	5.4
SF91.nlsCre	iRV	B	eco	193.0	93.6	92.4
SF91.nlsCre	iRV	N	eco	291.0	94.6	85.5
SF91aPBS.nlsCre	RMT	B	eco	26.6	95.6	7.6
SF91aPBS.nlsCre	RMT	N	eco	199.0	90.2	48.3

TABLE 1: Characterization of RMT and iRV particles. The results for eight different virus preparations are shown. t.U.: transducing units.

Importantly, comparison of the same pseudotypes (ecotropic and VSVg), revealed that the reverse transcriptase activity (Table 1; Fig. 2) were similar for iRV and RMT particles but did not correlate as nicely as the retroviral genomic RNA content with the biological titer. Furthermore, Western blot analysis of the retroviral supernatants used for the experiments whose results are shown in Table 1 revealed comparable capsid (p30) and Gag precursor protein (p65) levels for RMT and iRV particles (Fig.

2). We conclude that RMT particles have a composition similar to that of iRV particles, a conclusion which is supported by the results of previous studies demonstrating that MLV particle assembly occurs independently of the packaged retroviral RNA (19, 16).

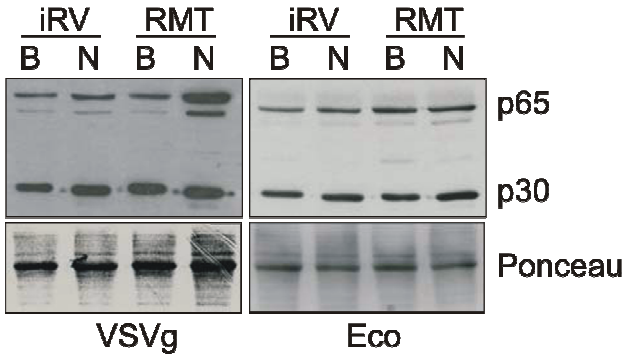

Fig. 2: Capsid Western blot of viral supernatants of iRV and RMT vectors. Denatured ecotropic (Eco) and VSVg-pseudotyped particle supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted, and retroviral capsid protein was detected with anti-capsid antibody and chemiluminescence. Sizes for p65 (Gag precursor) and p30 (processed CA) are given on the right. Ponceau stain served as loading control.

RMT is restricted by inhibitory RNA expressed in target cells

The above results and our previously published experiments provide indirect evidence for the mechanisms underlying RMT (8). Further supporting the hypothesis that genomic RNA containing the packaging signal mediates the biological activity of RMT, we found that RMT activity depends on the amount of unspliced RNA expressed in packaging cells, whereas high expression of spliced, subgenomic RNA did not contribute to RMT activity (data not shown). To directly address whether mRNA delivered by retroviral particles is the cause of RMT, we tested whether it is sensitive to inhibition by shRNA as a form of an engineered restriction. We mixed unmodified control cells (shRNA negative) with cells coexpressing shRNA (either shGFP, directed against EGFP sequences on the vector mRNA, or scrambled shControl) and DsRed fluorescent protein as a marker from the same lentiviral construct. DsRed expression therefore indicates cells expressing the shRNA. We transduced the mixed two cell populations (shRNA negative plus shGFP and shRNA negative plus shControl) using RT-proficient (iRV) and RT-deficient (RMT) EGFP encoding vectors and analyzed them 36 h posttransduction. shRNA directed against EGFP specifically and significantly (P<0.0001; n=9) inhibited the RMT-mediated

expression of this protein, whereas the expression of the scrambled control shRNA had no effect (Fig. 3).

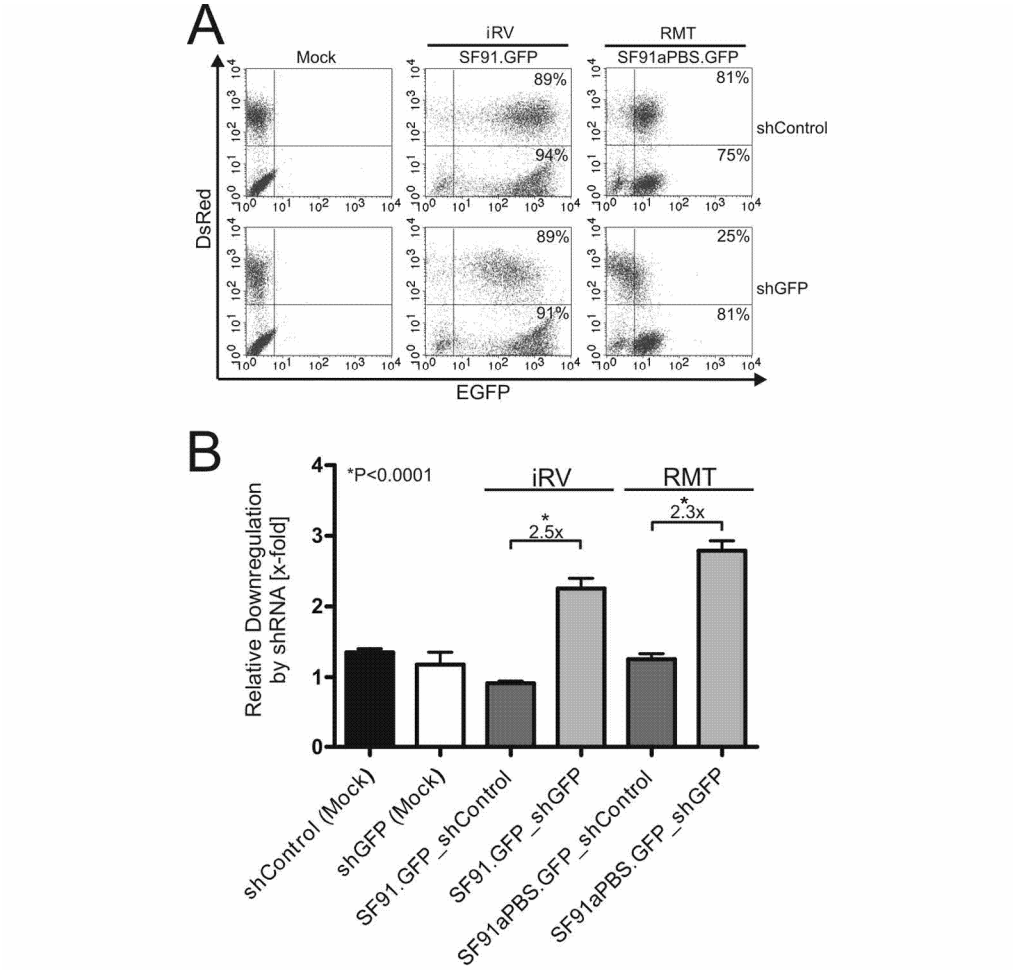

Fig. 3: RMT is susceptible to ectopically expressed shRNAs. (A) SC-1 mouse fibroblasts were engineered to stably express either an shRNA directed against EGFP (shGFP) or a scrambled shRNA (shControl), marked by coexpression of the DsRed fluorescent protein. Before transduction, shRNA-expressing cells (either shGFP or shControl) were mixed with control cells and transduced with either SF91.eGFP (iRV; RT-competent) or SF91aPBS.eGFP (RMT; RT-deficient) ecotropic particles. After 36 h, transduction efficiencies and mean fluorescence intensities (MFI) were determined via FACS. Percentages of EGFP-positive cells are shown for the DsRed-positive (shRNA expressing cells) and the DsRed-negative population (control cells not expressing shRNAs). (B) Same experimental setting as described for panel A, but the results of several experiments (n=9; P<0.0001) are displayed in the bar chart. To calculate the relative factor of downregulation by shRNAs, the MFI of cells not expressing shRNAs was divided by the MFI of cells expressing either shControl (dark gray) or shGFP (light gray). The black bar and the white bar indicate the background MFI levels of untransduced (mock) mixed cell populations: shControl and SC-1 or shGFP and SC-1, respectively. Error bars show standard errors of the mean.

In contrast, the transduction efficiency of iRV particles was not significantly altered by shRNAs targeting the retroviral RNA genome, although we found a clear reduction of *de novo* synthesized RNA in cells transduced with iRV (Fig. 3A, lower dot blot of iRV, compare upper right quadrant showing cells expressing the shRNA and lower right

quadrant showing unmodified cells). The DsRed-negative, shGFP-negative cell population served as an internal positive control and expressed high levels of EGFP after transduction. The degrees of shRNA-mediated inhibition of EGFP expression were similar for RMT and integrating vectors (~2.4-fold) (Fig. 3B). Together with data shown in Table 1 and our previous findings (8), these data imply that retroviral particles can deliver unspliced retroviral RNA containing a packaging signal into target cells for immediate ribosomal translation.

RMT is sensitive to TRIM5α

To consider the role of the retroviral particle in RMT, we analyzed sensitivity to cellular restriction factors targeting the capsid protein. If RMT is mediated by specifically packaged mRNA contained in retroviral particles, then those formed by the N-tropic MLV capsid should be sensitive to restriction by huTRIM5α. To address this question, we packaged RT-proficient (iRV) and RT-deficient (RMT) forms of the Cre vector into B-tropic or N-tropic VSVg-pseudotyped MLV virions and transduced our previously described human (HT1080) and mouse (SC-1) Cre reporter cells (38). To avoid saturation of huTRIM5α restriction, retroviral supernatants were used at MOIs lower than 1 (MOI 0.05 to 1). In permissive murine Cre reporter cells, the potencies of the two virus supernatants to express Cre were comparable (Fig. 4A). However, when transducing human Cre reporter cells (which endogenously express huTRIM5α), the N-tropic vector particles were strongly restricted, independently of their ability to reverse transcribe. Both N-tropic vector particles were inhibited by huTRIM5α. SF91aPBS.nlsCre (RMT) was inhibited by around fourfold (Fig. 4D and E) and SF91.nlsCre (which can initiate RT and form integrating retroviral DNA) by around 10-fold compared to the inhibition of their B-tropic counterparts (Fig. 4C and E). Figure 4F shows the potencies (Cre activity in mouse Cre reporter cells) of all retroviral supernatants used for the experiments whose results are illustrated in Fig. 4C to E. Together, these experiments reveal that RMT is sensitive to restriction by huTRIM5α and that the restriction occurs independently of RT.

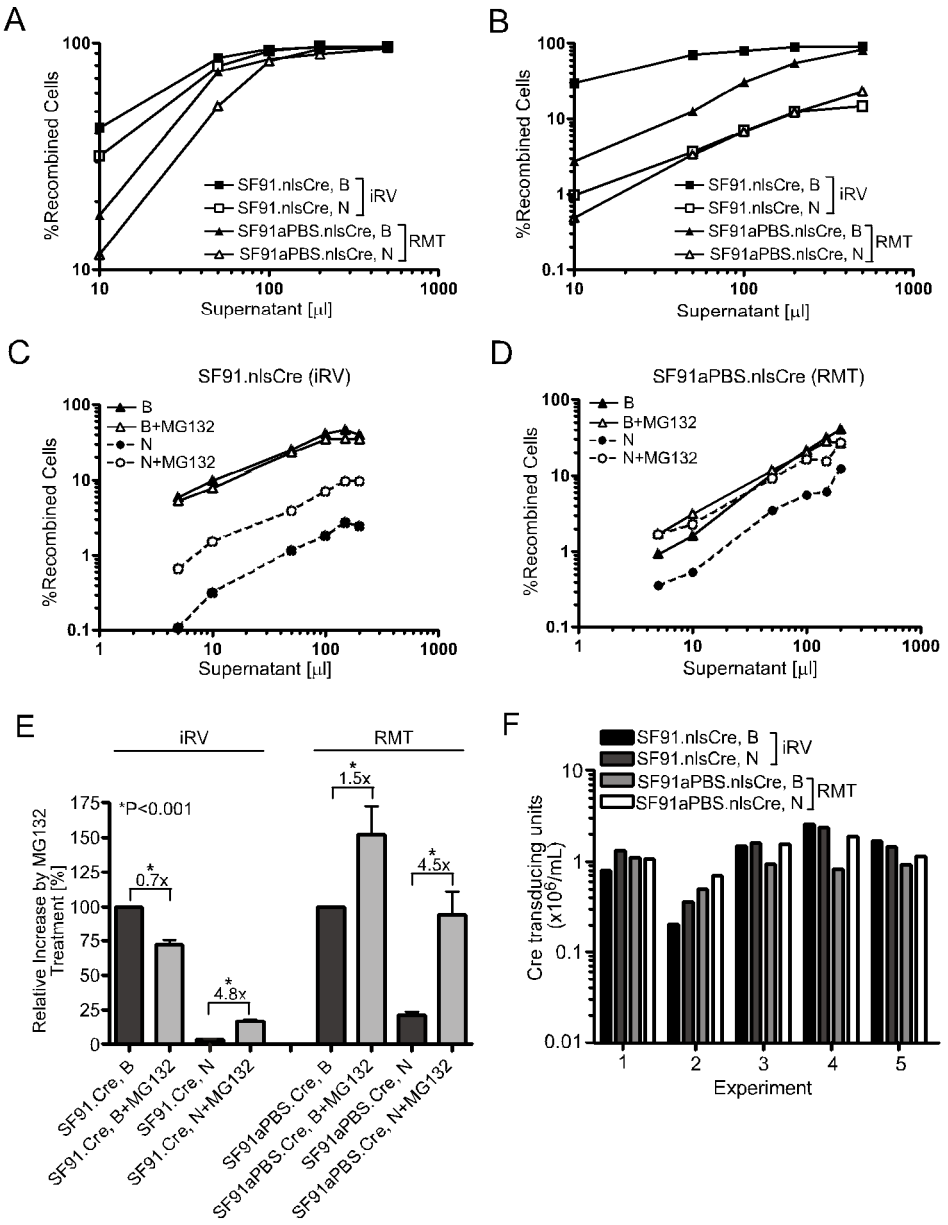

Fig. 4: RMT is susceptible to TRIM5α restriction but can be rescued by the inhibition of proteasomes. All experiments were performed with Cre reporter cells (38). (A) Comparable efficacies of all supernatants to recombine mouse Cre reporter cells. RT-competent (SF91.nlsCre) and RT-deficient (SF91aPBS.nlsCre) vectors encoding Cre were packaged into N- or B-tropic VSVg particles. (B) huTRIM5α restricts N-tropic particles. Supernatants tested in experiments whose results are shown in panel A were used for the infection of human Cre reporter cells. (C) Human Cre reporter cells were transduced with the indicated amounts of N- or B-tropic RT-competent supernatants (SF91.nlsCre) either in the presence or in the absence of MG132. The graph displays the recombination efficiencies from five independent experiments. All supernatants used in this experiment were checked for comparable Cre activity on mouse Cre reporter cells (Fig. 4F). (D) Experimental setup was as described for panel C, but RT-deficient particles were used (SF91aPBS.nlsCre). Absolute recombination efficiencies from five independent experiments are shown. (E) Relative display of the data sets shown in panels C and D. The proteasomal inhibitor MG132 efficiently antagonizes restriction of RMT by huTRIM5α. Human Cre reporter cells werte transduced with N- or B-tropic supernatants (RT-competent as well as RT-deficient) either in the presence (light gray bars) or in the absence of MG132 (dark gray bars). The bar chart reflects the relative increase of recombined cells from MG132 treatment (n=12; P<0.001) in relation to nonrestricted B-tropic particles. Error bars show standard errors of the mean. (F) Recombination efficacies of the supernatants used for the experiments whose results are shown in panels C to E. The bar chart displays the Cre-transducing units per ml of the indicated supernatants for five independent experiments, determined on permissive mouse Cre reporter cells.

We next addressed whether the restriction by huTRIM5α can be overcome by treatment with the proteasome inhibitor MG132. Since MG132 increases the efficiency of lentiviral infection in a cell-type dependent manner (27), we examined the influence of MG132 on restricted particles in comparison to its influence on non-restricted particles. To minimize unspecific toxicity, we used MG132 at a relatively low concentration (0.5 µmol/liter), which is at least four times lower than the concentration used in related studies on retroviral restriction (5, 29). Both the RT-proficient (iRV) (Fig. 4C and E) and RT-deficient (RMT) (Fig. 4D and E) N-tropic vector particles were partially rescued by MG132. Rescue occurred at all doses of virus tested, revealing that the doses used did not result in a saturation of either restriction or proteasomal degradation. Importantly, treatment with MG132 significantly increased the efficiency of Cre delivery by N-tropic particles, with similar values for RT-proficient and RT-deficient particles (4.8-fold and 4.5-fold, respectively) (Fig. 4E). This increase of infectivity mediated by MG132 was greater in the context of restricted particles (P<0.001; n=12) (Fig. 4E). MG132 only lead to a slight increase in the infectivity of unrestricted RT-deficient particles (1.5-fold), whereas it even reduced the infectivity of RT-proficient particles, possibly due to a residual cytotoxic effect. Nevertheless, proteasome inhibition did not allow a complete rescue of RMT following restriction by huTRIM5α.

The restriction factor *Fv1* also inhibits RMT

Previous work has demonstrated that restriction by the mouse *gag*-like restriction factor *Fv1* occurs after RT (13)**.** However, *Fv1* can compete with TRIM5α for restricted virus, suggesting that it interacts with the virion at the same time as TRIM5α, before significant RT has occurred (21). To address whether restriction by *Fv1* depends upon initiation of RT, we packaged RT-proficient (iRV) and RT-deficient (RMT) Cre vectors with the B-tropic *gag-pol* and transduced human Cre-reporter cells that were engineered to express $Fv1^n$. Interestingly, $Fv1^n$ clearly reduced RMT (mediated by SF91aPBS.nlsCre), although this restriction was less profound than that observed with the RT-proficient vector (SF91.nlsCre) (Fig. 5). We thus found that $Fv1^n$ partially inhibits RMT, a process which, as shown above, requires all retroviral proteins except reverse transcriptase and integrase. This reveals that restriction by $Fv1^n$ occurs irrespective of the initiation of RT, the formation of retroviral DNA and the subsequent maturation of the PIC.

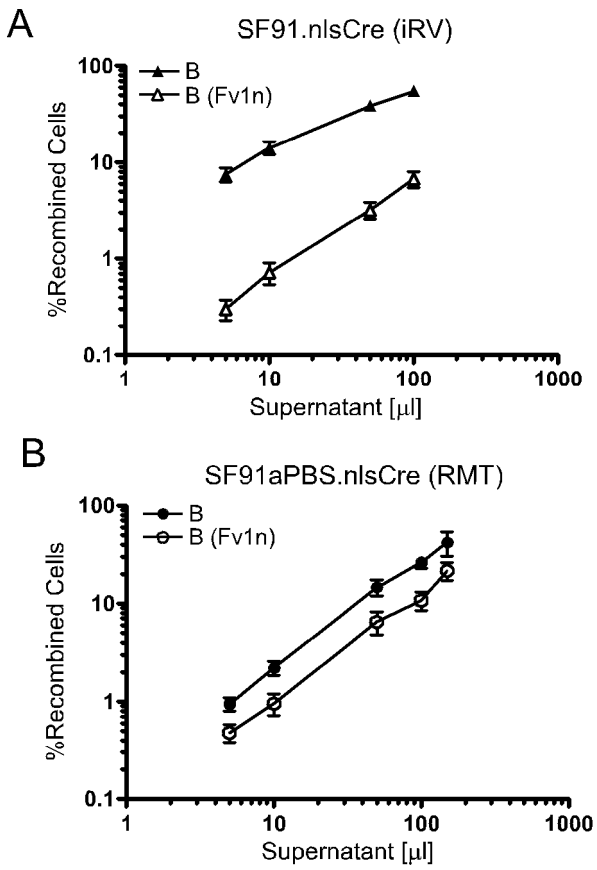

Fig. 5: RT-competent and RMT particles are sensitive to *Fv1* restriction. (A) Nonrestricting human Cre reporter cells ectopically expressing *Fv1*^{n} were transduced with increasing amounts of RT-competent (SF91.nlsCre), B-tropic particles. The mean values and standard errors of the mean of the results of six independent experiments are shown. (B) Same experimental setup as described for panel A, but RT-deficient (SF91aPBS.nlsCre) instead of RT-competent particles were used.

Restriction by TRIM5α, but not *Fv1*, is associated with reduced retroviral genomic RNA levels

Since RMT is mediated by packaged retroviral genomic RNA (Table 1; Fig. 3) and is restricted by TRIM5α (Fig. 4) and, to a lesser extent, by *Fv1*^{n} (Fig. 5), we wanted to know whether the restriction is associated with destruction of the retroviral genomic mRNA. To address this point, we transduced human Cre reporter cells endogenously expressing huTRIM5α with restricted (N-tropic) or nonrestricted (B-tropic) RMT particles, in the presence or absence of MG132. At 2, 4, 6 and 8 h postinfection, we harvested total RNA and performed quantitative real-time RT-PCR using primers targeting the retroviral genomic RNA. Real-time PCR analysis revealed that the restriction by TRIM5α was associated with the degradation of the retroviral genomic mRNA (Fig. 6A). Strikingly, 8 h posttransduction, the RNA levels of the restricted N-

tropic particles were 10.8 times lower than for the nonrestricted B-tropic particles. In contrast, we found no significant difference for N- and B-tropic particles in nonrestrictive mouse Cre reporter cells (data not shown). Interestingly, proteasome inhibition with MG132 allowed partial recovery of the retroviral genomic RNA (4.5-fold), suggesting that it is lost through recruitment to the proteasome by TRIM5α (Fig. 6A). The reduction of mRNA was predominantly detectable at the later time point (>2h), suggesting that degradation does not immediately follow particle uptake. Furthermore, we monitored, in the same cell populations, the fate of the retroviral capsid (p30) in restrictive and nonrestrictive cells (Fig. 6C) up to 8 h after transduction. Similar to the RNA data, we saw reduced capsid levels for the restricted N-tropic particles, which could be compensated by addition of MG132.

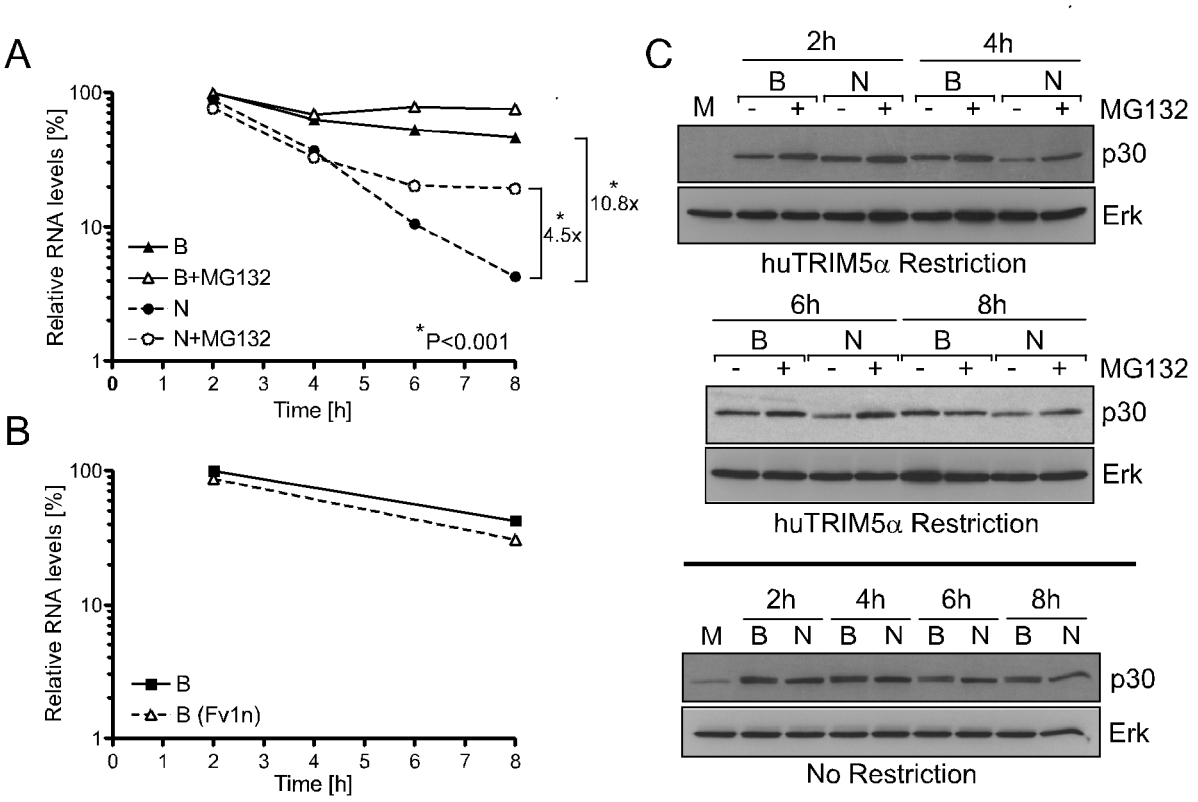

Fig. 6: Retroviral genomic RNA and capsid levels of restricted and nonrestricted particles from 2 to 8 h postinfection. (A) Human Cre reporter cells were transduced with N- or B-tropic RMT particles either in the presence or absence of the proteasomal inhibitor MG132. Genomic retroviral RNA levels were determined via real-time RT-PCR at the indicated time points (2, 4, 6, and 8 h postinfection) and normalized to the (nonrestricted) B-tropic value (2 h, no MG132, 100%). RNA preparations used in this experiment were treated three times with DNAse. (B) Nonrestrictive human Cre reporter cells or human Cre reporter cells ectopically expressing *Fv1^n* were transduced with B-tropic RMT particles. The genomic retroviral RNA levels at the indicated time points are shown. Quantitative real-time RT-PCR was performed as described for panel A. (C) Capsid (p30) levels of retroviral particles in human Cre reporter cells endogenously expressing huTRIM5α (upper panel) and in nonrestrictive mouse Cre reporter cells (lower panel). Western blot of protein samples harvested 2 to 8 h posttransduction is shown. Presence (+) or absence (-) of MG132 is indicated. Normalized to cellular Erk protein, densitometry revealed 50% reduction of N-tropic p30 at 4 h in the absence of MG132 and restoration of N-tropic p30 to the level of B-tropic p30 by the addition of MG132.

Restriction by *Fv1*^{n} did not alter the levels of retroviral genomic RNA (Fig. 6B). This is consistent with the observation that *Fv1*-restricted particles can still undergo RT but are blocked at a later step (13). Together, the results of these experiments reveal that RMT is mediated by retroviral particles and is thus dependent on the amount of mRNA made accessible to ribosomes in the target cells. Furthermore, these observations support the notion that *Fv1* and TRIM5α interact with the particle independently of initiation of RT and that restriction by TRIM5α leads possibly to proteasomal degradation of the particle.

d. Discussion

Our experiments have established that RT-deficient retroviral particles are able to make their genome accessible for translation in the unspliced *gag-pol* reading frame. Retroviruses are thus capable of RMT, resulting in low-level, transient expression of virally encoded gene products in transduced cells. RMT may occur if retroviral particles have not packaged a tRNA primer, if reverse transcriptase is mutated, or, as used in our experimental approach, if retroviral vectors are generated that are unable to bind the tRNA primer. Previously, we have demonstrated that retroviral RMT depends upon the presence of the packaging motif in the transduced mRNA, *gag*, and *env*, but not reverse transcriptase. The passive transfer of protein and the contamination of retrovirus-conditioned medium with plasmid DNA have been excluded as underlying this phenomenon (8). Here, we have demonstrated that the efficiency of RMT correlates with the expression of packaged retroviral mRNA rather than the amount of protein encoded in the *gag-pol* reading frame in viral producer cells (Table 1). Finally, the sensitivity to shRNA expressed in target cells (Fig. 3) clearly shows that the viral mRNA is responsible for RMT.

We went on to show that RMT is restricted by cytoplasmic restriction factors TRIM5α and *Fv1*, both of which are directed against the retroviral capsid. The side-by-side comparison of RT-deficient (i.e., RMT) with RT-competent integrating virus (iRV) reveals that TRIM5α restricts RMT particles to a lesser extent than iRV. This implies that RMT-competent virions are partially able to escape restriction and release their nucleic acids for translation. In other words, we hypothesize that the somewhat weaker restriction of RMT particles than of iRV particles by restriction factors targeting the capsid reflects the fact that iRV particles still have to complete a number of complicated steps in their life cycle (RT, formation of a PIC, and integration),

whereas RMT particles only have to release their mRNA for subsequent translation. Importantly, data obtained in functional assays of biological activity mediated by RMT correlated well with RNA levels determined by real-time PCR.

Furthermore, restriction of RMT particles could be rescued more efficiently than restriction of RT-proficient particles by inhibition of the proteasome with MG132, suggesting that the restriction of RMT is more dependent on the proteasome. The RNA data correlated with the capsid levels determined by Western blot analysis (Fig. 6).

We also found that RMT is sensitive to restriction by *Fv1* when delivered by the appropriately *Fv1* sensitive capsid. Again, RMT vectors were less sensitive to this form of restriction than reverse-transcribing vectors. Strikingly, we found that restriction by TRIM5α, but not by *Fv1*, leads to clear destruction of the viral RNA (Fig. 6). These data are consistent with recent observations that inhibition of the proteasome during restriction by TRIM5α rescues RT and support the notion that the RT block is due to destruction of the particle, and the RNA by the proteasome (1, 39). While the proteasome might not degrade the RNA directly, we imagine that degradation of the virion protein would render the genome sensitive to degradation by cellular nucleases. These observations are inconsistent with an uncoating mechanism for TRIM5α, which might be expected to increase the release of the genome and RMT (5, 22, 23). Finally, our use of retroviral vectors which cannot reverse transcribe due to modification of the PBS demonstrates that sensitivity to TRIM5α and proteasomal degradation of the mRNA do not depend on initiation of RT.

In summary, RMT suggests a potential evolutionary role of immediate early translation of retroviral nucleic acids. As shown here, this by-product of the retroviral life cycle can be exploited to study cytoplasmic restriction of retroviral particles, using both biological activity and biochemical parameters as readouts. We thus found that the sensitivity to TRIM5α does not depend on the initiation of RT and that degradation of RNA and capsid is correlated with restriction mediated by TRIM5α. Our data also support a hypothesis that, in nonrestrictive cells, retroviral nucleic acids become accessible to host factors, including ribosomes, as a result of particle remodeling during cytoplasmic trafficking. Particle modifications that trigger mRNA release after entry are thus expected to further increase the efficiency of RMT.

e. Materials and Methods

Retroviral vectors and plasmids

Gammaretroviral vectors termed SF91 were derived from SF91.GFP (11, 31). The vector SF91aPBS.GFP.pre was generated by introducing the artificial PBS as an *Xba*I/*Apa*I fragment from SF91aPBS.GFP (8, 15) in SF91.GFP.pre (31). The retroviral vector used for engineering human Cre reporter cells to express *Fv1^n* was derived from pCFCR (21). The red fluorescent protein of this construct was first excised by *Age*I and *Not*I, and the Zeocin resistance gene inserted as a blunt *Nco*I/*Sal*I fragment from pT/Zeo (kindly provided by Z. Ivics, Max-Delbrück-Center, Berlin, Germany).

The basic lentiviral construct pRRL.PPT.SF.GFPpre has been previously described (30) and is a derivative of pRRL.PPT.PGK.GFPpre (kindly provided by L. Naldini, Milano, Italy). For the construction of a lentiviral shRNA construct, an shRNA cassette consisting of an H1 (Pol. III) promoter and an shRNA coding sequence directed against EGFP were introduced into the 3' dU3 region using a previously introduced unique *SnaB*I site. The shRNA sequence was created using primer 5' GFP (5'-GATCCCCGCGGCAAGCTGACCCTGAAGTTCATTTCAAGAGAATGAACTT CAGGGTCAGCTTGCCGTTTTTGGAAA-3') and 3' GFP (5'-AGCTTTTCCAAAAACG GCAAGCTGACCCTGAAGTTCATTCTCTTGAAATGAACTTCAGGGTCAGCTTGCC GCGGG-3'), self-annealed and cloned as a *Bgl*II/*Hind*III fragment into pSuper (Oligoengine, Seattle, WA, USA). From there, the H1 promoter plus shRNA were cloned as a *Sma*I/*Hinc*II fragment into the *SnaB*I site of the lentiviral vector (see above).

To create integration-defective lentiviral vectors, an integrase-deficient *gag-pol* construct (pcDNA3.gpD64V.4xCTE) harboring a D64V point mutation in integrase was used (kindly provided by M. Milsom, Cincinnati Children's Research Foundation, Cincinnati, OH).

Gammaretroviral and lentiviral particle production

Gammaretroviral and lentiviral vector supernatants were produced in human 293T packaging cells using the calcium phosphate precipitation method (Calcium phosphate transfection kit, Sigma Aldrich, Munich, Germany), assisted by 25 µM chloroquine (Sigma Aldrich). The day before transfection, 5 x 10^6 293T cells were

seeded in a 10-cm dish. For gammaretrovirus production, the retroviral vector expression plasmid (5 µg) was cotransfected with expression plasmids for Moloney-MLV *gag-pol* (M57DAW, 15 µg) and either ecotropic (K73, 3 µg; kindly provided by T. Kitamura, Tokio, Japan) or VSVg (pMD.G, 2 µg) envelope. For the production of N- or B-tropic gammaretroviral particles, we used 5 µg of either pCIG3N or pCIG3B *gag-pol* expression plasmids (4). To ensure equal transfection efficiencies of gammaretroviral nlsCre vectors, 1 µg of the pEGFP-C1 expression plasmid (BD Clontech, Heidelberg, Germany) was cotransfected. For lentivirus particle production, 5 µg of the lentiviral vector expression plasmids were cotransfected with 12 µg lentiviral *gag-pol* (pcDNA3.gp.4xCTE), 5 µg Rev (RSV-Rev kindly provided by T. Hope, Chicago, Northwestern University, IL, USA) and 2 µg VSVg envelope expression plasmids. Supernatants were harvested 36 h, 48 h, and 60 h post-transfection, filtered through a 0.22-µm filter (Millipore, Schwalbach, Germany), and stored at -80°C until use. For comparison of RMT particles with episomal lentiviral particles (Fig. 1), supernatants were concentrated via ultracentrifugation as previously described (30).

Cell culture and transduction

293T, SC-1, HT1080, and previously described human (HT1080 derived) and mouse (SC-1 derived) Cre reporter cells (8, 38) were grown in Dulbecco´s modified Eagle´s medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum. Human Cre reporter cells ectopically expressing *Fv1*^{n} were cultured in the presence of 150 µg/ml Zeocin (Invitrogen, Karlsruhe, Germany). The day before transduction, 5 x 10^4 cells were seeded. Serial dilutions of retroviral or lentiviral supernatants were applied to the cells either in the presence or in the absence of 0.5 µM MG132 (Calbiochem, Bad Soden, Germany). The transduction procedure was assisted by protamine sulfate (4 µg/ml, Sigma Aldrich) and centrifugation for 60 min at 400 x g and 32°C. After 14 h of incubation, the virus-containing medium was replaced with fresh medium. The percentage of EGFP-positive or recombined cells was determined by flow cytometry (fluorescence-activated cell sorting [FACS]) analysis at the indicated time points.

Western blotting

Human or mouse Cre reporter cells were infected with B- or N-tropic nlsCre-encoding RMT particles (MOI of 1) either in the presence or absence of 0.5 µM MG132 (Calbiochem). Ninety minutes posttransduction, the supernatant-containing medium was removed, the cells were washed three times with phosphate-buffered saline, and fresh medium either with or without MG132 was added. At the indicated time points, cells were harvested and cell lysates prepared using proteinase inhibitors (Complete Mini, Roche, Mannheim, Germany) containing radioimmunoprecipitation assay buffer. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%), transferred to nitrocellulose membranes (Bio-Rad, Munich, Germany), and probed with goat anti-RLV p30 serum (final concentration, 2 µg/ml, kindly provided by S. K. Ruscetti, National Cancer Institute at Frederick [NCI-Frederick], Frederick, MD) in Tris-buffered saline with 0.05% Tween and 3% milk powder (TBST-3% dry milk). A donkey anti-goat horseradish peroxidase conjugate (Santa-Cruz, Heidelberg, Germany) diluted 1:2,000 in TBST/3% dry milk served as the secondary antibody. Detection was carried out by chemiluminescence (ECL, Pierce, Bonn, Germany). For the detection of Erk protein, membranes were incubated with polyclonal rabbit anti-Erk-2 (1:2,000 in TBST/3% dry milk; Santa-Cruz), followed by incubation with goat anti-rabbit horseradish peroxidase (Santa-Cruz) diluted 1:2,000 in TBST/3% dry milk.

Real-time RT-PCR quantification

On the day of transduction, 3 x 10^6 murine or human Cre reporter cells or human Cre-reporter cells ectopically expressing *Fv1*^{n} were infected by B- or N-tropic nlsCre-encoding retroviral particles (supernatants were adjusted to Cre activity determined on permissive cells) either in the presence or absence of 0.5 µM MG132 (Calbiochem). At 2, 4, 6 and 8 h posttransduction, cells were washed three times with phosphate-buffered saline and harvested, and their total RNA was prepared using the RNAzol extraction method (WAK Chemicals, Steinbach Germany). Before RT-PCR, RNA samples were treated two times with RNase-free TURBO DNase (Ambion, Dresden, Germany) and purified (Qiagen RNeasy Mini Kit) according to the manufacturer´s protocol (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed with Quanti-Tect RT Kit (Qiagen) using Oligo(dT) and random hexamer primers (MBI Fermentas, St.-Leon-Rot, Germany) in the same molecular ratio.

Quantitative PCR was performed with an Applied Biosystems 7300 Real-Time PCR system (Foster City, CA, USA) using a Quanti-Tect SYBR Green Kit (Qiagen). The amplification of the Cre DNA sequence was carried out by using oligonucleotides 5`-AACATTTGGGCCAGCTAAACA-3´ and 5´-AGAGCCTGTTTTGCACGTTCA-3´. The Cre-specific signal was normalized to the signal obtained by the amplification of mouse or human β-actin DNA with oligonucleotides 5`-CCTCCCTGGAGAAGAGCT-A-3´ and 5´-TCCATGCCCAGGAAGGAAG-3´. Results were quantified using the comparative threshold cycle method.

Characterization of RMT vector and wild-type retroviral supernatants

Retroviral SF91.nlsCre and SF91aPBS.nlsCre supernatants were produced, harvested and concentrated via ultracentrifugation (32). The obtained retrovirus pellets were resuspended in phosphate-buffered saline, aliquotted, and stored at -80ºC. For determination of the RNA content, concentrated supernatants were pretreated with RNase-free TURBO DNase (Ambion). Retroviral RNA was extracted with an RNeasy Micro Kit (Qiagen) according to the manufacturer´s protocol, including an additional DNAse treatment. First-strand cDNA synthesis and real-time PCR quantification were performed as described above. An *in vitro*-transcribed retroviral RNA derived from SF91.nlsCre served as the standard for the quantification of RNA. All samples were checked for plasmid DNA contamination. Western blot analysis for retroviral CA (p30) was performed as described above using denatured supernatants. The levels of reverse transcriptase activity of retroviral supernatants were determined using a RetroSys C-type RT activity kit (Innovagen, Lund, Sweden) according to manufacturer's instructions.

Statistical analysis

Data from the experiments are expressed as mean ± standard deviations. Student´s paired *t* test was used for the comparison of differences between indicated groups. A $P<0.05$ was considered significant.

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E. Publication 3

Overcoming Promoter Competition in Packaging Cells Improves Production of Self-Inactivating Retroviral Vectors

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Author's contribution statement:
Axel Schambach designed and cloned the monocistronic SIN vectors for the Sin, SCS, SRS and SERS series. Furthermore, AS acquired and analyzed the data for Figs. 1, 2, 4C & D and 5, designed the dual fluorescent vector series, generated the SRS11.SF.MGMT supernatant used for the experiment shown in Fig. 3 and participated in writing the paper. Daniela Müller cloned the dual fluorescent vector series, aquired additional northern blot and titer data (not shown), and performed the FACS analysis for the dual fluorescent vectors (Fig. 4B). Melanie Galla constructed RSF91.nlsCre and RSFaPBS.nlsCre, aquired and analyzed all data for Suppl. Fig. 1. Monique M. A. Verstegen and Gerald Wagemaker transduced primary rhesus CD34+ cells with SRS11.SF.MGMT and performed the FACS analysis of these cells (Fig. 3). Rainer Loew provided the tet-responsive element of vector Tet11.SF. Christopher Baum developed the overall concept of the study and participated in writing the paper. Jens Bohne participated in the northern blot analysis for Fig. 1B, analyzed the phosphoimager data for all shown northern blots, developed the concept of promoter interference in retroviral SIN vectors and participated in writing the paper.

Overcoming Promoter Competition in Packaging Cells Improves Production of Self-Inactivating Retroviral Vectors

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a. Abstract

Retroviral vectors with self-inactivating (SIN) long terminal repeats not only increase the autonomy of the internal promoter but may also, reduce the risk of insertional upregulation of neighboring alleles. However, gammaretroviral as opposed to lentiviral packaging systems produce suboptimal SIN vector titers, a major limitation for their clinical use. Northern blot data revealed that low SIN titers were associated with abundant transcription of internal rather than full-length transcripts in transfected packaging cells. When using the promoter of Rous sarcoma virus or a tetracycline-inducible promoter to generate full-length transcripts, we obtained a strong enhancement in titer (up to 4 x 10^7 transducing units per ml of unconcentrated supernatant). Dual fluorescence vectors and Northern blots revealed that promoter competition is a rate-limiting step of SIN vector production. SIN vector stocks pseudotyped with RD114 envelope protein had high transduction efficiency in human and non-human primate cells. This study introduces a new generation of efficient gammaretroviral SIN vectors as a platform for further optimizations of retroviral vector performance.

b. Introduction

Self-inactivating (SIN) retroviral vectors lack enhancer-promoter sequences in the U3 region of their long terminal repeats (LTRs) and use internal *cis*-regulatory sequences to initiate transcription of a gene of interest.[1] The SIN design has several important advantages: it reduces the risk of recombination to replication-competent retroviruses (RCR), impedes the mobilization of vector RNA in case of RCR superinfection, increases the autonomy of the internal promoter[1] and theoretically reduces the risk of insertional upregulation of neighboring alleles depending on the choice of the internal enhancer/promoter. These features can be achieved without compromising the potency of the integrated transgene allele. Although the deletion of enhancer sequences from the LTR impairs overall transcript levels and increases 3' read-through,[2] improved RNA processing of the internal transcript still allows the generation of SIN vectors that mediate comparable transgene expression levels as their LTR counterparts,[3] SIN vectors are thus of interest for a variety of applications in human gene therapy.

On the basis of foamyvirus (FV) or lentiviruses such as the human immunodeficiency virus (HIV), SIN vectors can be produced from transiently transfected packaging cells, without substantial loss of titers compared to constructs containing intact LTR sequences.[4,5] In contrast, the first generations of gammaretroviral SIN vectors based on murine leukemia virus (MLV) suffered from strongly reduced titers.[1] While MLV vectors cannot transduce non-dividing cells, they still represent important tools for human gene therapy, because they do not require the incorporation of any sequences overlapping with coding sequences of *gag*, *pol*, *env* or accessory genes,[6] in contrast to the most common forms of vectors based on HIV or FV.[4,5] In addition, MLV SIN vectors are likely to increase the safety of human gene therapy protocols when used under conditions where MLV-based LTR vectors already show therapeutic efficiency.[7-10]

Before the present study, the mechanisms responsible for the severe titer reduction of early generations of gammaretroviral SIN vectors were unclear. When we introduced the post-transcriptional element (PRE) from woodchuck hepatitis virus into the 3'-untranslated region of SIN vectors, we were able to increase infectious titers above 10^6 transducing units per ml of unconcentrated cell-free culture supernatant.[3,11] Owing to the mode of action of the PRE,[12,13] this suggested that insufficient 3' RNA processing of retroviral transcripts was partially responsible for

reduced SIN vector yields. However, SIN titers still remained substantially reduced in comparison with LTR-driven counterparts, representing a potential limitation for clinical use. These observations suggested at least three hypotheses: (1) the gammaretroviral packaging signal is bipartite and involves sequences overlapping with the enhancer-promoter of the U3 region; (2) the interaction of the Rev-responsive element (RRE) with Rev-protein generated in producer cells is responsible for the superior titer of lentiviral SIN vectors; or (3) promoter differences between gammaretroviral and lentiviral SIN vectors lead to a superior generation of full-length transcript in the lentiviral context.

In the present study, we addressed these hypotheses by incorporating modules derived from state-of-the-art lentiviral vector plasmids[14,15] into gammaretroviral constructs. As underlined by a novel dual fluorescence reporter assay, we found that promoter competition was the major limitation for the production of gammaretroviral SIN vectors. This could be overcome by using the promoter of Rous sarcoma virus (RSV) or a tetracycline-inducible promoter to drive expression of the full-length RNA. We demonstrate the potency of this new gammaretroviral design for highly efficient transduction of rhesus monkey CD34+ cells with SIN vectors encoding a clinically relevant selection marker.

c. Results

Gammaretroviral SIN vectors produce abundant internal transcripts in transfected packaging cells

In previous work, we have shown that gammaretroviral SIN vectors, are as potent as their lentiviral counterparts in terms of transgene expression.[16] Gammaretroviral SIN vectors, however, did not reach the same titers as their LTR-driven counterparts.[3,16] The use of the PRE from Woodchuck hepatitis virus restored SIN vector titers only partially.[3]

To address the underlying mechanisms, we initially compared three vector backbones, all containing the P140K mutant of methylguanine-methyltransferase (MGMT; Ragg *et al.*[17]) followed by the PRE (Figure 1A). We compared our standard gammaretroviral LTR vector SF91,[6,18] the gammaretroviral SIN vector Sin11.SF,[16] and two third-generation lentiviral vectors (Figure 1A) containing the central polypurine tract (cPPT).[14,15] Lentiviral vectors used the internal SF promoter[19] (as Sin11.SF) or the weaker phosphoglycerate kinase (PGK) promoter. Gammaretroviral

supernatants were produced in 293T cell-based Phoenix-gp packaging cells,[20] and lentiviral vectors in 293T cells.[15] All particles contained ecotropic Env proteins[21,22] to avoid re-infection and RNA production from integrated proviruses.[23]

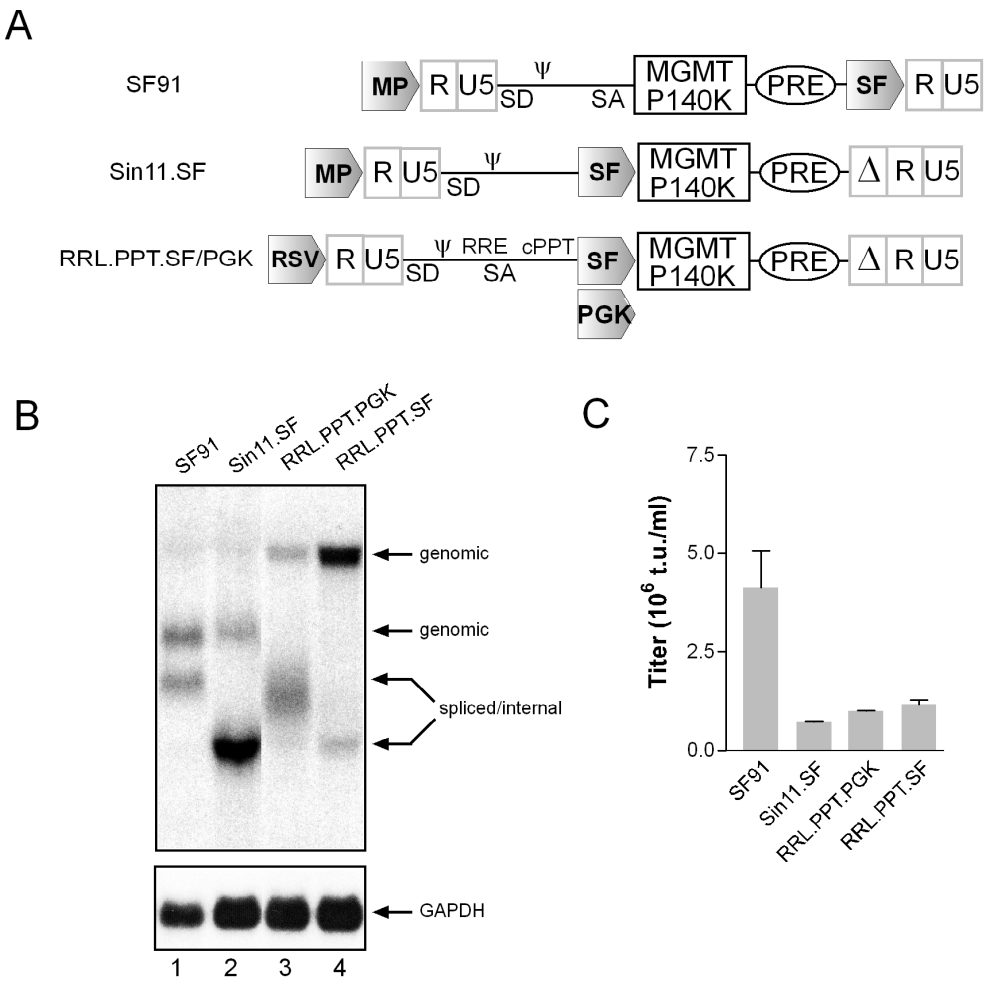

Fig. 1: Gammaretroviral SIN vectors show lower titers compared to LTR-driven counterparts. (A) Vectors used for comparison of gammaretro- and lentiviral backbones (plasmid configuration). pSF91 is an LTR-driven gammaretroviral vector. pSin11.SF is the corresponding SIN gammaretroviral vector (majority of U3 deleted in the 3'-LTR), with an internal SF promoter. pRRL.PPT.SF/PGK are the lentiviral counterparts, whith internal SF or PGK promoters. The U3 promoter-enhancer sequences are named after their virus origin. MP stands for myeloproliferative sarcoma virus (MPSV), SF for spleen focus-forming virus (SFFV), and RSV for Rous sarcoma virus. Within the leader regions, the primer binding site (not shown), the packaging signal (ψ), and splice donors and acceptors (SD, SA) are marked. In addition, the lentiviral vector RRL.PPT.SF/PGK contains 400 bp gag-sequences (not shown), RRE and cPPT.[15] All vectors carry the MGMT (methylguanine-methyltransferase) transgene and the PRE. (B) Northern blot analysis of 10 µg total RNA from Phoenix-gp (SF91, Sin11.SF) and 293T cells (RRL.PPT.SF/PGK). A PRE probe was used to detect all RNA species. The blot was reprobed with glyceraldehyde-3-phosphate dehydrogenase (GADPH) as a loading control. (C) The supernatants produced by the packaging cells were used to infect SC-1 murine fibroblasts in serial dilutions. Four days post-transduction MGMT-positive cells were analyzed by flow cytometry for intracellular FACS staining.[17] Titer is expressed as transducing units (t.u.) per ml unconcentrated supernatant. Values represent three independent transfection/ transduction procedures.

The retroviral LTR vector harboring a splice-competent leader region produced both spliced and unspliced RNAs (Figure 1B, lane 1), the latter containing the packaging signal. In total RNA, a 1:1 ratio of both transcripts was reproducibly observed.[3] In

contrast, Sin11.SF showed a predominant signal for the internal RNA produced from the internal promoter (Figure 1B, lane 2), in expense of the amount of genomic RNA available for packaging. As this correlated with the titer determined by stable transfer of the MGMT expression cassette into SC-1 fibroblast cells (Figure 1C), promoter competition was a likely explanation for the titer reduction of gammaretroviral SIN vectors. Surprisingly, although bearing the identical internal expression cassette, lentiviral vectors showed a much better ratio of genomic vs internal RNA (Figure 1B, lane 4). However, lentiviral titers were only slightly increased, possibly because we pseudotyped the particles with the ecotropic envelope (Figure 1C).

Use of the RSV promoter improves the production of SIN genomic RNA

The 5' region of the lentiviral backbone differs from the gammaretroviral with respect to several features: (i) R/U5, (ii) the cPPT, (iii) the RRE, and (iv) the RSV promoter driving expression of the genomic RNA. We incorporated the last three modules into Sin11.SF encoding enhanced green fluorescent (eGFP). We found that neither the cPPT nor the RRE significantly influenced the ratio of genomic vs internal RNAs (data not shown).

We then thus focused on modifications of the 5' promoter driving the expression of full-length RNA in the packaging cells. A new set of vectors was constructed (Figure 2A), to evaluate the extent of promoter competition with four different promoter configurations at the 5' end, and three internal promoters (Figure 2A). The titer produced in Phoenix-gp cells is shown in Figure 2B and C provides a direct comparison of the RNA from packaging cells analyzed by Northern blot. In the Sin11.SF context, the internal SF promoter gave the highest titer in comparison to the promoters derived from PGK or cytomegalovirus (CMV). This suggests that a strong internal promoter also activates the upstream enhancer (Sin11.CMV and Sin11.SF in Figure 2C, lane 4). Although CMV was the strongest promoter in the internal position (Figure 2C), its transfer to the 5' end (SCS vectors) did not increase titers (Figure 2B).

Using the RSV promoter to drive expression of the genomic RNA (SRS vectors) resulted in a substantial increase in titer (Figure 2C). Depending on the internal promoter, titers increased up to 40-fold (Figure 2B). This correlated with an increase in the total amount of genomic RNA (Figure 2C, lanes 8-10).

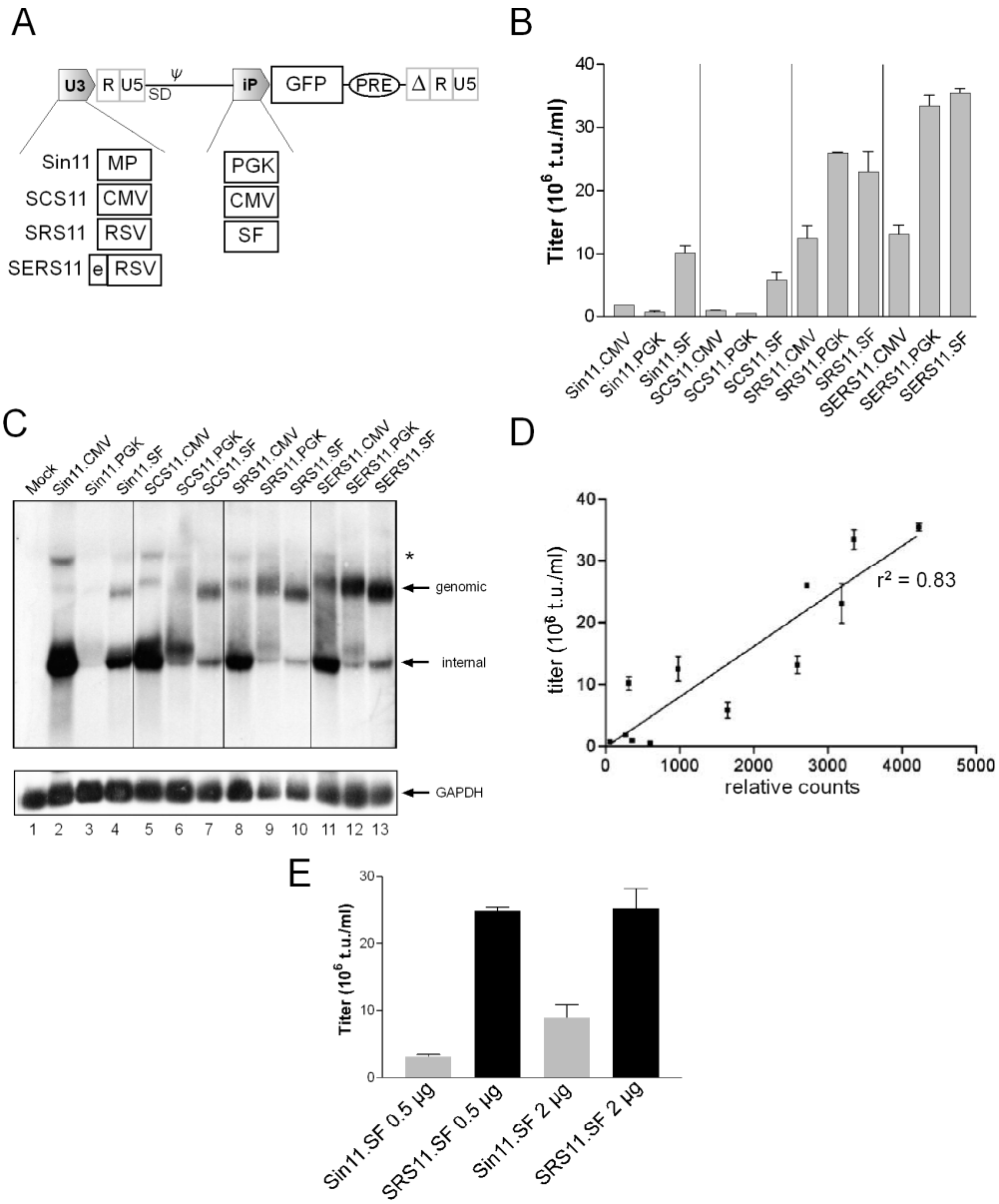

Fig. 2: Presence of the RSV promoter in the 5'-LTR leads to a substantial titer increase. (A) Schematic drawing of the SIN vector backbone (compare Fig. 1A), used to evaluate the different promoter elements. 5' modifications included four different promoter-enhancer elements (MPSV U3, CMV, RSV, RSV+SV40 enhancer). At the internal position (iP), three different promoters were analyzed driving expression of eGFP. (B) Titer analysis of the indicated constructs performed as in Fig. 1C. Note that the scale is different to Fig. 1C and that titers up to 4 x 10^7 t.u./ml are reached. Two micrograms of transfer vector were used per transfection. Error bars indicate the standard deviations from three independent experiments. (C) Northern blot analysis of 10 µg total RNA from Phoenix-gp packaging cells. The RNA species are named on the right side. The asterisk indicates a higher molecular weight band in case of the CMV promoter. This band might represent read-through and usage of a potential polyA signal in the bacterial plasmid backbone as indicated by sequence analysis (data not shown). A PRE probe as in Fig. 1 was used. The blot was reprobed with GAPDH. (D) Phosphoimager analysis. On the *x* axis, the radioactive signals in relative counts for the genomic RNA (normalized according to GAPDH levels) of the Northern blot analysis shown in Fig. 2C are given, and were plotted against the corresponding titers (*y* axis, compare Fig. 2B) as determined in triplicates. The correlation coefficient is shown within the graph. (E) Titer analysis of different amount of transfected transfer vectors.

In the context of the internal SF promoter, the ratio of genomic vs internal RNA became similar to that observed for lentiviral vectors (compare Figure 1B, lane 4 to Figure 2C, lane 10). Thus, lentiviral and gammaretroviral SIN vectors showed equivalent results when containing the upstream RSV promoter increasing the total amount of packageable genomic RNA.

To further strengthen the 5' promoter, we inserted the SV40 enhancer[24,25] upstream of the RSV promoter (SERS series, Figure 2A). This modification further increased the amount of genomic RNA (Figure 2C, lanes 11-13). Titers thus reached levels of 3-4 x 10^7 infectious units per ml unconcentrated cell-free supernatant (Figure 2B), concomitant with a further increased amount of genomic RNA (Figure 2C, lanes 11-13). To further investigate the correlation between the amount of genomic RNA and the increase in titer, we quantitated the Northern blot data by phosphoimager analysis (Figure 2D). We observed an almost linear correlation between the amount of genomic RNA and the resulting titer.

We thus reached the maximum titer that we achieved with LTR-driven vectors under our packaging conditions, indicating that even higher titers might be achievable when improving other components of the packaging systems. Of note, the vector modifications used to increase the retroviral titer leave the sequence of the integrated provirus unchanged.

We then lowered the amount of transfer vector from 2 µg to 0.5 µg, revealing greater differences in titer upon modification of the 5' end (Figure 2E). The upstream RSV promoter led to 10-fold enhancement in titer when the amount of transfer vector was limiting and only to a 2.5-fold increase when the transfer vector was provided in excess (Figure 2E). Furthermore, the threshold at which the amount of genomic RNA becomes limiting was reached with the SRS vectors at 0.5 µg, in contrast to the conventional SIN vectors (5 µg of transfer vector; Figure 2E and data not shown).

In addition, the use of the RSV promoter also showed beneficial effect in the LTR context as shown by retroviral pseudotransduction[26] (Supplementary Figure 1) and by integration-competent LTR vectors encoding eGFP, which showed a 3-fold titer increase (data not shown).

Performance of the new SIN vectors in primary hematopoietic cells

Using the SRS backbones, we designed efficient vectors expressing the clinically relevant selection marker MGMT transgene (Figure 3).[16] The substitution of the

MPSV (SIN vectors) for the RSV promoter (SRS vectors) led to a 3-fold relative increase in vector titers (determined on HT1080 cells, data now shown).

In order to test the performance of the vector supernatants on primary cells, we transduced rhesus CD34+ cells with RD114/TR pseudotypes using multiplicities of infection (MOIs) of 1, 5 and 10 (Figure 3). Using an MOI of 10, productive transduction of more than 90% of Rhesus CD34+ cells was obtained (Figure 3, one representative experiment is shown). Furthermore, we transduced human CD34+ cells with MGMT encoding vectors at an MOI of 1 resulting in 43% MGMT expressing human hematopoietic cells (data not shown).

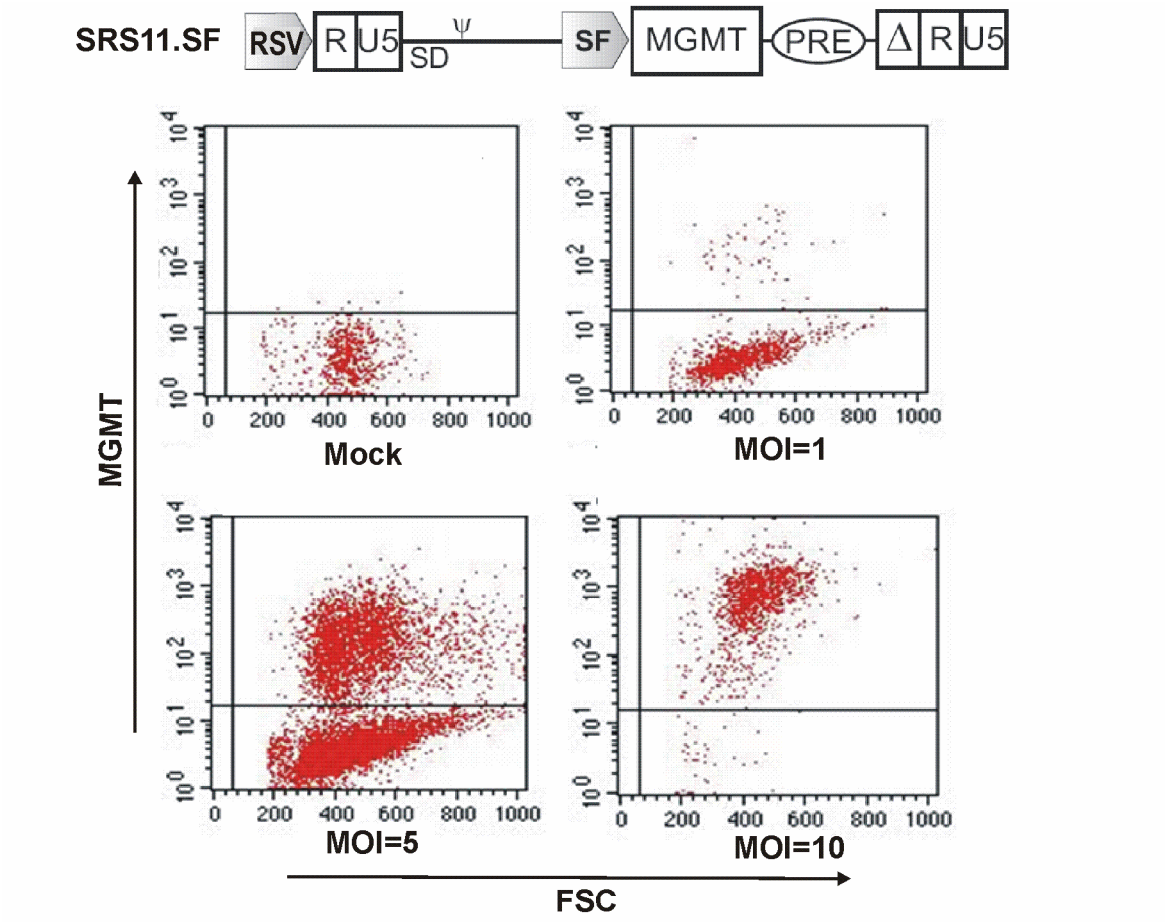

Fig. 3: Performance of the new SIN vector in primary rhesus CD34+ cells. To evaluate the efficiency of the new SIN vectors in a clinically relevant setting, the transgene was changed to MGMT (SRS11.SF.MGMT). Transduction of rhesus CD34+ cells with RD114/TR pseudotypes was shown in four independent experiments using intracellular FACS staining with a monoclonal MGMT antibody. One representative example with mock-transduced (upper left) or SRS11.SF-transduced cells using MOIs of 1, 5 and 10 (upper right, lower left and lower right, respectively) is displayed. A gate was set to separate MGMT-positive and -negative cells. The forward scatter (FSC) is given on the *x* axis, and MGMT fluorescence intensity on the *y* axis.

A dual fluorescent vector system suggests a Pol II occupation model for promoter competition

To address the mechanism of promoter competition, we developed a dual fluorescent vector by introducing the cDNA for the Discosoma red fluorescent protein Express (DsRed)[27] upstream of the internal promoter driving eGFP (Figure 4A). The amount of DsRed should correlate with the amount of genomic RNA, and eGFP should mirror the quantity of the internal RNA. Both fluorescent proteins allow fast and quantitative analysis in single cells owing to similar maturation kinetics (Clontechniques XVII: 3, 2002) as opposed to the Northern analysis that reflects the average RNA production in a cell population.

We started by comparing the basic retroviral SIN vectors with the RSV-modified vectors harboring two different internal promoters. Figure 4B shows a representative fluorescence-activated cell sorter (FACS) analysis of transfected Phoenix-gp cells. Standard SIN vectors showed an unfavorable ratio of green (internal RNA) vs red fluorescence (genomic transcript), as expressed by the quotient of the *y* vs *x* mean fluorescence intensity (Figure 4B, upper left panel). In contrast, the RSV modification increased this ratio six-fold; the dot plot analyses revealed that the effect of the RSV promoter was independent of the expression level (Figure 4B, upper right panel). These data are in line with titer determinations (Figure 4C) and RNA levels (Northern blot in Figure 4D, compare lanes 2, 3 to 6, 7). We observed a direct correlation between the amount of genomic RNA and increase in titer. However, for the SRS constructs, the amount of genomic RNA increased in case of the internal CMV promoter, but it led only to minor titer increase, probably because in this setting saturating levels were already reached (Figure 4D and C, lanes 6 and 7).

We next used the dual fluorescence vectors to study the mechanisms of promoter competition. The downstream promoter might be occupied by read-through transcription of the RNA polymerase II (pol II) transcription complex originating at the upstream promoter.[28,29] Besides, epigenetic promoter modifications could occur, which are probably more important following transgene integration.[30-32] If promoter occupation by RNA polymerase II is the relevant mechanism, inserting a transcriptional termination signal (polyA signal) 5' of the internal promoter should rescue its activity by reducing the probability of transcriptional read-through.[33] We thus cloned the bovine growth hormone (BGH) polyA in front of the internal promoter resulting in pSRS.Red.pA.SF or pSRS.Red.pA.CMV (Figure 4A). FACS analysis of the parental vectors confirmed our previous findings (Figure 4B, lower left panel).

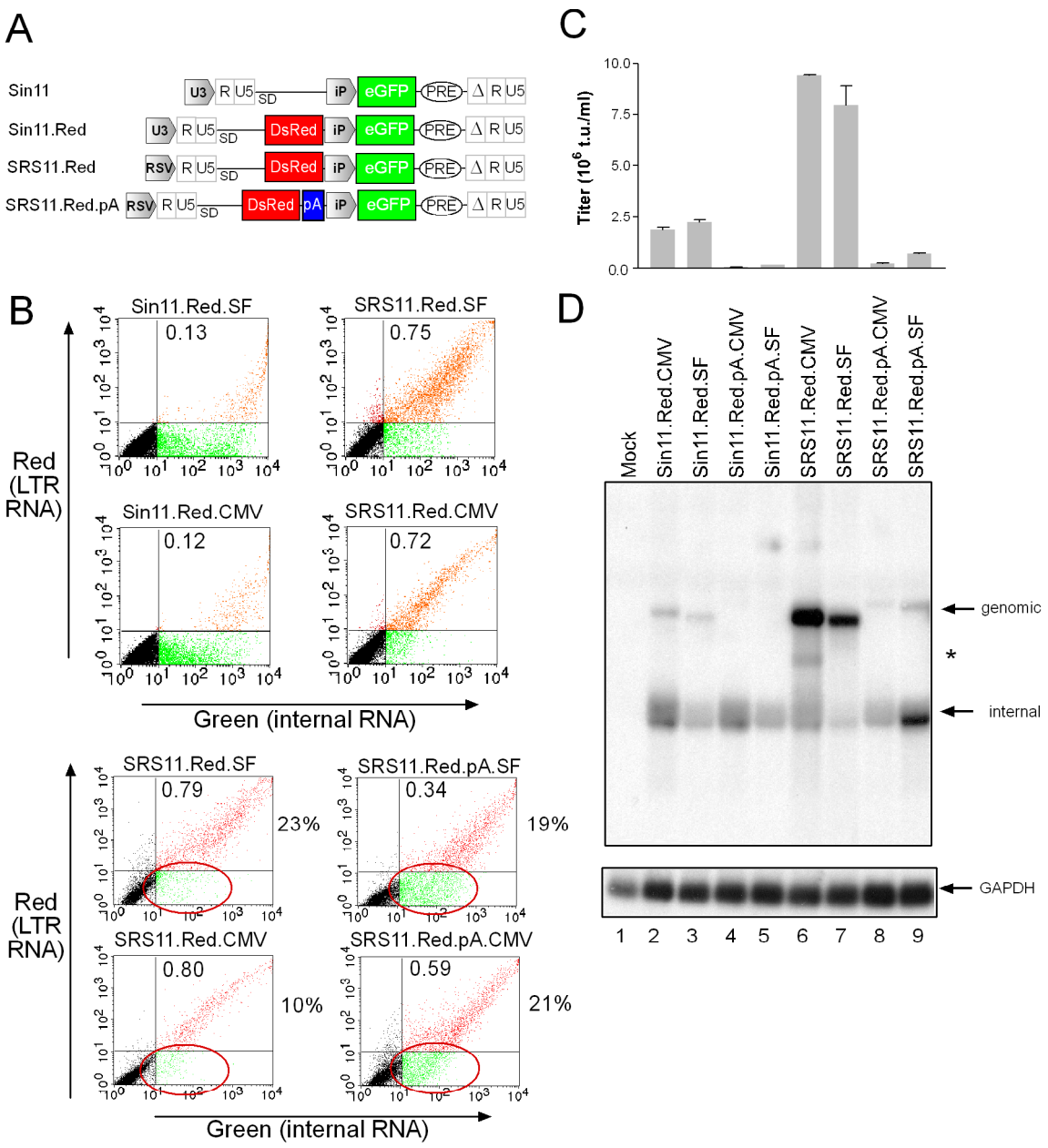

Fig. 4: Dual fluorescent vectors as a tool to analyze promoter competition. (A) Vectors encoding the DsRed Express cDNA on the genomic RNA and eGFP on the internal RNA, driven by the internal promoter (iP). The 5' MP promoter (U3) was substituted by the RSV promoter and an additional polyA signal, derived from BGH (pA), was added upstream of the internal promoter. (B) FACS analysis of transfected Phoenix-gp cells. Cells were analyzed 48 h post-transfection. The *y* axis represents DsRed fluorescence correlating with the genomic RNA and the *x* axis shows eGFP expression from the internal promoter. The values in the upper right quadrant give the quotient of *y* vs *x* mean. The circle in the middle panel marks cells that intensify their green fluorescence after insertion of the polyA. On the right side, the transfection efficiencies of this particular experiment are given. (C) Titer analysis of the indicated constructs. Standard deviations represent three independent experiments. (D) Northern blot analysis of 10 µg total RNA from transfected Phoenix-gp cells. A PRE probe was used to detect the genomic and the internal RNA. The short RNA initiating from the 5' LTR and terminating at the polyA can not be detected with this probe. The asterisk marks a cryptic splice event from an efficient splice donor within DsRed to a weak acceptor site within the CMV promoter as predicted by a splice site prediction program (http//www.fruitfly.org/seq_tools/splice.html).

The presence of the BGH polyA increased the eGFP signal (reflecting the internal transcript) as expressed by the ratio of mean fluorescence intensities (Figure 4B, lower right panel). The BGH polyA reduced vector titers strongly (Figure 4C and D), but not completely; this suggested that residual polymerase read-through did occur, especially when transcripts were driven by the RSV promoter (Figure 4D).

Taken together, these studies reveal promoter competition in SIN vector plasmids as a mechanism that potentially reduces the yield of genomic RNA. This can be overcome by the choice of a suitable 5' enhancer/promoter.

Tetracycline-inducible promoters also drive high levels of genomic vector transcripts for high titer SIN vector production

Tetracycline-inducible promoters (Tet) in combination with the respective trans-activators (TAs) are very potent promoter/enhancer combinations that mediate high and robust expression.[34] Tet-inducible promoters have already been successfully used in the LTRs of lentiviral vectors to create all-in-one vectors with tet-promoter and TA[35,36] and to drive the genomic RNA of lentiviral vectors in inducible packaging cells.[37] Therefore, it was tempting to test whether a Tet-inducible promoter incorporated into the retroviral 5' LTR (Figure 5A) is also capable of generating sufficient titers comparable to the SERS vector series (Figure 2). Potentially, this would allow the stable production of vectors with transgenes whose over-expression is toxic for producer cells. Furthermore, high titer virus production would be possible in cell lines where the RSV promoter is not active enough (in light of promoter competition). Figure 5B shows the results of a representative experiment. The Tet-inducible SIN vector Tet11.SF was transfected with or without the transactivator (TA) and set into comparison with the SERS11.SF vector (without TA). Titers of the Tet-inducible vector reached almost 2 x 10^7 transducing units per ml supernatant. Figure 5C shows the corresponding Northern blot of the packaging cell line. Interestingly, the amount of internal transcript also increased implying an interaction between the 5' and the internal promoter. The combination of the RSV promoter and SV40 enhancer still produced more genomic RNA (Figure 5C, lane 4), but this did not translate into titer, probably because gag/pol or env were limiting (experiment conducted with saturating plasmid amounts) (Figure 2D). In summary, this indicates that Tet-inducible promoters are useful for high-titer production of gammaretroviral SIN vectors.

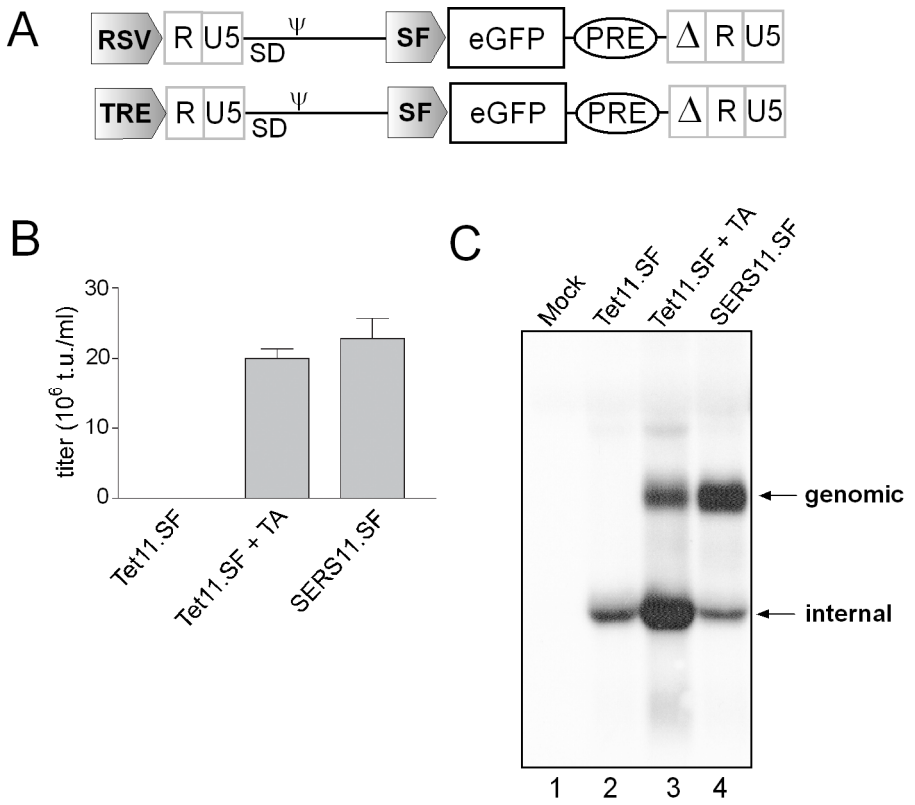

Fig. 5: A tetracycline-inducible promoter allows high-titer gammaretroviral SIN vector production. (A) Scheme of the vector backbone of Tet11.SF with the tet-responsive element (TRE) incorporated into the 5'-LTR U3 in comparison to the SRS vector (Fig. 2A). (B) Titers of the depicted constructs in t.u./ml as determined on SC-1 cells. For production of Tet11.SF, a transactivator plasmid (TA, 5 µg) was co-transfected. (C) Northern blot of 10 µg total RNA from Phoenix-gp packaging cells. RNA species are indicated on the right side. Loading was controlled via 28S rRNA comparison on an ethidium bromide agarose gel (data not shown).

d. Discussion

The present study was undertaken to overcome a major limitation of gammaretroviral SIN vectors, which is the loss of titer observed upon deletion of the 3' U3 region. Theoretically, all classes of SIN vectors are faced with the problem that a non-specific internal promoter will be active in the packaging cells and generate RNAs that do not contribute to the titer but rather reduce the amount of genomic RNA. However, the use of a strong internal promoter is often desirable in the target cell to reach a therapeutic threshold, as for the expression of metabolic selection markers,[38] genes antagonizing viral infections[39] or recombinant T-cell receptors.[40]

Our data obtained upon transient transfection in packaging cells revealed that insufficient production of full-length transcript from the 5' promoter is a major

limitation of gammaretroviral SIN vectors. As we found that currently used lentiviral vectors produced greater amounts of genomic RNA, we screened through different modules present in the lentiviral backbone (RRE, cPPT, 5' promoter). Interestingly, neither the RRE nor the cPPT were able to produce higher titers in the gammaretroviral background, not even in the presence of Rev (data not shown).

We rather find that high production of genomic RNA mainly depends on the choice of the 5' promoter. Both RSV and tetracycline-inducible promoters led to high SIN vector titers. Why these promoters lead mediate production of genomic RNA might be explained by studies with lentiviral vectors. One aim during construction of third-generation lentiviral vectors was to become Tat-independent during the production process.[15] The RSV promoter was found to perform this function in the so-called third generation lentiviral vectors and to be superior to CMV[15] as in our context (Figure 2). Also, Kafri *et al.* successfully generated lentiviral SIN vectors using a tetracycline-inducible promoter to drive the genomic transcript.[35] Our data obtained in the context of gammaretroviral vectors, which are Tat-independent *a priori*, implies that recruiting an elongation competent pol II complex like the Tat-dependent HIV LTR and not only the basal promoter strength is important to overrule the internal promoter. Accordingly, use of the even "stronger" CMV promoter to drive genomic retroviral RNA expression did not give rise to higher titers.

If the elongation rate of RNA pol II is important, the density of transcriptionally engaged polymerases on the internal promoter suppresses its activity.[28,41,42] Accordingly, we found that an efficient cellular termination signal placed upstream of the internal promoter activated the internal promoter, although residual read-through over the cellular polyA signal was still observed. Secondly, we enhanced transcriptional elongation by adding the 72 bp enhancer repeats from SV40 upstream of the RSV promoter,[24,43] which further increased the amount of genomic RNA and vector titers.

Competition between neighboring promoters could also occur at the level of enhancer interactions, with the stronger promoter attracting the enhancers of its neighbor. This might explain why the strong internal SFFV enhancer-promoter led to a higher SIN titer than the internal PGK promoter being the weakest of the promoters tested.[17] However, the SV40 enhancer modification together with the usage of the RSV promoter makes the vectors less dependent on interactions with the internal promoter (Figure 2C).

We conclude that promoter competition is a major hindrance for the production of gammaretroviral SIN vectors and that both enhancer competition and promoter occupation need to be addressed when attempting to improve SIN vector titers. Increasing the processivity of the 5' promoter enhanced vector titers by up to 40-fold, dependent on the cDNAs and internal promoters used. This enables the production of high titer supernatants using relatively low amounts of transfected plasmid, as required for efficient clinical-scale gene transfer. For a clinical study that explores the feasibility and safety of gene transfer into hematopoietic cells in adult patients, roughly 2 x 10^9 infectious particles would be required (5 x 10^6 CD34+ cells/kg, 70 kg body weight, two transductions with three infectious units per cell). When pseudotyping SRS and SERS vectors with human-infectious envelopes GALV and RD114, we reproducibly obtained titers of 1 x 10^7 infectious units per ml of unconcentrated cell culture supernatant (data not shown). Two hundred milliliters supernatant would thus be sufficient for the treatment of a single patient, and 2.4 liters for an entire phase I clinical trial including preclinical safety tests. Because 10^6 packaging cells yield about 1 ml supernatant per harvest, and at least three high titer harvests can be obtained following transient transfection, not more than 8 x 10^8 packaging cells would have to be transfected to obtain sufficient material for a phase I study. As 0.5 µg of SERS plasmid suffices for high titer transient production from 5 x 10^6 packaging cells (Figure 2E), less than 100 µg plasmid DNA would be required for clinical-grade vector production. This reduces the costs of GMP-grade plasmid production and lowers the risk of plasmid contamination of retroviral supernatants. Based on a better understanding of other limiting components of the vector production system, further improvements will likely be possible.

e. Materials and Methods

Plasmids

MGMT-encoding gammaretroviral vectors (pSF91, pSin11.SF) and lentiviral vectors (pRRL.PPT.PGK and pRRL.PPT.SF) have been previously described.[16] In brief, pSF91 encodes an LTR-driven vector,[6,18] and pSin11.SF a corresponding SIN vector using the same SFFVp U3 region (SF; including the enhancer; -342 to +18, relative to the transcriptional start site, GenBank no. AJ224005) as an internal promoter. pRRL.PPT.PGK and pRRL.PPT.SF are lentiviral SIN vectors with internal promoters

human PGK and SF, respectively. The basic lentiviral construct pRRL.PPT.PGK. eGFP.PRE was kindly provided by Luigi Naldini (Milano, Italy).

For a functional comparison of different promoters in the 5' LTR, we constructed a modular vector set using 4 different promoters in the 5' LTR in relation to three different internal promoters. As internal promoters we used CMV, PGK (GenBank no. M11958, nt. 5-516) or SF. The 4 versions of the 5' LTR were as follows:

Our former SIN series uses the MPSV U3 region to transcribe the full-length RNA in transfected packaging cells.[3] The SCS series represents SIN vectors containing the CMV promoter fused to the start site (+1) of the full-length RNA. SRS constructs are SIN vectors that use the RSV U3 fused to the start site of the full-length RNA. The SERS series consists of SIN vectors that use a combination of the SV40 enhancer[24,25] and the RSV U3 fused to the start site of the full-length RNA.

Modified 5' LTRs were cloned by overlap-polymerase chain reaction (PCRs). For the amplification of the CMV promoter (GenBank no. K03104, nt. -582 to -1, relative to transcriptional start site), primers 5'CMVafl (5'-CGATCTTAAGTAGTTATTAATAGT AATCAA-3') and 3'CMVR (5'-GTCAATCGGAGGACTGGCGCCGGTTCACTAAACCA GCTCTG-3'), 5'CMVR (5'-CAGAGCTGGTTTAGTGAACCGGCGCCAGTCCTCCGAT TGAC-3') and 3'Leaderbgl (5'-CCAGATACAGATCTAGTTAGCCAA-3') were used. PCR templates were pcDNA3 (Invitrogen, Karlsruhe, Germany) and pSF91,[6] respectively. The PCR fragment was cloned into pSin11SF using *Afl*II and *Bgl*II sites. For amplification of the RSV promoter (GenBank no. J02342, nt. -233 to -1, relative to transcriptional start site) primers 5'RSVscaafl (5'-GCTTAGTACTCTAGCTTA AGAATGTAGTCTTATGCAATACT-3') and 3'RSVRoverlap (5'-AGTCAATCGGAGGA CTGGCGCGTTTATTGTATCGAGCTAGGC-3'), 5'RSVRoverlap (5'-GCCTAGCTCG ATACAATAAACGCGCCAGTCCTCCGATTGACT-3') and 3'LeaderBgl (see above) were used. Templates for this overlap-PCR were pRSV-Rev (kindly provided by Tom Hope, Chicago, IL, USA) and pSF91, respectively. The PCR-fragment was cloned into the pSin11.SF using *Afl*II and *Bgl*II restriction sites. The SV40 enhancer (GenBank no. AF025845, nt. 18-252), which includes two 72 bp tandem repeats was amplified using primers 5'SV40enh (5'-CTACTTAAGACGCGTGGCCTGAAATAAC CTCTGAA-3') and 3'SV40enh (5'-GCTACTTAAGGGACTATGGTTGCTGACTA-3') and the pRL-SV40 plasmid (Promega, Mannheim, Germany) as a template. The PCR product was transferred into the *Afl*II site of pSRS11.SF (upstream of the RSV promoter). To clone a tetracycline-inducible promoter into the 5' LTR to drive the full length vector RNA, we amplified the promoter fragment via PCR using primers

5'Tet11afl (5'-GCTACTTAAGCTTCTTTCACTTTTCTCTGTCA-3') and 3'Rkpn (5'-GAGAACACGGGTACCCGGGC-3') and plasmid ptES_1-1(g)p. The tetracycline-inducible promoter consists of a tet-operator hexamer with 4C specificity fused to the Moloney murine leukemia virus (MMLV) minimal promoter. The resulting PCR fragment was cloned into the *Afl*II and *Kpn*I sites of the 5' LTR of pSRS11.SF. All PCR fragments were confirmed by sequencing.

To insert the DsRed Express (Clontech, Mountain View, CA,USA) cDNA and the BGH polyadenylation signal (polyA) downstream of the packaging signal and upstream of the internal promoter, a new multiple cloning site (MCS) was constructed, in which successively the DsRed Express sequence and the BGH polyA were included. The phosphorylated oligonucleotides 5'leaderMCS (5'-GCTGACGCG TACTAGCGCTGACTTCGAAGC-3') and 3'leaderMCS (5'-GGCCGCTTCGAAGTCA GCGCTAGTACGCGTCAGCTGCA-3') were annealed and ligated into the *Pst*I/*Not*I opened sites of the retroviral leader region of pMP71-CD34-2A-eGFP to introduce *Afl*II, *Eco47*III and *BstB*I restriction sites. The DsRed Express cDNA (Clontech) was PCR-amplified with primers 5'DsRedmlu (5'-GCCTACGCGTGTCGCCACCATGGCC TCCTCCGA-3') and 3'DsRedeco47III (5'-GTCTAGCGCTCTACAGGAACAGGTGG TGGC-3') and cloned into the respective sites of the leader MCS. The BGH polyA (232 bp, template pcDNA3, Invitrogen) was amplified via PCR with primers 5'BGHpolyAcla (5'-GCTAATCGATACTGTGCCTTCTAGTTGCCA-3') and 3' BGH polyAsal (5'-GCATGTCGACCATAGAGCCCACCGCATC-3'), digested with *Sal*I and *Cla*I, treated with Klenow polymerase and ligated into the *Eco47*III opened leader MCS.

Cell lines, transfections and transductions

Phoenix-gp packaging cells *(*kindly provided by G. Nolan, Stanford, CA, USA) and 293T cells were used for retroviral and lentiviral supernatant production, respectively. Phoenix-gp, 293T, HT1080 and murine fibroblast SC-1 cells were maintained in Dulbecco's modified Eagles medium (DMEM, Gibco, Gaithersburg, MD, USA) supplemented with 10% FCS, 100 U/ml penicillin/ streptomycin, and 2 mM glutamine. The day before transfection, 5 x 10^6 Phoenix-gp or 293T cells were plated on a 10 cm dish. The medium was exchanged and 25 µM chloroquine (Sigma-Aldrich, Munich, Germany) was added. 0.5-5 µg transfer vector DNA, 1 µg of a eGFP reporter plasmid to determine transfection efficiencies (if eGFP was not the cDNA of the

transfer vector), and 2 µg of an ecotropic envelope plasmid[44] or 5 µg of an RD114/TR envelope plasmid (kindly provided by F.-L. Cosset, Lyon, France) were used. In addition, 10 µg of a retroviral gag/pol plasmid (M57-DAW) were transfected using the calcium phosphate precipitation method. M57 is an MLV gag/pol expression plasmid (kindly provided by Harald Wodrich, Montpellier, France) and its derivative M57-DAW is devoid of residual overlaps with the transfer vector. When producing lentiviral vectors, 5 µg of a Rev plasmid (pRSV-Rev) were co-transfected. For vector production of the tet-inducible vector 5 µg of the expression plasmid pPGK.TP, harboring the authentic TA with 4C DNA-binding specificity, was co-transfected.

The medium was changed after 10-12 h. Equal transfection efficiency was controlled by FACS analysis. Supernatants containing the viral particles were collected 24-72 h after transfection, filtered through a 0.22 µm filter, and stored at -80℃ until usage.

SC-1 cells were transduced by centrifugation for 60 min at 2000 rpm at 32℃ in the presence of 4 µg/ml protamine sulfate (Sigma-Aldrich). After transduction, cells were grown for 4-5 days and subsequently analyzed by flow cytometry and Northern blot. Titration of the vector supernatants on SC-1 cells was performed as described previously.[3]

Rhesus monkey primary cells

Purpose-bred male rhesus monkeys (*Macaca mulatta*), each weighing 2.5 to 4 kg and cynomolgus monkeys (*Macaca fascicularis*) weighing 4 to 6 kg, aged 2 to 3 years old, were used. Housing, experiments, and all other conditions were approved by an ethics committee in conformity with legal regulations in The Netherlands.

Purification of $CD34^+$ rhesus cells was performed by positive selection using Dynalbeads (Dynal, Oslo, Norway[45]) Briefly, low-density cells were incubated with an IgG2A antibody against CD34 (mAb 561; from G. Gaudernack and T. Egeland, Rikshospitalet, Oslo, Norway) covalently linked to rat anti-mouse IgG2A beads. $CD34^+$ cells devoid of the CD34-antibody were recovered using polyclonal antibodies against the Fab part of the CD34 antibody (Detachebead, Dynal Biotech, Hamburg, Germany). Purified $CD34^+$ cells were analyzed by flow cytometry and prestimulated at a concentration of 10^5/ml for 2 days prior transduction in serum-free enriched DMEM supplemented with human recombinant growth factors fetal liver tyrosine kinase 3-ligand (Flt3-L; 50 ng/ml, kindly provided by Amgen, Thousand Oaks, CA, USA), thrombopoietin (rhTPO; 10 ng/ml, kindly provided by Genentech, South San

Francisco, CA, USA) and stem cell factor (SCF; 100 ng/ml) as previously described.[46]

Retroviral transduction of Rhesus CD34+ cells

To enhance the transduction efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 µg/cm^2.[47] Before adding the prestimulated purified rhesus BM to the fibronectin-coated dishes, the CH-296 fragment was preincubated with virus supernatant for 1 hour at 37°C.[47] Subsequently, nucleated cells were resuspended in the vector-containing supernatant (MOI as indicated in Results) supplemented with hematopoietic growth factors (Flt3-L, TPO and SCF) and added to the coated and preloaded dishes in a concentration of 1-3 x 10^{5}cells/ml. Over a period of 2 days, culture supernatant was replaced completely by resuspending non-adherent cells into fresh retrovirus supernatant and growth factors. After 2 days the cells were harvested, the transduction efficiency was analyzed by flow cytometry.

Flow cytometry

For intracellular staining of MGMT, the Cytofix/Cytoperm Kit (Becton Dickinson, Heidelberg, Germany) was used according to manufacturer's instructions. In brief, at least 3 x 10^5 cells were harvested and washed in phosphate-buffered saline. Cytofix/Cytoperm fixative (4% paraformaldehyde: 250 µl) was added for 20 min at 20℃. Washing with 1 ml Perm/Wash buffer was follow ed by incubation 30 min at 4℃ with 0.25 µg of a murine anti-MGMT monoclonal antibody (Chemicon, Hampshire, UK). After two washing steps with Perm/Wash buffer, 1 µg of a goat-anti-mouse PE-conjugated secondary antibody (Becton Dickinson) was added for 30 min at 4℃. After two additional washing steps, the samples were analyzed in a FACScalibur using CellQuest software (Becton Dickinson). A gate was set on a homogenous cell population, as determined by scatter characteristics, and 20,000 events were monitored. A marker was set to calculate the percentage and mean fluorescence intensity of positive cells. For the dual fluorescence assay with eGFP and DsRed Express, 2 µg transfer vector, 10 µg M57-DAW, 2 µg ecotropic MLV env were transfected into Phoenix-gp cells using the calcium phosphate technique. Three days

post transfection, the packaging cells were analyzed by FACS. Compensation of FL-1 (eGFP) and FL-2 (DsRed Express) was performed using monofluorescent constructs. A marker gate was set and the mean fluorescence intensities for eGFP- and DsRed Express-positive cells were calculated accordingly.

Northern blot

Total RNA preparation and Northern blot analysis was performed as described before.[18] Specific probes (100 ng) corresponding to the PRE fragment, present in the respective retroviral and lentiviral vectors and the eGFP cDNA were radiolabeled using the DecaLabel DNA labeling kit (Fermentas, St. Leon-Rot, Germany). Membranes were washed, sealed, and exposed to X-ray film (Kodak X-Omat-AR, Kodak, Stuttgart, Germany) and quantified by Phosphoimager (Amersham, Freiburg, Germany) analysis.

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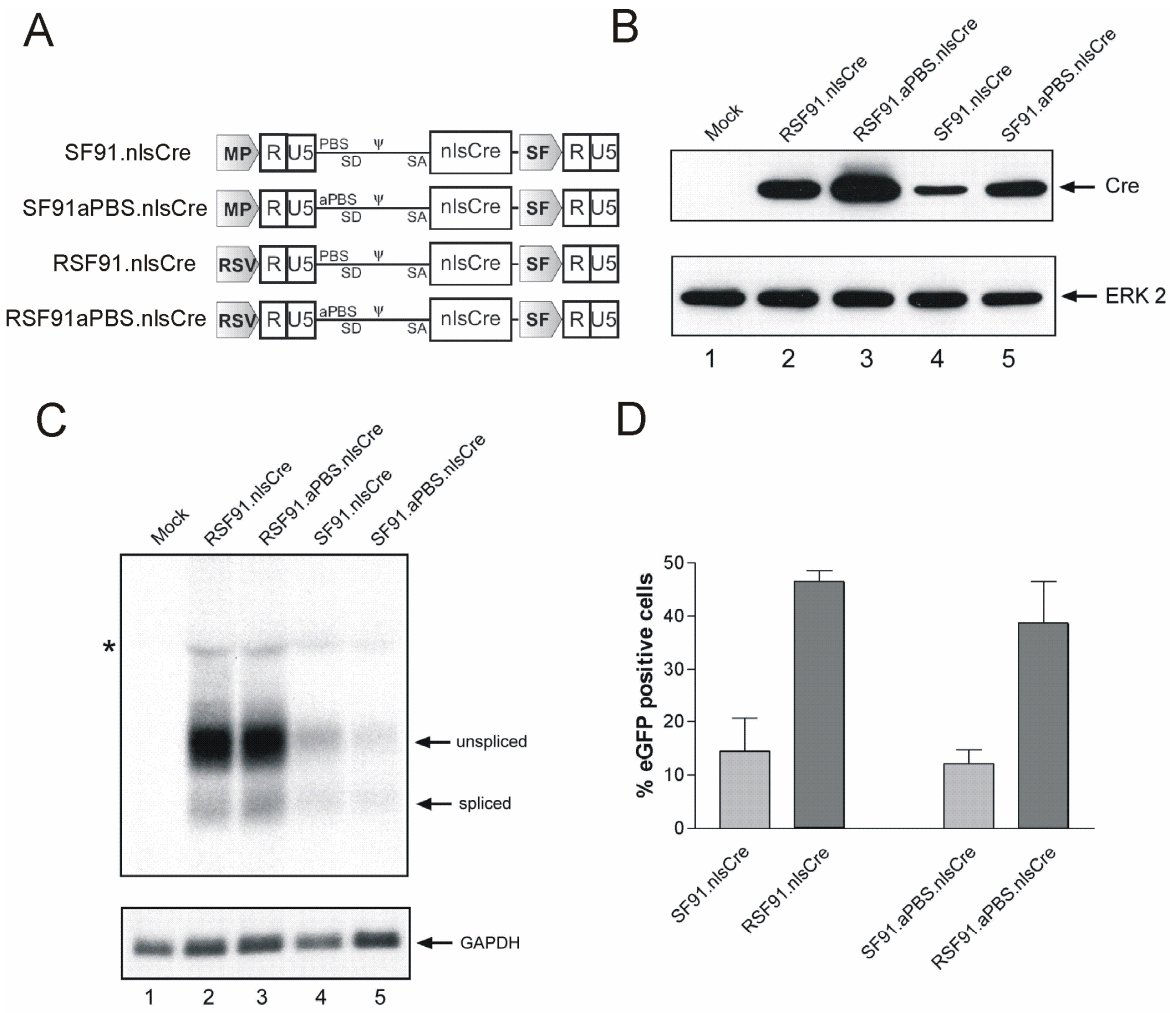
g. Supplementary Figures

Supplementary figure 1



Suppl. Fig. 1: The RSV promoter enhances particle mediated mRNA-transfer. (A) Depicted are the LTR vectors used for particle mediated mRNA transfer (RMT) based on the LTR vector SF91. The vectors encode nuclear localizing Cre (nlsCre), a derivative harbors an artificial primer binding sites (aPBS).[26] The 5' U3 region was either MP or RSV. (B) 293T cells were transfected with 5 µg of the indicated plasmids plus gag/pol and ecotropic envelope packaging constructs. Cell lysates were prepared after 60 h. Protein detection was carried out with antibodies directed against Cre and ERK 2 as loading control. (C) Northern blot using 15 µg of total RNA from transfected 293T cells. A probe corresponding to the Cre cDNA was used. The blot was reprobed with GAPDH. The RNA species are marked on the right. The asterisk marks a potential read-through band on the plasmid. (D) Supernatants of packaging cells were used to pseudotransduce reporter cells carrying the SFr-2 allele. RMT of Cre-induced eGFP expression that was detected by flow cytometry after 48 h. Error bars represent three independent experiments.

F. Discussion & Outlook

The general aim of the present study was the exploitation of modified murine leukemia virus-based vectors for targeted and transient cell manipulation. To accomplish this, three different vector mutants (ΔU5, ΔPBS and aPBS) were constructed and tested for their capability to transiently express the gene of interest (publication 1). Proof of principle was established with the reversible transfer of nucleic acids of the site-directed recombinase Cre into human and mouse fibroblasts carrying Cre indicator alleles (Will et al. 2002). Although all three vector mutants were clearly disabled in stable gene transfer (up to 10,000-fold reduction compared to the wild-type vector), their capability to recombine Cre indicator cells was highly efficient (publication 1, Fig. 1 and Fig. 2). Interestingly, the recombination efficacy of the aPBS vector mutant was always superior to that seen for the ΔU5 or ΔPBS vector mutants (publication 1, Fig. 2A), and was therefore primarily used in the experiments contributing to this work.

Plasmid	Packaging signal	Gag/Pol	Env	Cre expression in packaging cell	Recombination event in Cre indicator cell
aPBS.nlsCre	Yes	Yes	Yes	Yes	Yes
aPBS.nlsCre	Yes	Yes	No	Yes	No
aPBS.nlsCre	Yes	No	Yes	Yes	No
CMV.nlsCre	No	Yes	Yes	Yes	No

TABLE 1: RMT requires the formation of infectious particles and depends on the retroviral packaging signal. The gammaretroviral aPBS.nlsCre (RMT) vector plasmid was co-transfected into human 293T packaging cells with both Gag/Pol and Env expression plasmids or Gag/Pol and Env alone. To test the necessity of the retroviral packaging signal Ψ, a CMV.nlsCre expression plasmid, lacking the Ψ signal, was transfected together with Gag/Pol and Env. Two days post-transfection, the supernatants of the transfected cells were harvested, concentrated via ultracentrifugation and tested for their recombination efficacies on mouse Cre indicator cells. To ensure equal transfection efficiencies, packaging cells were checked for Cre protein levels via western blot technique.

Persistent intracellular expression of Cre recombinase is known to have genotoxic side effects (Loonstra et al. 2001; Pfeifer et al. 2001; Silver and Livingston 2001) and led to the counterselection of cells harboring stable integrated wild-type SF91.nlsCre vectors (publication 1, Fig. 2B). However, these side effects were not observed for cells treated with aPBS.nlsCre vector particles, strongly arguing for a dose-controlled and reversible expression of Cre recombinase in these cells. Further (and to some extent more indirect) analysis demonstrated that this type of transient Cre expression requires particle assembly, is receptor-mediated (publication 1, Fig. 4) and depends on the presence of the packaging signal (Ψ) within the retroviral vector genome

(TABLE 1). For that reason we named this technique retroviral particle-mediated mRNA transfer (RMT).

The characterization of RMT particles did not reveal any obvious differences concerning genomic RNA content, reverse transcriptase activity or capsid load when compared to wild-type particles (publication 2, Table 1 and Fig. 2). In addition, both RMT and wild-type retroviral Cre supernatants showed a clear correlation between genomic RNA content and the efficacy to recombine Cre indicator cells (biological titer) (Fig. 10).

Replacement of the RMT vector plasmid´s myeloproliferative sarcoma virus (MPSV) enhancer-promoter region with sequences from Rous sarcoma virus led to increased levels of packageable genomic RNAs in viral producer cells and clearly improved RMT in target cells (publication 3, Suppl. Fig. 1).

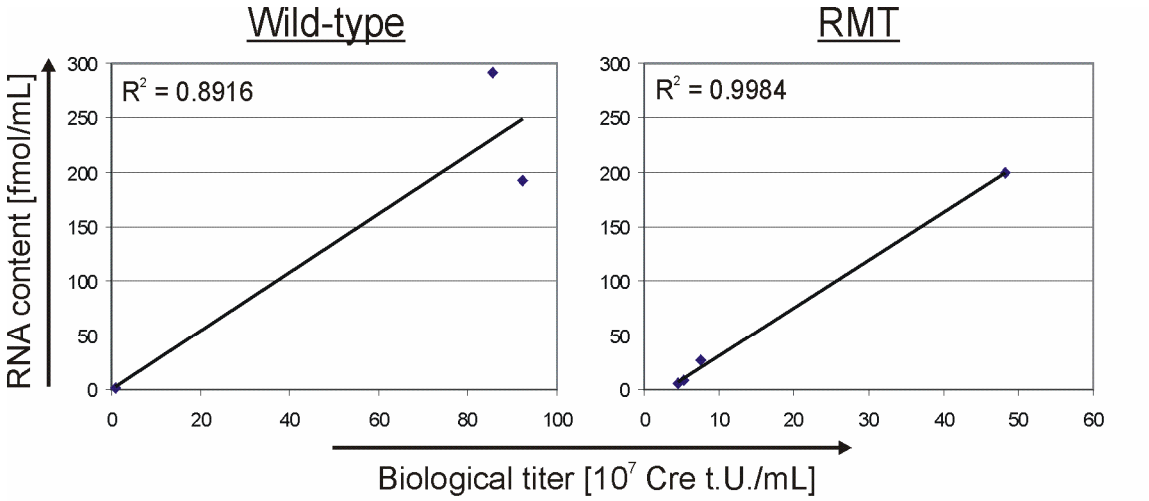


Fig. 10: The RNA content of retroviral supernatants correlates with the biological titer. Regression calculated from data shown in table 1 of publication 2. The values of four different supernatants were plotted for wild-type and RMT Cre vectors, respectively. Interestingly, the correlation is even stronger for the RMT supernatants. t.U.: transducing units.

To further study the mechanism of RMT, the susceptibility of RMT to cellular restriction factors (huTRIM5α and Fv1) and short hairpin RNAs (shRNAs) was investigated (publication 2). Both huTRIM5α and to a lesser extent Fv1 were capable of effectively restricting RMT (publication 2, Fig. 4 and Fig. 5). Since both factors are suggested to interact directly with the retroviral capsid at an early post-entry step (Towers et al. 2000; Goff 2004; Stremlau et al. 2004; Passerini et al. 2006), the sensitivity of RMT to huTRIM5α and Fv1 supports the hypothesis that the phenomenon underlying RMT is particle-mediated rather than due to passive protein transfer and/or contamination of retroviral particles by transfected plasmid DNA.

Interestingly, the restriction of RMT by huTRIM5α resulted in reduced levels of retroviral mRNA in target cells, whereas restriction by Fv1 did not significantly affect the incoming retroviral RNA genome (publication 2, Fig. 6A and 6B). Furthermore, treatment with the proteasomal inhibitor MG132 partially relieved huTRIM5α-mediated restriction of RMT (publication 2, Fig. 4D and 4E), which was also reflected in partial retroviral mRNA recovery (publication 2, Fig. 6A). Finally, cells ectopically expressing shRNAs targeting the retroviral RNA genome inhibited RMT (publication 2, Fig. 3), also clearly showing that the transferred viral mRNA is the main component responsible for the transient expression of foreign proteins via RMT in target cells. Thus, these data indicate that as a result of particle remodeling during cytoplasmic trafficking, the retroviral mRNA genome may become accessible to ribosomes and serve as a translation template if it is not undergoing reverse transcription (Fig. 11).

1. RMT in comparison to current transient expression methods: Advantages and limitations

The present study introduces RMT as a potential tool for the transient and reversible expression of proteins in target cells. As demonstrated for nlsCre, we showed that this type of transient expression method was equally efficient, but did not show any cytotoxic side effects in human or mouse fibroblasts when compared to the integrating vectors. Noteworthy, this new mode of particle-mediated mRNA transfer does not lead to stable integration events, as observed for lentiviral vectors with blocked integrase activity (publication 2, Fig. 1C).

In side by side comparison with alternative transient expression methods, such as physicochemical transfection (e.g. lipofection) of transgene expression plasmids or the use of adenoviral vectors, RMT was superior for the transient expression of nlsCre in Cre indicator cells (publication 1, Suppl. Fig. 2). Thus, in contrast to RMT, delivery of nlsCre via episomal adenoviral vectors caused counterselection of cells due to persistent Cre expression similar to the integrating gammaretroviral nlsCre vector. Furthermore, cells which were physicochemically transfected with an nlsCre expression plasmid were not significantly counterselected over time, but showed a high mortality rate early after transfection, probably due to the transfection reagent (Lipofectamine 2000, Invitrogen). Therefore, RMT represents a dose-controlled and non-cytotoxic technique which facilitates cytoplasmic mRNA delivery into a target cell

by utilization of all non-cytopathic properties supporting efficient cell entry and cytoplasmic trafficking.

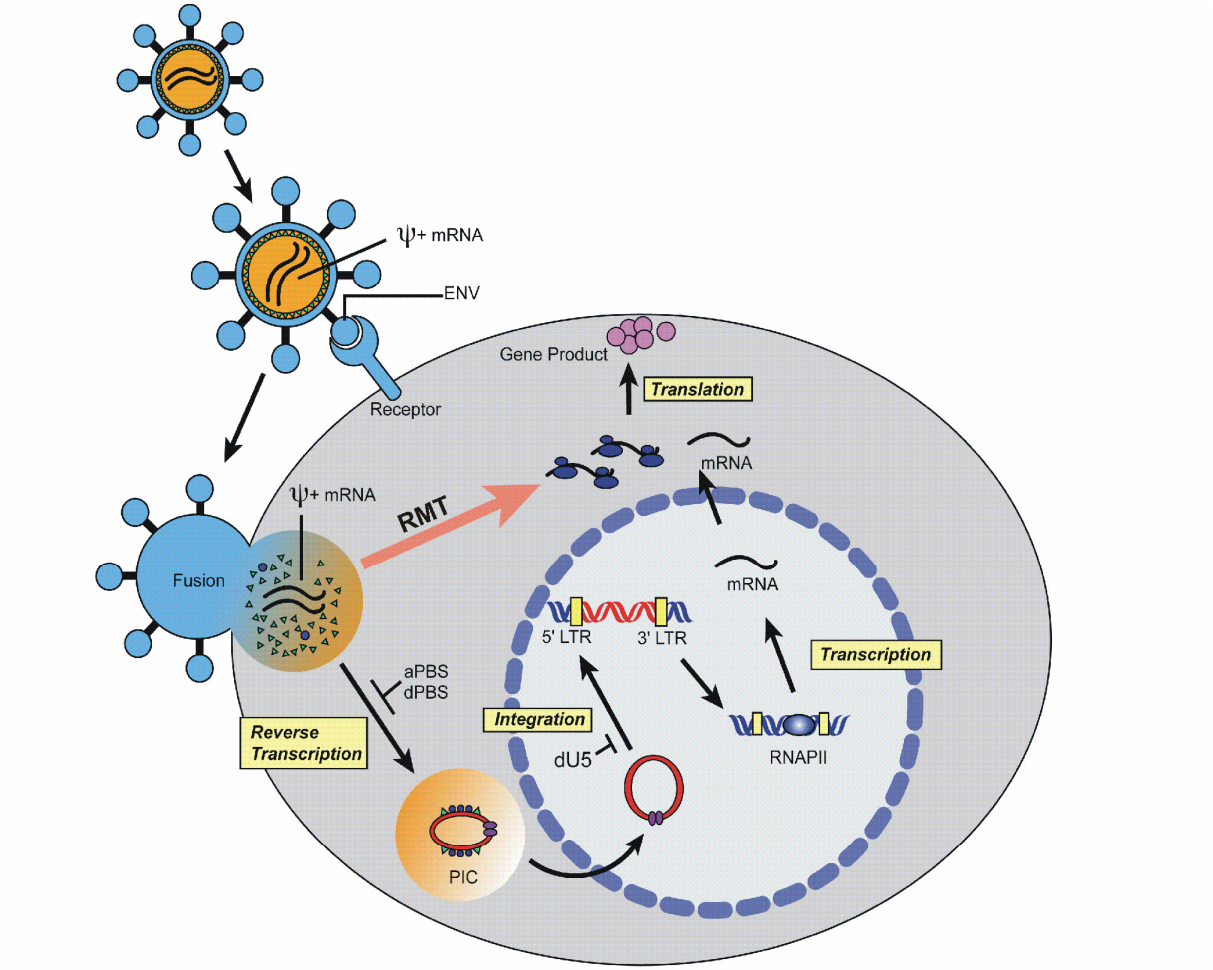


Fig. 11: The proposed "early translation" of retroviral vector genomes shortly after entry. Retroviral vector particles enter the cell cytoplasm in a receptor-mediated manner (fusion at the cell surface or endosomal membranes, depending on the type of Env protein). Subsequently, the retroviral vector particle undergoes an uncoating process and initiates reverse transcription of the retroviral vector genome (Ψ+mRNA) into double stranded DNA, resulting in the formation of a preintegration complex (PIC). After nuclear entry, the proviral DNA stably integrates into the host´s genome and *de novo* synthesis of retroviral vector mRNA via the RNA polymerase II (RNAP II) is initiated. The newly synthesized mRNA is then transported into the cytoplasm and serves as a translation template for the generation of the encoded transgene. *De novo* transcription of mRNA is most efficient from the integrated provirus, but may also occur from circular intermediates, as expected for the integration-disabled ΔU5 mutant . The present study indicates that translation of the proviral mRNA does not necessarily require *de novo* transcription, but may also occur from the incoming "maternal" genomic Ψ+mRNA, if reverse transcription is inhibited (ΔPBS and aPBS mutant). Thus, the orange arrow indicates a novel pathway relevant for retroviral particle-mediated mRNAtransfer (RMT).

Since we could show that the entry of RMT particles into cells is receptor-mediated and depends on the type of the chosen viral envelope protein (publication 1, Fig. 4), specific pseudotyping of RMT particles would allow targeting of distinct cells in a mixed population. For example, measles virus, which has two types of glycoproteins, the hemagglutinin (H) protein responsible for receptor recognition, and the fusion (F)

protein which mediates membrane fusion, can be efficiently retargeted by mutating the H protein binding sites for its native receptors and fusing single-chain antibody fragments to its ectodomain (Nakamura et al. 2005). In a more recent study, the successful cell entry targeting of lentiviral vectors through pseudotyping with engineered measles glycoproteins was shown (S. Funke et al., American Society of Gene Therapy´s (ASGT), 10th annual meeting, 2007). Thus, pseudotyping of RMT particles using the envelope proteins of measles virus might also be promising for targeted and reversible manipulation of cells.

However, RMT also has limitations. Taking into consideration that RMT-mediated transient expression of proteins is low and only present for a short time span (if compared to integrating or episomal retroviral/ lentiviral vectors; publication 2, Fig. 1), the protein levels might be not sufficient for applications which require high expression levels to show a phenotypic effect. However, one way of increasing the intensity and duration of RMT would be to administer the viral particles repetitively. In addition, manipulation of the retroviral disassembly process might be a further possibility to improve RMT. Furthermore, RMT vector plasmids containing a 5' RSV promoter (see publication 3, Suppl. Fig. 1) or a wPRE element (compare publication 1, Fig. 1C to publication 2, Fig. 1B) increased RMT titers by more efficient viral mRNA load of particles in packaging cells, and thus transgene expression in target cells. Thus, it might be reasonable that combination of both elements on the same vector plasmid will lead to a further improvement of RMT.

As shown in this study, RMT can be inhibited by cellular restriction factors and shRNAs (publication 2, Fig. 3, 4 and 5). This might also be the case for other forms of innate immunity. Thus, potential recognition of retroviral proteins and/or RNA genomes by toll-like receptors or cytoplasmic helicases, such as RIG-I (retinoic acid-inducible gene I) or MDA5 (melanoma-differentiation-associated gene 5), may also impair RMT (Yoneyama et al. 2004; Akira et al. 2006; West et al. 2006). However, in case of huTRIM5α restriction, inhibition of RMT can be almost completely released by blocking the proteasome (publication 2, Fig. 4D). This might also be true for other unknown cellular restriction factors, as has been demonstrated by the lab of Luigi Naldini. The authors of this paper could show that proteasome inhibition improved stable lentiviral gene transfer into hematopoietic stem cells (Santoni de Sio et al. 2006). However, proteasome inhibitors are known to be cytotoxic and care must be taken regarding dose concentration and duration. Importantly, restriction by huTRIM5α can be circumvented by choosing NB-tropic (from MoMLV) or B-tropic

versions of MLV *gag* for particle formation, or in the case of lentiviral vectors, *gag* sequences derived from HIV-1.

Due to low expression levels of RMT, its administration *in vivo* might require more than one application to show the desired phenotype. Although it has been shown that retroviruses – in contrast to adenoviruses – are relative weak immunogens (Jooss and Chirmule 2003; Thomas et al. 2003; Dalba et al. 2007), the development of innate as well as adaptive immune responses has to be considered. In addition, as shown for EGFP and β-galactosidase, the nature of the transgene may also induce immunity (Stripecke et al. 1999; Jooss and Chirmule 2003; Mian et al. 2005). However, the degree to which viral vectors induce harmful immune-mediated and inflammatory responses depends upon the amount of antigens presented and must exceed a certain threshold (Thomas et al. 2001; Thomas et al. 2003). Due to low and short-term expression levels of RMT, this potential risk might be negligible, but needs to be more precisely clarified.

2. What makes the aPBS mutant superior to the ΔPBS and ΔU5 mutants?

To establish a murine leukemia virus-based gammaretroviral vector system that inhibits stable gene transfer, three different vector mutants were generated (aPBS, ΔPBS and ΔU5, publication 1, Fig. 1A). These vectors were tested for their potential to transiently deliver the site-directed recombinase Cre into Cre indicator cells. The vector aPBS contains an artificial PBS that was designed not to match any naturally occuring tRNA molecule (Lund et al. 1997), whereas the vector ΔPBS completely lacks the 18 base pairs being necessary for primer binding. Thus, both vector mutants cannot prime RT and are disabled in forming proviral DNA. In contrast, vector ΔU5 contains all elements required for reverse transcription into double-stranded DNA, but lacks the *att* recognition motif of the retroviral integrase (Basu and Varmus 1990), along with flanking sequences of the U5 region. The efficacy of the aPBS vector mutant to recombine Cre indicator cells was found to be superior to that seen for ΔPBS or ΔU5 mutants (publication 1, Fig. 2A). Interestingly, although the ΔU5 mutant was expected to result in high recombination efficiencies (formation of extrachromosomal retroviral DNA from which nlsCre is transiently expressed over a distinct time period), its recombination efficacy was the lowest when compared to aPBS and ΔPBS vector mutants. How can this be explained? A recent publication

from our laboratory investigated the role of sequences upstream of the 5´ splice site (SD) in MLV alternative splice regulation (Kraunus et al. 2006). Detailed analysis of the first untranslated exon showed that the primer binding site activates splicing, whereas flanking sequences either downstream or upstream of the primer binding site are inhibitory. Retroviruses usually exhibit a well-balanced expression of their full-length genomic mRNA and their spliced *env* mRNA variant and thereby ensure equal expression of structural and enzymatic proteins for the generation of new virus progeny. However, deletion of the 5´ U5 region within murine leukemia-based gammaretroviral EGFP vectors (whose splicing pattern is comparable to wild-type MLV) strongly enhanced splicing, leading almost exclusively to the accumulation of spliced message in the cell cytoplasm (Kraunus et al. 2006). Since the unspliced genomic retroviral mRNA is used not only for the production of Gag and Gag/Pol precursor proteins, but also represents the packageable retroviral RNA genome, the shift to more spliced RNA also results in a greatly decreased titer. In addition, deletion of the 5´ U5 region tremendously impairs translational utilization and reduces the mean fluorescence intensity of EGFP by 80% when compared with the wild-type EGFP vector (Kraunus et al. 2006). Thus, the decrease in titer and impaired translational utilization could both contribute to the unexpected low recombination efficacy of the ΔU5 mutant. Furthermore, it was shown for avian sarcoma virus (ASV), Rous sarcoma virus (RSV) and HIV-1 that sequences within the 5´ U5 region are important for primer binding and reverse transcription initiation (Aiyar et al. 1992; Morris and Leis 1999; Beerens et al. 2000a; Beerens et al. 2000b; Beerens et al. 2001; Morris et al. 2002). As this might be also the case for MLV, the impairment of reverse transcription could be an additional reason for the poor performance of the ΔU5 mutant. To further improve the technology of this type of nonintegrating extrachromosomal gammaretroviral vector, it might be advisable to introduce only mutations that are limited to the *att* sequences (Nightingale et al. 2006), thereby possibly avoiding alterations in splicing ratio, translational utilization and reverse transcription. Another step towards episomal retroviral vectors would be the use of integrase-mutated (e.g. in the DDE motif of the integrase core domain) gammaretroviral vector particles (Philpott and Thrasher 2007) and/or the application of integrase inhibitors (e.g. raltegravir).

Compared to the aPBS variant, the ΔPBS mutant also showed lower nlsCre recombination efficacy. The first untranslated exon of MLV is highly structured and forms, with U5 and 5’ leader sequences, two stem-loop RNA structures which are

separated by the unpaired primer binding site sequences (Mougel et al. 1993). The deletion of the primer binding site, as determined by an RNA folding prediction program (MFOLD) (Zuker 1989), results in fusion of the two separated stems to an elongated, more stabilized RNA stem loop structure (Kraunus et al. 2006) and therefore could potentially affect the ribosomal scanning mechanism.

In contrast to the ΔPBS and ΔU5 mutants, the aPBS mutant demonstrated a similar splicing pattern and no loss of translatability as compared to wild-type vectors (publication 3, Suppl. Fig.1). Interestingly, western blot analysis of equally transfected cells showed even higher translatability of the aPBS vector mutant as compared to wild-type vector (publication 3, Suppl. Fig. 1B), probably due to the absence of a bound primer (see also below).

Taken together, the aPBS vector mutant is clearly superior to the other tested variants and is the preferred vector construct to achieve RMT. Furthermore, it also has the advantage that one and the same construct can be used for RMT or the generation of integrating retroviral vectors depending on the co-transfection of the artificial tRNA that matches aPBS in packaging cells (Lund et al. 1997).

3. Translation or reverse transcription? Two potential pathways for retroviral mRNAs after cell entry

The reverse transcription process is proposed to begin immediately after entry during uncoating of the virion core in the cytoplasm (Telesnitsky and Goff 1997). Similary to cellular mRNA, the encapsidated RNA genome of retroviruses harbors a 5´ Cap structure and a 3´ PolyA-tail. Thus, in addition to reverse transcription, retroviral genomes theoretically represent a template for translation.

The supplementary figure 3 of publication 1 shows the susceptibility of RMT to the reverse transcription inhibitor Azidodeoxythymidine (AZT or zidovudine). AZT is a nucleoside analog that acts as a chain terminator if incorporated into DNA because the deoxyribose 3´OH residue is replaced by an azido (N3) group. Since retroviral reverse transcriptases lack a proof-reading activity, which is reflected by low DNA synthesis accuracy (Preston et al. 1988; Goodenow et al. 1989), incorporation of AZT into the growing DNA chain results in an irreversible block of reverse transcription.

In this experiment, SC-1 derived mouse Cre indicator cells were infected with either RT-deficient RMT (aPBS.nlsCre) or intact wild-type (SF91.nlsCre) retroviral particles in the presence of different AZT concentrations. The recombination efficiencies were

determined via FACS analysis three days post transduction. SC-1 cells transduced with wild-type SF91.EGFP particles at the same AZT concentrations served as internal controls for the efficacy of AZT to block reverse transcription. FACS analysis of SF91.EGFP treated SC-1 cells 3 days post transduction revealed that an AZT concentration of 5 µM was sufficient to efficiently inhibit stable EGFP transfer. As expected, nls.Cre encoding RMT particles (aPBS.nlsCre) were completely insensitive to AZT and their efficacy to recombine Cre indicator cells was not even impaired by AZT concentrations up to 100 µM. In contrast, the recombination efficiency of wild-type SF91.nlsCre particles in the presence of AZT was reduced by up to 50%. Interestingly, although 5 µM AZT efficiently inhibited stable EGFP transfer of SF91.EGFP particles in SC-1 cells, the efficacy of wild-type SF91.nlsCre particles to recombine Cre indicator cells was not completely abolished or even decreased at this or higher AZT concentrations. These data strongly argue for the presence of AZT-insensitive, translatable wild-type retroviral RNA genomes within incoming particles that led to transient nlsCre expression early after entry. Indeed, at early timepoints (5-24 hrs post infection), wild-type particles of SF91.EGFP also showed transient EGFP expression in the presence of AZT, but at a lower intensity than the corresponding RMT particles (data not shown). These data imply the possibility that two types of RNA exist within wild-type particles (vectors SF91.nlsCre or SF91.EGFP): translatable, AZT-insensitive and non-translatable, AZT-sensitive RNAs. Interestingly, an RMT particle (vector aPBS.nlsCre), complemented by the corresponding artificial tRNA (Lund et al. 1997) had a phenotype similar to wild-type in the presence of AZT (own observation, data not shown).

Noteworthy, in the absence of AZT we observed equally increasing EGFP expression until 13 hrs after exposure for both SF91.EGFP and aPBS.EGFP vectors (publication 1, Fig. 1; publication 2, Fig. 1). Since reverse transcription of wild-type particles is generally not completed before 8 to 12 h after viral penetration (Telesnitsky and Goff 1997), one can probably exclude *de novo* synthesized RNA as the underlying cause of GFP expression in the early hours of infection. These data indicate that reverse transcription competent particles also harbor translatable genomic mRNA.

All data taken together, it is tempting to speculate that translatability of the retroviral mRNA depends upon the absence of a bound tRNA and/or lack of reverse transcription. This would argue for potentially competing pathways (translation vs. reverse transcription) for the fate of the retroviral mRNA. In addition, RMT vectors (harboring the aPBS) transiently expressed in equally transfected packaging cells

(verified by co-transfection of an EGFP expression plasmid, data not shown) produced higher transgene protein levels than their corresponding wild-type derivatives (publication 3, Suppl. Fig. 1B), further supporting the hypothesis that a bound tRNA primer may impede translation. However, further and more extensive experiments are needed to address this question.

4. By-product or necessity? Biological purpose of RMT for the retroviral life cycle

We have shown that wild-type vector genomes of incoming retroviral particles may also become accessible to ribosomes, leading to a transient transgene expression early after entry (publication 1, Fig. 1C and Suppl. Fig. 3; publication 2, Fig. 1C). However, this type of RMT is not as efficient as that seen for aPBS vector mutants, probably due to viral reverse transcriptase and cellular translation factors competing for the same template. There are two possible explanations why RMT can also be observed during infection with wild-type particles. First, RMT is a by-product during infection, and is mediated by RT-defective particles releasing their RNA genomes for translation after disassembly (defective particle hypothesis). However, the recombination efficiency of the AZT blocked wild-type virus (SF91.nlsCre) (i.e. the RMT proportion) always corresponds to approximately 50% of the value achieved in the absence of AZT, independent of the MOI (multiplicity of infections) and the retroviral preparation. This stoichiometry was observed in multiple independent experiments and for several virus preparations (even for aPBS.nlsCre complemented with the artificial corresponding tRNA). This argues against the defective particle hypothesis. A second explanation would be that RMT of wild-type particles could have a biological purpose and may be necessary for the retroviral life cycle at early or late post-entry phases (necessity hypothesis). From an evolutionary perspective, the latter would presuppose that the viral genomic RNA necessary for RMT as well as reverse transcription originates from a single particle. Interestingly, despite the fact that two genomes are encapsidated within an infectious retroviral particle, only one provirus is detected after infection with single virions (Hu et al. 1990; Hu and Temin 1990). Based on this phenomenon, retrovirions are considered pseudodiploid in character. Currently, one popular notion is that the availability of two RNA templates during reverse transcription can help retroviruses survive extensive damage to their genomes, by intermolecular switching of the RNA templates if

necessary (Coffin 1979). Indeed, intermolecular transfer of minus strand strong stop DNA (minus strand transfer) and/or plus strand strong stop DNA (plus strand transfer) has been observed during reverse transcription (Yu et al. 1998; van Wamel and Berkhout 1998), presumably contributing to the preservation of the retroviral genetic information (Coffin 1979) as well as dispersing useful mutations and thereby promoting viral evolution (Katz and Skalka 1990; Temin 1991). However, only one proviral DNA is formed as a result of one infection event and intermolecular minus or plus strand transfer has been shown not to be essential for successful reverse transcription (Jones et al. 1994). What happens to the RNA genome which is not reverse transcribed? The data of the present study suggest that the second RNA genome may serve as a template for translation. If both processes (translation and reverse transcription) take place in close proximity, one could further speculate that translation of the genome which does not undergo reverse transcription has a biological purpose and leads to the expression of viral proteins or the recruitment of cellular proteins that are necessary for a successful completion of the infectious life cycle of retroviruses.

5. Do eukaryotic translation initiation factors assist during reverse transcription?

Reverse transcription is proposed to begin subsequently after entry into the cytoplasm. However, there are reports that reverse transcription can initiate in extracellular virions, but at rather low frequencies. It was shown that not more than 1 in 1000 particles possess minus strand strong-stop DNA (Lori et al. 1992; Trono 1992; Zhang et al. 1993), therefore the significance of this in infection processes is questionable. These observations are be supported by our data, where the presence of AZT during packaging of wild-type vector particles had no significant influence on transduction efficiencies of target cells (data not shown). Currently, it is thought that the most limiting factor of reverse transcription is the availability of dNTPs, a resource which is found in large quantities in the cytoplasm of an infected cell. This view is based on the observations that reverse transcription proceeds poorly in quiescent cells where intracellular dNTP concentrations are low (Zack et al. 1990; Zack et al. 1992), and simply increasing the concentration of dNTPs permits accumulation of full-length retroviral DNA (O'Brien et al. 1994). In addition, it is possible that the size and structure of extracellular virions preclude significant DNA synthesis before

cytoplasmic entry, so that core disassembly may be necessary for reverse transcription to occur.

In this study, RMT was also observed in cells infected with wild-type retroviral particles (publication 1, Fig. 1C and Suppl. Fig. 3; publication 2, Fig. 1). This implies, as already discussed, that the capped and polyadenylated wild-type genome is accessible to ribosomes for translation. However, the translatability of retroviral genomic mRNAs seems to depend on the absence of a bound primer and/or lack of reverse transcription, hence indicating that reverse transcription and translation of retroviral wild-type genomes are two competing processes. Furthermore, the observation that approximately 50% of retroviral RNA genomes (publication 1, Suppl. Fig. 3) (e.g. one out of two RNA genomes per virion) is capable of RMT within SF91.nlsCre wild-type particles, argues against RT-defective particles being responsible for RMT. If the template for RMT and reverse transcription may originate from the same wild-type particle, it is tempting to speculate that the disassembled particles become accessible to translational initiation factors, thereby causing a competition of translation and initiation of reverse transcription (as already discussed above). Noteworthy, during translational initiation, eIF4G (eukaryotic initiation factor 4G) bridges the cap binding protein eIF4E with the polyA binding protein (PABP) and circularizes the mRNA to facilitate translation (Fig. 12) (Gebauer and Hentze 2004). This process brings the 3’ UTR in close proximity to the 5’ end of the mRNA (Wells et al. 1998). Interestingly, this close proximity of both mRNA ends - induced by translational initation factors – might also be beneficial during reverse transcription and could theoretically assist in successful plus and minus strand transfer (Fig. 5). Of note, the PABP has been found in purified retroviral vector particles by proteomic analysis (Segura et al. 2008). This discussion points the way to further experiments addressing the “necessity hypothesis” in that, besides the availability of dNTPs, eukaryotic translational initiation factors might be involved in successful retroviral reverse transcription.

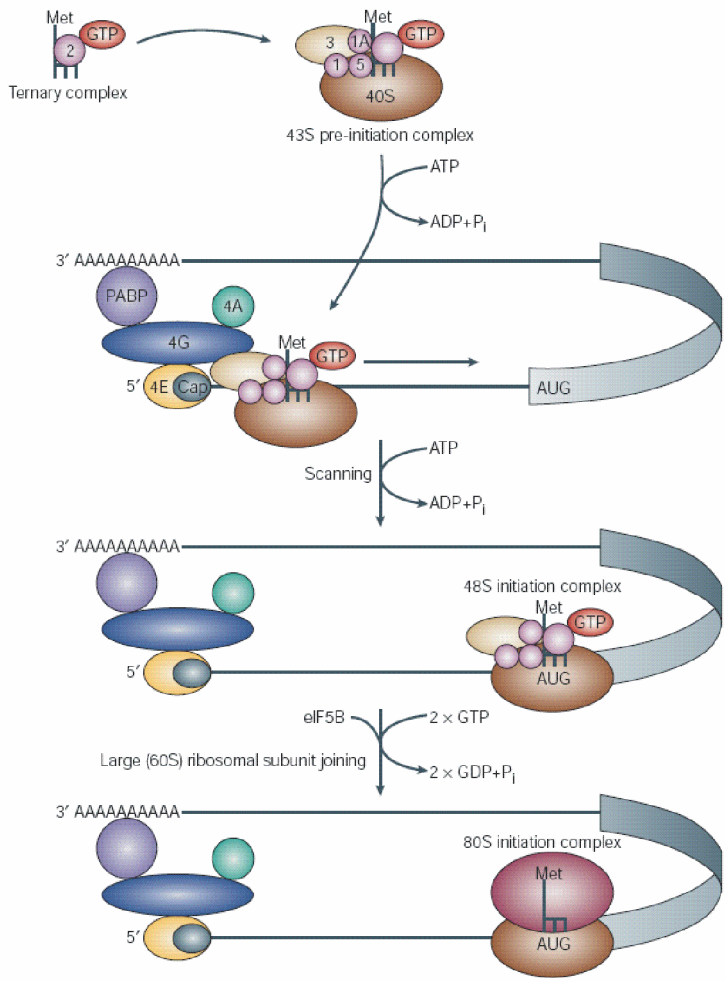


Fig. 12: The initiation of translation in eukaryotes. The figure shows a simplified model of eukaryotic translational initiation. Eukaryotic initiation factors (eIFs) are depicted as colored, numbered shapes. The methionine-loaded initiator tRNA (L-shaped symbol) binds to GTP-coupled eIF 2, yielding the ternary complex. The ternary complex then binds to the small ribosomal subunit (40S), eIF 3 and other initiation factors to form the 43S pre-initiation complex. The latter recognizes the mRNA by the binding of eIF 3 to the eIF 4G subunit of the Cap-binding complex. Besides the eIF 4G protein, the Cap-binding complex contains eIF 4E, which directly binds the Cap, and the RNA helicase eIF 4A. The interaction of eIF 4G with the polyA binding protein (PABP) is believed to circulize the mRNA, thereby bringing the 3´UTR in close proximity to the 5´end of the mRNA. The 43S pre-integration complex scans the mRNA from the 5´-end until it identifies the AUG start codon. Scanning is assisted by the factors eIF 1 and eIF 1A. Stable binding of the 43S pre-initiation complex to the AUG codon forms the 48S initiation complex. Subsequent joining of the large ribosomal subunit (60S) results in the formation of the 80S initiation complex. Both AUG recognition and joining of the formation of the large ribosomal subunit trigger GTP hydrolysis on eIF 2 and eIF 5B, respectively. Finally, the obtained 80S complex is competent to catalyze the formation of the first peptide bond. P: inorganic phosphate. *The figure is adapted from Gebauer et al., Molecular Cell Biology, 2004.*

6. The inhibition of RMT via endogenously expressed human TRIM5α is caused by mRNA degradation

The human cytoplasmic restriction factor TRIM5α blocks N-tropic MLV at an early post-entry step and prevents the accumlation of reverse transcription products in infected cells (Himathongkham and Luciw 1996; Towers et al. 2000; Cowan et al.

2002; Besnier et al. 2003; Stremlau et al. 2004; Passerini et al. 2006). The main retroviral determinant which confers susceptibility to huTRIM5α is located within the N-tropic retroviral capsid protein (arginine 110) and is suggested to be recognized by the C-terminal PRYSPRY domain of huTRIM5α (Towers et al. 2000; Perron et al. 2004).

Although extensive studies have been persued over the last years, less is known about the exact mechanism by which TRIM5α proteins restrict retroviral infection after capsid recognition. One study supports the idea that TRIM5α intervenes with the normal uncoating step of the viral capsid, and that this leads to an accelerated disassembly of the capsid structure, leaving the retroviral RNA genome unprotected (Perron et al. 2004; Perron et al. 2007). In addition, these authors argue that ubiquitin ligation, proteasome degradation, and/or massive degradation of the viral core are not essential components of TRIM5α restriction (Perron et al. 2007). Moreover, N-tropic MLV infection was efficiently blocked by huTRIM5α in a Chinese hamster E36 cell line expressing a temperature sensitive E1 ubiquitin ligase, even at the nonpermissive temperature (Perez-Caballero et al. 2005b). However, the laboratory of Thomas J. Hope found that proteasome inhibition abrogates the ability of human and rhesus TRIM5α proteins to prevent the accumulation of RT products, but that this does not relieve the ability of TRIM5α proteins to restrict viral infection (Anderson et al. 2006; Wu et al. 2006). A more recent study by the same laboratory showed that proteasome inhibition prevents the clearance of HIV-1 viral complexes from the cytoplasm, leading to the stable sequestration of these complexes in cytoplasmic bodies (Campbell et al. 2008).

In the present study, the susceptibility of reverse transcription-deficient aPBS (RMT) vector particles to endogenously expressed huTRIM5α was investigated (publication 2, Fig. 4 and 6). We found that N-tropic RMT vector particles are sensitive to huTRIM5α and that restriction is accompanied by a clear loss of retroviral genomic mRNA as well as an accelerated degradation of retroviral capsid proteins in restrictive human Cre indicator cells. Furthermore, proteasome inhibition with MG132 almost completely abrogates huTRIM5α-mediated restriction of RMT vector particles and is reflected in the partial recovery of retroviral mRNA genomes. Since transient protein expression via RMT depends not only on the accessibility but also the availability (amount) of translatable retroviral mRNA genomes within the cytoplasm of target cells, the data obtained with MG132 indicate that the main limiting factor during restriction of RMT by endogenous huTRIM5α is the loss of retroviral mRNA genomes

rather than their reduced accessibility (trapping in huTRIM5α cytoplasmic bodies). Interestingly, in contrast to human Cre indicator cells, the degradation of retroviral genomic mRNA derived from N-tropic aPBS.nlsCre particles is 5 times less pronounced in feline Cre indicator cells ectopically expressing huTRIM5α. However, western blot analysis of these cells still showed accelerated degradation of N-tropic capsid proteins. The result of these observations is an approximately 2 times higher recombination efficacy of N-tropic aPBS.nlsCre particles when compared to their B-tropic counterparts in feline Cre indicator cells. Interestingly, MG132 could not further improve N-tropic-mediated RMT in this cellular background (unpublished observations, data not shown). These data further support the hypothesis that the observed inhibition of N-tropic RMT particles in human Cre indicator cells (endogenously expressing huTRIM5α) is mainly due to subsequent degradation of the retroviral RNA genomes. Furthermore, the different behavior of N-tropic RMT particles in human and feline fibroblasts expressing huTRIM5α may argue for the existence of one or multiple cellular cofactors which are responsible for the proteasome dependent degradation of retroviral RNA genomes in human cells.

The current understanding of TRIM5α-mediated restriction does not include the targeted degradation of retroviral RNA genomes derived from restricted wild-type (RT-competent) particles (Chatterji et al. 2006). However, the data of the present study revealed that RNA genomes from N-tropic particles underlie targeted and proteasome dependent degradation in human cells endogenously expressing TRIM5α. The fact that human and rhesus TRIM5α prevent accumulation of retroviral RT products and that proteasome inhibition abrogates this ability (Anderson et al. 2006; Wu et al. 2006), suggests that inhibition of RT is simply a consequence of retroviral RNA genome degradation.

Combining results from our laboratory with those published by others led us to propose the following model (Fig. 13). Shortly after entry, the capsid of an incoming N-tropic retroviral RMT particle is recognized by huTRIM5α. Subsequently, the particle becomes sequestered in huTRIM5α cytoplasmic bodies, where, potentially, accelerated uncoating and/or degradation of the retroviral capsid occurs. When endogenously expressed in human fibroblasts, huTRIM5α leads to a proteasome dependent degradation (probably triggered by a cofactor X) of the retroviral genomic RNA, resulting in inhibition of RMT. In contrast, ectopically expressed huTRIM5α in feline fibroblasts causes only moderate decreases in genomic RNA levels, as

reflected in higher transient expression levels when compared to the unrestricted counterpart.

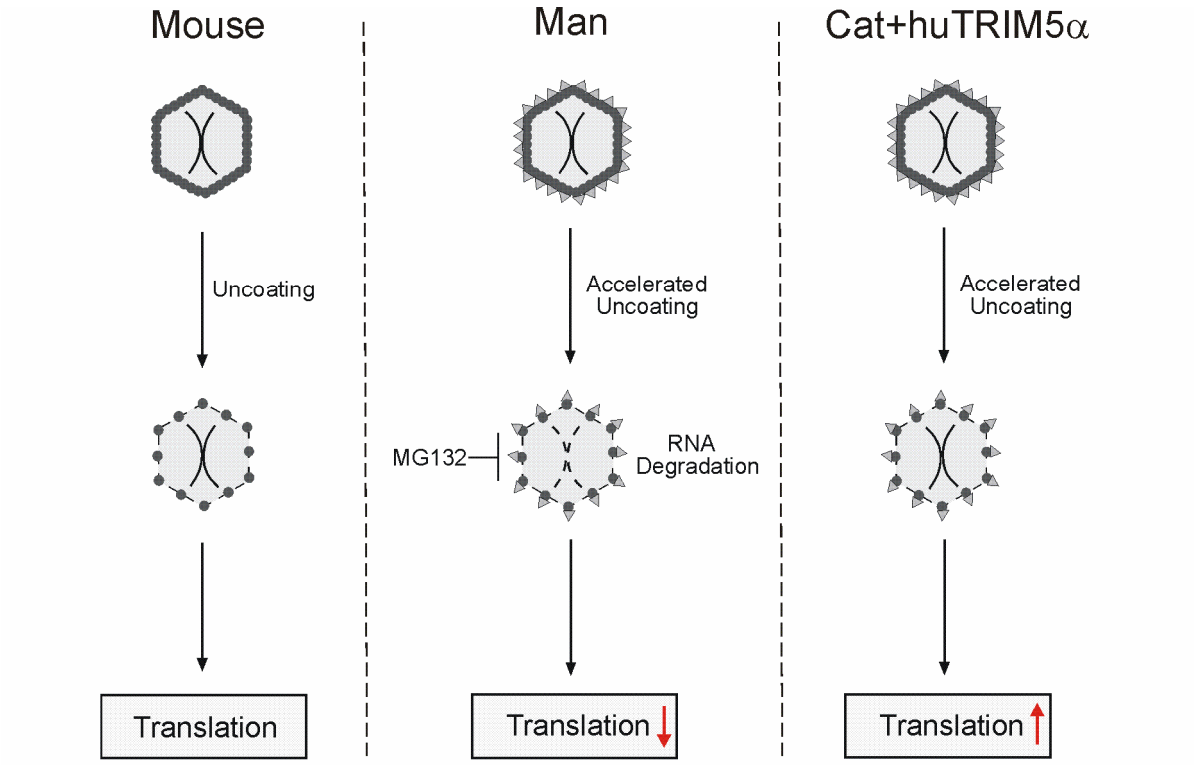


Fig. 13: Human TRIM5α-mediated restriction of RMT: A proposed model. In non-restricting mouse cells (left), the N-tropic RMT capsid uncoats and releases the RNA genomes into the cytoplasm for translation. In contrast, huTRIM5α (triangle) endogenously expressed in human cells (middle) binds to the incoming N-tropic capsid and initiates its accelerated uncoating. Subsequently, the RNA genomes become degraded in a proteasome dependent manner, resulting in less translation templates and inhibition of RMT. The application of the proteasomal inhibitor MG132 impairs degradation of the retroviral RNA genomes and almost completely rescues RMT. However, huTRIM5α ectopically expressed in feline cells (right) improves N-tropic RMT, probably due to an accelerated liberation of the retroviral genomes and an inadequate degradation of the latter. Of note, B-tropic and NB-tropic particles are not restricted by huTRIM5α.

7. Outlook and implications for future applications

The present study introduces RMT as an engineered gammaretroviral vector system, which allows the reversible manipulation of cells. The transferred retroviral mRNA – packaged into retroviral particles – follows the fate of retroviral entry, and delivers the mRNA genome encoding the gene of interest as a template for translation in the cytoplasm of target cells. Since retroviral entry is receptor-mediated, distinct cell populations can be targeted for dose-controlled transient protein expression.

Noteworthy, gene transfer via RMT prevents the problem of insertional mutagenesis, the dysregulation of neighboring genes by integration of the vector cassette into the

host cell chromatin (Li et al. 2002; Hacein-Bey-Abina et al. 2003a; Hacein-Bey-Abina et al. 2003b). Therefore, retroviral non-integrating and transient gene transfer approaches, such as episomal DNA (e.g. 2-LTR circles initiated by integrase-deficient lentiviral vectors) and the transfer of translatable mRNA are desirable and represent useful alternatives to the conventionally used integrating vector systems.

Generally, RMT might be particularly useful and of relevance in applications where relatively low and transient expression of proteins confers striking biological effects. One interesting area of application would be to modify cell fate by transiently introducing proteins that lead to defined changes in cell behavior. Examples are the expression of receptors involved in homing of circulating stem cells, transcription factors or other cellular proteins regulating cell expansion and differentiation, and recombinases (e.g. nlsCre) or integrases for targeted genetic interventions. The following paragraphs will give a few examples of possible scenarios.

The ectopic expression of the homeobox transcription factors HoxB4 (Antonchuk et al. 2002) or Nup-HoxA10 fusion protein (Pineault et al. 2005) are useful tools for expansion of hematopoietic stem cells (HSC) *in vitro*. However, their stable ectopic expression can drastically alter lineage repopulation ability (Schiedlmeier et al. 2003) *in vivo* and even contribute to leukemogenesis (Zhang et al. 2008). Thus, HoxB4 expression via RMT would be a safer alternative.

Recently, transcription factors (Oct3/4, Sox2, Klf4 and optionally also c-Myc) needed for genetic reprogramming of somatic (differentiated) cells to an embryonic stem cell-like state (so called induced pluripotent stem cells, iPS) have been identified (Takahashi and Yamanaka 2006; Meissner et al. 2007). Induction of pluripotent stem cells was demonstrated from murine and human fibroblasts. Interestingly, the four factors necessary for reprogramming the fibroblasts into iPS cells only had to be expressed for 10-12 days (Brambrink et al. 2008; Stadtfeld et al. 2008). Since the shutdown of the three (four) introduced factors after 10-12 days is a prerequisite for normal cell differentiation (Brambrink et al. 2008), RMT could be a useful tool for reprogramming differentiated cells to iPS cells.

A third example may be the use of RMT for cancer gene therapy. The transient expression of apoptosis inducing proteins in cancer cells, such as Bax (BCL-2-associated X protein) and Bak (BCL-2-antagonist/killer-1), which are involved in mitochondrial fragmentation and thereby release of apoptogenic (cytochrome C) and apoptosis inducing factors (AIF), could be sufficient to trigger cell death (Tsujimoto 1998; Kuwana et al. 2005). An alternative would be the transient expression of

cytotoxic proteins, such as diphtheria toxin A or plant-derived toxins Aralin (from *Aralia elata*) or Ricin (from *Ricinus communis*), which are known to efficiently inactivate protein synthesis, thereby causing cell death (Olsnes 1978; Maxwell et al. 1986; Lord et al. 1994; Tomatsu et al. 2003; Michl and Gress 2004).

Provided that RMT-mediated transgene expression levels are sufficient to induce adequate immune responses, it might be feasible to use RMT particles as either prophylactic or therapeutic tools for vaccination against infectious diseases or cancer. So far, conditionally replicating vectors (replicating properties are under the control of a tetracycline-regulated promoter) and genome free particles (so-called virus-like particles, VLPs) have been developed for different types of protective cellular immunity (Dalba et al. 2007). Whereas conditionally replicating vectors still suffer from some level of leakiness (intrinsic activity refractory to doxycycline) and thereby impair safety (Pluta et al. 2005), VLPs have demonstrated safe and efficient induction of humoral and cellular immune responses in animal studies (Boisgerault et al. 2002) as well as in phase II and III clinical trials (Harper et al. 2004; Villa 2006). Thus, the expression of specific antigens via RMT in dendritic or other antigen presenting cells could be an interesting tool for vaccination.

Besides this multitude of practical implications associated with our discovery of the RMT process, this thesis also introduces RMT as a novel tool to obtain insights into unsolved processes of the retroviral life cycle. Here, the interaction of cellular host factors (huTRIM5α, Fv1) with RMT particles has been studied. In the case of huTRIM5α-mediated restriction, our data has led to a more complete picture of huTRIM5α-mediated restriction of N-tropic gammaretroviral particles. Therefore, RMT might be an interesting tool to study other retrovirus-host interactions, especially those that are upstream and independent of reverse transcription (e.g. retroviral disassembly, RTC formation). The gain of further insights into retrovirus-host interactions and/or the retroviral life cycle will allow the development of new antiretroviral therapies. Furthermore, the more detailed understanding of processes within the retroviral life cycle can be used for the enhancement of retroviral or lentiviral vector technologies. Conversely, further and deeper insights into retroviral entry and disassembly mechanisms may allow further improvement of RMT, leading to higher protein expression levels in target cells.

In conclusion, RMT vector particles hold great promise for applications in which low and transient expression of proteins achieves striking biological effects, and are

useful tools to decipher retrovirus-host interactions early after entry before proviral DNA synthesis.

G. Appendix

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2. List of own publications

1. **Galla M.**, Schambach A., Towers G. J., and Baum C.; Cellular Restriction of Particle-Mediated mRNA Transfer; Journal of Virology 2008, Vol. 82, p. 3069-3077.
2. Schambach A., **Galla M.**, Maetzig T., Loew R., and Baum C.; Improving Transcriptional Termination of Self-Inactivating Gammaretroviral and Lentiviral Vectors, Molecular Therapy 2007, Vol. 15, p. 1167-1173
3. Kraunus J., Zychlinski D., Heise T., **Galla M.**, Bohne J., and Baum C.; Journal of Biological Chemistry 2006, Vol. 281, p. 37381-37390.
4. Schambach A., Müller D., **Galla M.**, Verstegen M. M. A., Wagemaker G., Baum C., Bohne J.; Overcoming Promoter Competition in Packaging Cells Improves Production of Self-Inactivating Retroviral Vectors; Gene Therapy 2006, Vol. 13, p. 1524-1533.
5. Baum C., Schambach A., Bohne J., **Galla M.**; Retrovirus Vectors: Toward the Plentivirus?; Molecular Therapy 2006, Vol. 13, p. 1050-1063.
6. Schambach A., **Galla M.**, Modlich U., Will E., Chandra E., Reeves L., Colbert M., Williams D. A., von Kallle C., and Baum C.; Lentiviral Vectors Pseudotyped with Murine Ecotropic Envelope: Increased Biosafety and Convenience in Preclinical Research; Experimental Hematology, Vol. 34, p. 588-592.
7. **Galla M.**, Will E., Kraunus J., Chen L., and Baum C.; Retroviral Pseudotransduction for targeted Cell Manipulation; Molecular Cell 2004, Vol. 16, p. 309-315.

3. Curriculum vitae

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1980-1984:	Gesamtschule Horn, Hamburg.
1985-1993:	Gymnasium St. Georg, Hamburg: Abitur.
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Diploma thesis

Nov 2001- Aug 2002: "Entwicklung von Techniken zur reversiblen Expansion von Blutstammzellen", Heinrich-Pette Institute for Experimental Virology and Immunology, Hamburg, Germany. Department of Cell and Virus Genetics, Prof. Dr. W. Ostertag.

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5. Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation “Retroviral particle-mediated mRNA transfer” selbständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 16. Mai 2008

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Melanie Galla