Targeted lentiviral vectors pseudotyped with the *Tupaia paramyxovirus* glycoproteins

Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
Diplom-Biologin Theresa Enkirch
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Oral examination:

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DECLARATION BY THE CANDIDATE

I hereby declare that this thesis is my own work and effort.
Where other sources of information have been used,
they have been indicated or acknowledged.

Signature:

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1A. SUMMARY

Lentiviral vectors (LVs) are vectors of choice for many gene therapy applications since they mediate long term gene expression and can transduce dividing and non-dividing cells. Recently, efficient targeting of LVs pseudotyped with the *measles virus* (MV) glycoproteins has been reported. However, MV antibodies in patients might limit the clinical use of these vectors. Thus, aim of this study was the development of targeted LVs pseudotyped with the glycoproteins of *Tupaia paramyxovirus* (TPMV). Since this animal paramyxovirus does not infect humans, no TPMV antibodies in patients are expected. For efficient incorporation in LVs, the TPMV glycoproteins, the hemagglutinin (H) and fusion (F) protein, were modified by truncation of their cytoplasmic tails. Targeting was achieved by displaying a single-chain antibody against the B cell surface marker CD20 on the H protein. The modified proteins were biochemically characterized and tested for their functionality. Unexpectedly, it was observed that an additional proteolytic cleavage of the F protein occurs during activation, resulting in the fragments F1a, F1b and F2. The newly identified fragment F1a was detected in virions and in supernatant of transfected cells. The F1a/F1b cleavage site was mapped and a cysteine protease was identified as likely activating protease. The data indicate that F protein processing is more complex than expected.

After characterization, the modified TPMV glycoproteins were screened in all combinations for their ability to form functional pseudotyped LVs. Most efficient pseudotype formation was achieved with CT truncations of 80 amino acids (aa) for H (HΔ80αCD20) and 32 aa for F (FΔ32) (titers ~ 10^6 t.u./ml). The resulting vectors selectively transduced CD20-positive cells in a mixed cell population. Furthermore, they mediated efficient gene transfer into activated and quiescent primary human B cells. Neutralization assays showed that TPMV-pseudotyped vectors were not neutralized by human sera containing MV antibodies.

In conclusion, it was demonstrated that targeted LVs pseudotyped with TPMV glycoproteins can be generated and escape neutralization by MV antibodies. Remarkably, the vectors are able to efficiently transduce even quiescent B cells. Hence, they might be a valuable vector choice when systemic application of targeted lentiviral vectors in humans is required.

1B. ZUSAMMENFASSUNG

Lentivirale Vektoren (LV) sind für viele Anwendungen in der Gentherapie besonders gut geeignet, da das eingebrachte Gen über einen langen Zeitraum exprimiert wird und sie mitotisch aktive und inaktive Zellen transduzieren können. Kürzlich wurden zielgerichtete LV entwickelt, welche mit *Masernvirus* (MV)-Glykoproteinen pseudotypisiert sind. Allerdings würden MV-Antikörper in Patienten die klinische Anwendung dieser Vektoren wahrscheinlich erschweren. Deshalb wurden in dieser Arbeit LV entwickelt, welche mit den Glykoproteinen des *Tupaia paramyxovirus* (TPMV) pseudotypisiert sind. Da es sich dabei um ein für den Menschen nicht infektiöses Tier-Paramyxovirus handelt, werden keine Antikörper in Patienten gegen dieses Virus erwartet. Für einen effizienten Einbau der TPMV-Glykoproteine in LV, nämlich das Hämagglutintin (H) und Fusionsprotein (F), wurden die zytoplasmatischen Domänen (ZD) der Proteine verkürzt. Zielgerichteter Zelleintritt wurde ermöglicht, indem ein einkettiges Antikörper-Fragment (single chain antibody, scAb) gegen das B-Zell-Oberflächenmolekül CD20 an das H-Protein fusioniert wurde. Die modifizierten Proteine wurden biochemisch charakterisiert und auf ihre Funktionalität geprüft. Dabei wurde ein neues Fragment des F-Proteins detektiert (F1a), das aus einer unerwarteten zusätzlichen Spaltung des F-Proteins stammt und sowohl in Virionen als auch im Überstand transfizierter Zellen nachgewiesen wurde. Die entsprechende Spaltstelle wurde lokalisiert und eine Cystein-Protease als wahrscheinlich aktivierende Protease identifiziert. Die Daten deuten darauf hin, dass die Aktivierung des F-Proteins komplexer ist als ursprünglich gedacht.

Die modifizierten Glykoproteine wurden des Weiteren in allen Kombinationen darauf getestet, funktionale pseudotypisierte LV zu bilden. Am effizientesten war eine Verkürzung der ZD von 80 Aminosäuren (AS) für H (HΔ80αCD20) und 32 AS für F (FΔ32) (Titer ~ 106 t.u./ml). Die entsprechenden Vektoren transduzierten selektiv CD20-positive Zellen in einer gemischten Zellpopulation und außerdem aktivierte und ruhende primäre humane B-Zellen. Neutralisations-Experimente zeigten, dass die Vektoren nicht von Humanserum mit MV-Antikörpern neutralisiert werden.

Die in dieser Arbeit entwickelten Vektoren ermöglichen gezielten Zelleintritt und transduzieren bemerkenswerter Weise sogar ruhende B-Zellen. Folglich würden sie sich sehr für gentherapeutische Anwendungen in Menschen eignen.

2. ABBREVIATIONS

α	anti-
αCD20-scAb	single-chain antibody directed against CD20
A	absorption or amper
Aa	amino acids
Ac	acetate
Amp	ampicillin
APS	ammonium peroxydisulfate
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
AZT	azidothymidine
BCR	B cell antigen receptor
BSA	Bovine Serum Albumin
°C	degree Celcius
ca.	circa
CMV	cytomegalovirus
CT	cytoplasmic tail
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DTT	dithio-1,4-threithol
ECACC	European Collection of Cell Cultures
ECL	enhanced chemiluminescence
E. Coli	Escherichia Coli
EDTA	ethylene-diamine-tetra-acetate
e.g.	for example
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
Env	envelope protein
et. al.	and others

F	fusion protein
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescence isothiocyanate
FP	fusion peptide
g	gram or gravitational acceleration
gag	group specific antigen
GALV	gibbon ape leukemia virus
GFP	green fluorescent protein
h	hour
H	hemagglutinin
HEK	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HeV	Hendravirus
HIV-1	human immunodeficiency virus-1
HRP	horseradish peroxidase
ICLC	Interlab Cell Line Collection
IL	Interleukin
kDa	kilodalton
l	liter
LB	Luria-Bertani
LV	lentiviral vector
LTR	long terminal repeats
m	milli-
M	Molar
mAb	monoclonal antibody
MFI	mean fluorescence intensity
µ	micro-
min	minute
MLV	murine leukemia virus
MOI	multiplicity of infection
MV	measles virus
n	nano-

NEB	New England Biolabs
NiV	Nipahvirus
OD	optical density
p.a.	pro analysis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-Phycoerythrin
PEI	polyethylenimine
pol	polymerase
ψ	psi-packaging signal of retroviral genomic RNA
RNA	ribonucleic acid
rpm	rounds per minute
RPMI	culture medium developed in the "Roswell Park Memorial Institute"
RT	room temperature
scAb	single-chain antibody
SDS	sodium dodecyl sulfate
sec	seconds
SFFV	spleen focus forming virus
SIV	simian immunodeficiency virus
SLAM	signaling lymphocyte activation molecule
SP	signal peptide
TBF	Tupaia baby fibroblasts
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TM	transmembrane domain
TPMV	Tupaia paramyxovirus
Tris	tris(hydroxymethyl)aminomethane
t.u.	transducing units
U	unit
UV	ultraviolet
V	volt
VSV-G	vesicular stomatitis virus-glycoprotein
WB	Western Blot

3. INTRODUCTION

3.1. Lentiviral vectors for gene therapy

Gene therapy is the treatment of a disease or a medical disorder by delivering genes into appropriate cells (Verma and Weitzman, 2005). Lentiviral vectors (LVs) are becoming a more and more attractive gene transfer system for the therapy of a variety of diseases, as they mediate long term gene expression and transduce both dividing and non-dividing cells (Matrai, Chuah, and VandenDriessche, 2010). These properties are advantageous in many gene therapeutic applications, because they allow stable transduction of, for example, terminally differentiated cells, like neuronal cells. LVs can accommodate large transgenes (up to ~ 10 kb) (Kaiser, 2003) and for example, change gene expression by introducing a therapeutic gene or kill a certain cell type by introducing suicide genes. Another strategy for therapy is to mediate antigen expression and presentation by transduction of dendritic cells with lentiviral vectors to activate the immune response of the host. Other possible target tissues include different areas of the brain, liver, muscle cells and bone marrow. While some cell types, mainly hematopoietic stem cells, can be transduced *ex vivo* and returned to the patient (Cartier et al., 2009), other cell types, like brain cells, can currently only be reached by direct injection of the viral vector.

Vectors based on retroviruses have already been tested in clinical trials. In the year 2000, eleven children with X-linked severe combined immunodeficiency (X-SCID) were cured by transduction of bone marrow with a vector based on the mouse leukemia virus (MLV), leading to correction of the defective gene (Cavazzana-Calvo et al., 2000). Unfortunately, some of the children developed leukemia due to insertional mutagenesis (Kaiser, 2003). Lentiviral vectors have been shown to harbour a lower risk of mutagenesis from transgene integration and therefore, they may substitute retroviral vectors in the future (Hematti et al., 2004; Modlich et al., 2009).

Currently, lentiviral vectors are applied in about 2.3 % of clinical trials (http://www.wiley.com//legacy/wileychi/genmed/clinical/), and the number is increasing. Although these vectors are a promising tool, vector specificity and safety have to be improved. Targeted vectors that can be systemically administered to the patient and still only transduce selected cells would be a major improvement in vector technology (Waehler, Russell, and Curiel, 2007).

3.1.1. Vector design

The first retroviral vectors developed for gene transfer were based on γ-retroviruses such as the murine leukemia virus (MLV) (Mann, Mulligan, and Baltimore, 1983). These retroviral vectors were also used in the first approved human gene therapy trial to correct severe combined immunodeficiency (SCID) (Anderson, Blaese, and Culver, 1990; Blaese et al., 1993; Levine and Friedmann, 1991). In recent years, lentiviral vectors in contrast to γ-retrovirus-based vectors have become more and more popular. They are derived from lentiviruses that, like γ-retroviruses, belong to the *Retroviridae*, a family of enveloped single-stranded (ss) RNA viruses of around 80 to 120 nm diameter (Vogt and Simon, 1999). The best known example of lentiviruses is the human immunodeficiency virus-1 (HIV-1). The genome consists of two copies of positive single- stranded RNA which, together with the viral replication enzymes, is enclosed by the capsid (Fig. 1). The viral envelope, that surrounds the capsid, is composed of the host cell membrane and complexes of the viral envelope protein Env which mediate attachment of the virus to its receptors on the host cell surface, enabling pH-independent fusion of the viral and cellular membrane (Freed, 2007).
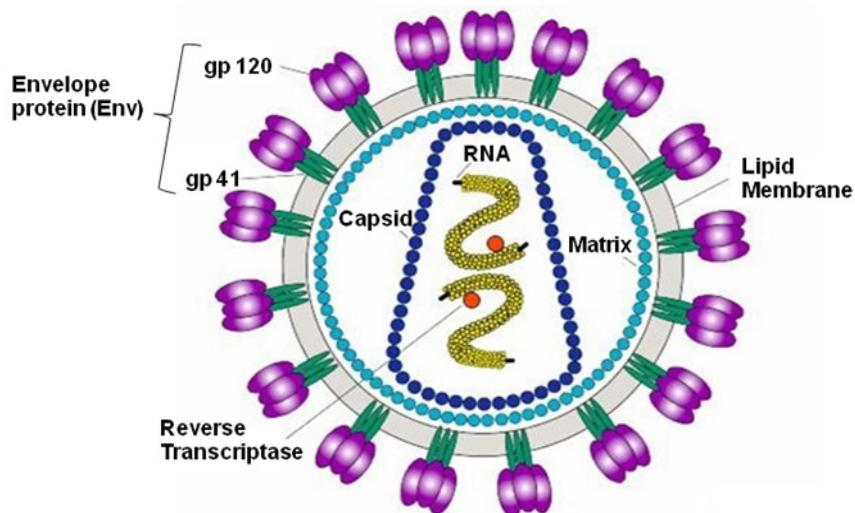
Figure 1: Schematic drawing of the lentivirus HIV-1
The two ssRNA molecules that are associated with the reverse transcriptase are indicated. They are surrounded by the capsid which is composed of the capsid protein p24. The membrane-associated matrix underlies the cell-derived lipid membrane with the inserted viral envelope proteins gp41 and gp120. (modified from http://www.charite.de/maximalmethodisch/e139/e84/hiv1.jpg

The lentiviral genome is more complex than that of other retroviruses. It is organized mainly in the *gag*, *pol* and *env* genes but compared to simple retroviruses, lentiviruses have additional accessory genes that regulate viral gene expression, assembly and replication (Freed, 2007), namely *tat*, *rev*, *nef, vif, vpu* and *vpr* (Fig. 2). The *Gag* gene encodes the structural proteins including the membrane associated matrix protein, the core forming capsid protein and the viral RNA-binding nucleocapsid protein. The viral enzymes that accompany the ssRNA are encoded by the *pol* gene, including the reverse transcriptase, which transcribes the viral RNA to DNA, the integrase, that catalysis integration of the proviral DNA into the host genome and the protease which is responsible for gag-pol cleavage and virion maturation (Katz and Skalka, 1994). The *env* gene encodes the viral envelope.

Figure 2: Schematic representation of the MLV and HIV-1 genome
A: Proviral structure of MLV genome; B: Proviral structure of HIV-1 genome LTR: long terminal repeats; ψ: encapsidation signal psi (modified from (Hu and Pathak, 2000))

On both ends of the retroviral genome there are redundant sequences called long terminal repeats (LTRs) (Coffin, 1996; Vogt, 1997) that can be further divided into U3 (unique 3'), R (repeat) and U5 (unique 5') regions (Fig. 2). The viral promoter, transcription enhancers, a transcription termination signal and polyadenylation signals are located in these regions. The LTRs also contain short sequences, the attachment sites that are important for integration of the viral DNA into the host

chromosomes (Coffin, 1996). The packaging signal ψ or encapsidation signal contains sequences that interact with viral proteins to mediate packaging of the genomic RNA into newly formed virions (Watanabe and Temin, 1982).

The life cycle of lentiviruses can be separated into different steps (Fig. 3): First, the virus binds to its receptors via the glycoproteins of the viral envelope and fuses with the cell membrane. Afterwards, the virus is uncoated and the viral core containing genomic RNA is released into the cytoplasm where the viral ssRNA is reverse transcribed into a dsDNA copy. The dsDNA within the core is then actively transported to the nucleus (Bukrinsky et al., 1993) and integrated into the host genome as a provirus. This active transport is one of the major advantages of lentiviruses over retroviruses, because this is the reason why they are able to infect not only dividing but also non-dividing cells. In contrast, other retroviruses are dependent on cell division and dissolution of the nucleus to integrate their reverse transcribed dsDNA copies. After integration of proviral DNA, the cellular transcription and translation machinery is used to express the viral genes and to generate RNA encoding all viral proteins. This newly generated viral RNA is packaged and a new virus is assembled which leaves the host cell by budding.
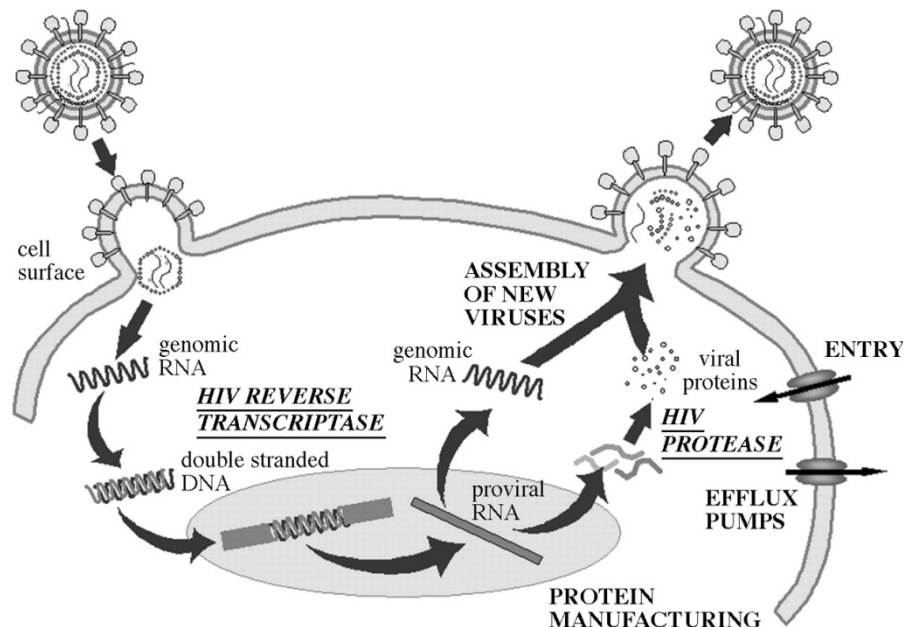
Figure 3: Life cycle of HIV-1
The virus binds to the cell surface and fuses with the host membrane. After entering the host cell, reverse transcriptase produces proviral double-stranded DNA that is transported to the nucleus. The DNA is integrated and viral RNA is synthesized for the production of viral proteins. At the cell membrane, assembly occurs and the virus leaves the cell by budding. (Hoggard and Owen, 2003)

Due to the lentiviral life cycle, it is obvious why they are becoming more and more popular as vectors in gene therapy. They are very efficient in gene transfer, and integration of the transferred genes into the host genome allows long-term gene expression. Vectors derived from lentiviruses are replication-deficient and can carry any gene of interest into a target cell, a process called transduction.

When generating lentiviral vectors some important safety issues have to be considered. First, all non-essential genes, like genes for the accessory proteins and for virulence should be removed from the vector sequence. Second, sequences necessary for vector RNA synthesis, packaging, reverse transcription and integration of cDNA should be split onto a different plasmid than elements encoding the viral enzymes and structural proteins. These so called *cis*- and *trans*-acting sequences should have a minimum of sequence overlap to reduce the risk of homologous recombination and thereby generation of replication-competent vectors. In *trans*, the genes for the polyproteins gag and pol have to be provided. The resulting plasmid is called packaging vector (Fig. 4, A). In *cis*-acting sequences, including the 5' and 3' LTR, the packaging signal ψ and the gene of interest which is under the control of a chosen promoter, are present on a plasmid called transfer vector (Fig. 4, B). The genes encoding the envelope proteins are provided on a third plasmid, the envelope vector (Fig. 4, C). The packaging vector and the envelope vector plasmids both lack a functional ψ-site and are therefore not packaged into vector particles.

The packaging vector plasmids are divided into "generations" depending on the viral sequences provided (Fig. 4, A). The first generation plasmid contains the *gag* and *pol* sequences, the viral regulatory genes *tat* and *rev* and all accessory genes (*vif*, *vpr*, *vpu* and *nef*). To improve lentiviral vector safety, in the second generation the four accessory genes were removed, leaving only the regulatory genes *tat* and *rev* (Zufferey et al., 1997). Tat protein increases the level of viral RNA during production by activating the LTRs and Rev protein interacts with viral RNA containing a Rev Responsive Element (RRE) to be transported from the cell nucleus to the cytoplasm. In the third generation, the *rev* gene is split from the *gag* and *pol* sequences and expressed from a separate plasmid (Dull et al., 1998).

The transfer vector plasmids can be modified by deleting promoter/enhancer sequences in the U3 region of the 3' LTR, resulting in a self-inactivating (SIN) vector (Miyoshi et al., 1997; Zufferey et al., 1997) (Fig. 4, B). During reverse transcription, this deletion is reproduced in the 5' LTR and therefore transcription of the provirus is

inactivated and no packagable RNAs can be produced (Fig. 4, D and E). This modification decreases the risk of replication competent lentiviruses. Additionally, LTR-mediated insertional activation of proto-oncogenes is reduced due to the deleting of the enhancer elements. One of the disadvantages of this vector system is the low transcription activity of the internal promoter of the transgene, in contrast to the promoter/enhancer sequences of the LTR. To overcome this problem and to further improve vector safety, the choice of the promoter is very important, for example, to set the transfer gene under control of an inducible or a cell-specific promoter (transcriptional targeting).
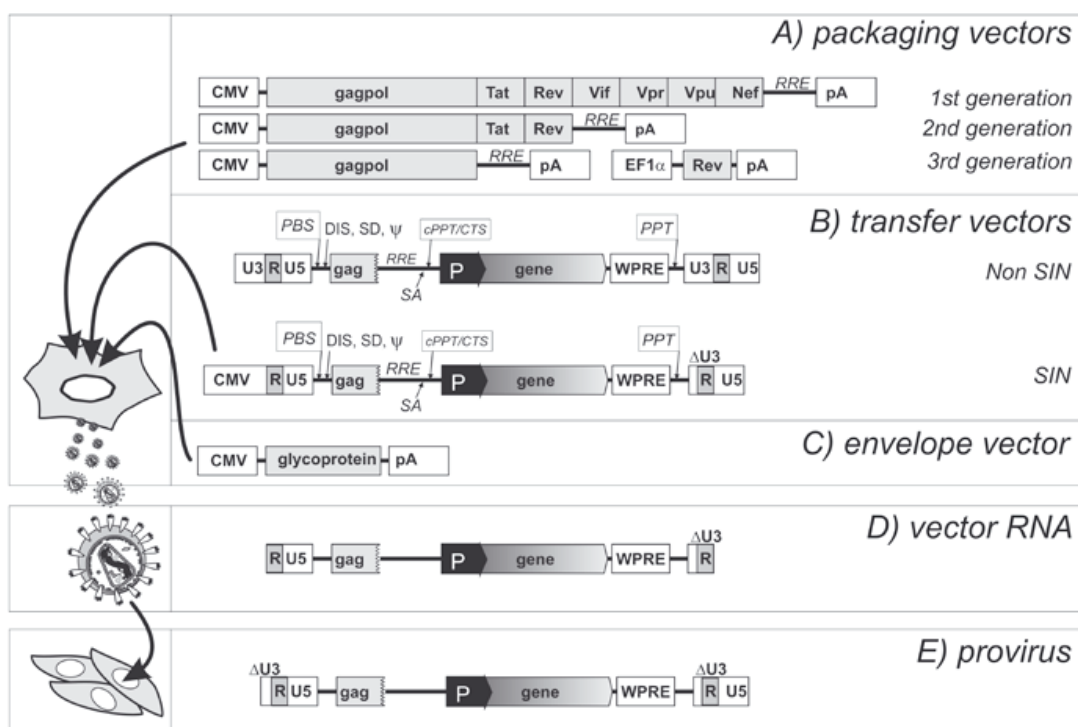
Figure 4: Gene delivery system based on lentiviral vectors
A-C: Different plasmids for production of lentiviral vectors and genes they contain are shown.
D: Schematic drawing of the RNA that the produced vector contains. E: Sequences integrated by the vector in host genome (provirus). CMV: cytomegalovirus immediate-early promoter; EF1α: human elongation factor 1-α promoter; gag: 5' portion of *gag* gene containing dimerization/packaging signals; PBS: primer binding site; DIS: dimerization signal; SD: splice donor site; SA: splice acceptor site; ψ: packaging signal; cPPT: central polypurine tract; CTS: central termination sequence; RRE: Rev response element; PPT: polypurine tract; pA: polyadenylation signal; ΔU3: SIN deletion in U3 region of 3' LTR; P: internal promoter for transgene expression; WPRE: woodchuck hepatitis virus (WHV) post-transcriptional regulatory element. (Pluta and Kacprzak, 2009)

For the production of lentiviral vectors, the packaging plasmid, the transfer plasmid carrying the transgene, e.g. an eGFP-encoding gene and a plasmid encoding the

envelope are transiently co-transfected in packaging cells, most commonly these are human embryonic kidney (HEK) 293T cells (Fig. 5) (Naldini et al., 1996).

Vector particles are released into the supernatant of the producer cells that have incorporated the RNA of the transfer vector but do not contain the genetic material for the core and envelope proteins. Since the viral vectors bud from the cell membrane, the viral envelope is composed of the cellular lipid-bilayer and the incorporated viral envelope proteins. The vector-containing cell supernatant can be used either immediately or concentrated for transduction.
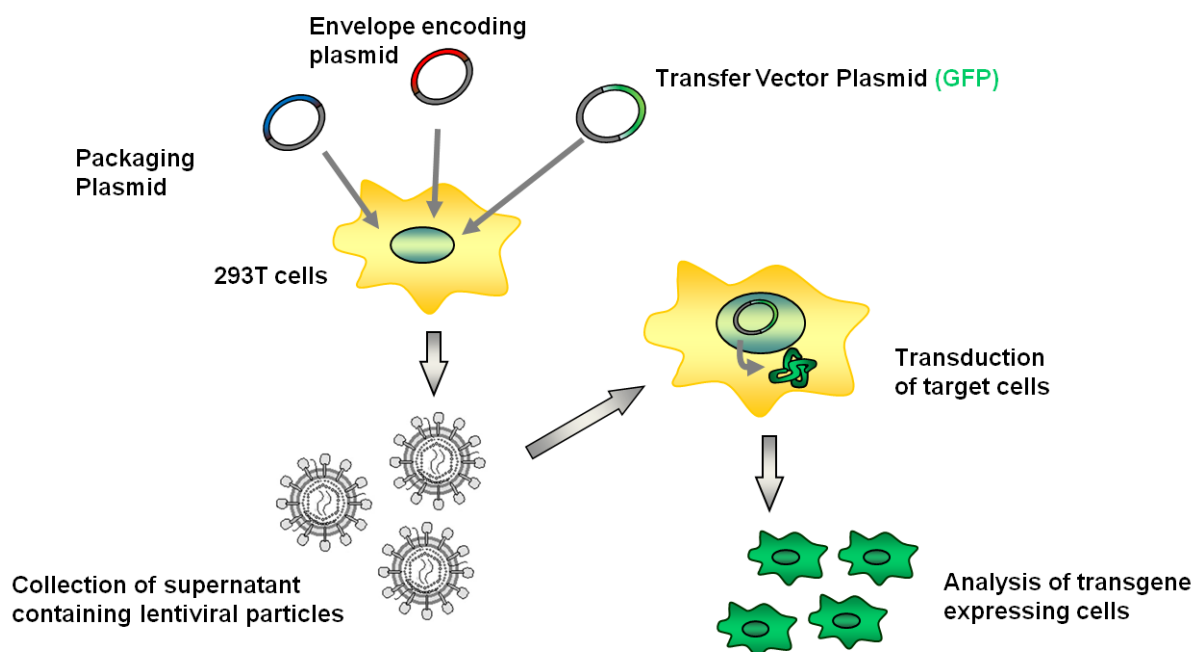
Figure 5: Production of lentiviral vectors
Packaging cells (here 293Tcells) are transfected with a packaging plasmid, a transfer vector plasmids and a plasmid encoding an envelope protein. After 24-48 hours, the viral particle containing supernatant of the producer cells is harvested and used for transduction of target cells. Transduced cells are finally analyzed for transgene expression, e.g. GFP. GFP: green fluorescence protein

Tropism of lentiviral vectors is determined by the viral envelope protein. It interacts with its receptor on the target cell membrane and triggers fusion. Since the HIV-1 wildtype envelope protein has only restricted tropism, glycoproteins derived from other viruses are often used for lentiviral production.

3.1.2. Pseudotyping of lentiviral vectors

One of the great advantages of lentiviral vectors is that their natural tropism can be altered by exchanging the native envelope protein with glycoproteins from different viruses, a process called pseudotyping (Fig. 6).
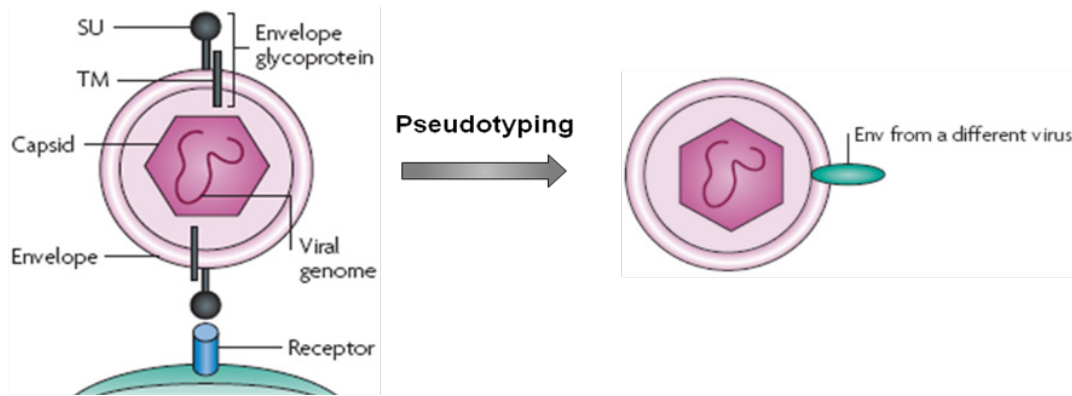
Figure 6: Pseudotyping of lentiviral vectors
A lentivirus binds to a receptor via specific glycoproteins. This interaction determines the host range of the virus. Pseudotyping means changing the tropism by replacing the viral attachment glycoprotein with that of a different virus. SU: surface subunit; TM: transmembrane subunit; Env: envelope glycoprotein (modified from (Waehler, Russell, and Curiel, 2007))

Lentiviral vectors are highly permissive for incorporation of glycoproteins derived from other viruses (Cronin, Zhang, and Reiser, 2005; Schnierle et al., 1997). The pseudotyped particles possess the ability to transduce target cells of the virus from which the envelope protein was derived. Consequently, pseudotyping allows infection of target cells that the virus does not naturally infect.

The key event for pseudotyping lentiviral vectors was the observation made when HIV-1 was produced in cells infected with different viruses, e.g. amphotropic murine leukemia virus (MLV) (Chesebro, Wehrly, and Maury, 1990; Spector et al., 1990) or herpes simplex virus (HSV) (Zhu, Chen, and Huang, 1990). The resulting virions showed an expanding host range, suggesting that envelope proteins of MLV and HSV were incorporated.

The molecular events occurring during pseudotyping of lentiviral vectors are still not fully understood. There are two mechanisms proposed that lead to incorporation of viral proteins in viral vectors, an active and a passive mechanism. For active incorporation, direct interactions of the cytoplasmic tail (CT) of the envelope protein

and components of the virion core or indirect interactions via cellular factors with viral gag proteins are required (Murakami and Freed, 2000a; Murakami and Freed, 2000b; Swanstrom and Wills, 1997). During passive incorporation, a nonspecific interaction between the cytoplasmic tail and the viral core takes place, assuming that there is no steric incompatibility of the CT with viral assembly and that there are sufficient amounts of envelope proteins presented at the site of virus budding (Swanstrom and Wills, 1997). Lipid rafts play an important role in pseudotyping. It was shown that many viruses such as C-type retrovirus, Lentivirus and Paramyxoviurs reassemble their envelope in these microdomains of the plasma membrane (Pickl, Pimentel-Muinos, and Seed, 2001). As a consequence, the envelope proteins of other viruses and cellular components are incorporated into viral particles during co-infection.

Today, the most common envelope protein used for pseudotyping is the glycoprotein of vesicular stomatitis virus, VSV-G. The possibility of pseudotyping LVs with VSV-G was first demonstrated 1996 independently by three different groups (Akkina et al., 1996; Naldini et al., 1996; Reiser et al., 1996). VSV-G-pseudotyped vectors have a high stability, a broad host range and high titers can be generated. However, since the aim of viral gene therapy is to target only specific cells or tissues, the broad tropism of VSV-G can be disadvantageous. For specific targeting of cells, glycoproteins of viruses that naturally infect only one cell type are often used. Examples are transduction of neuronal tissue by lyssavirus-pseudotyped LVs (Desmaris et al., 2001; Watson et al., 2002) or airway epithelium transduction by filovirus–pseudotypes (Chan et al., 2000; Kobinger et al., 2001).

One of the problems of pseudotyping LVs is that often the titers reached by pseudotyped lentiviral vectors are very low due to insufficient incorporation of heterologous glycoproteins. To overcome this problem the cytoplasmic tails of some glycoproteins have to be modified. As mentioned before, CT interactions with the viral core may play an important role in glycoprotein incorporation. It was shown that most mammalian and avian retroviruses such as HIV, MLV and ALV (avian leukemia virus) require proteolytic cleavage of the R-peptide, a short amino acid sequence (16 aa) at the C-terminus of CT of the envelope glycoprotein for activation (Bobkova et al., 2002; Green et al., 1981; Henderson et al., 1984; Schneider et al., 2011). Pseudotyping of retroviral vectors using paramyxovirus glycoproteins requires truncation of the proteins' cytoplasmic tails. The fusion (F) and attachment (H) proteins of the measles virus can only be incorporated into lentiviral vectors when

their cytoplasmic tails are truncated (Funke et al., 2008a; Funke et al., 2009). In another example the F (fusion) and HN (hemmagglutinin-neuraminidase) protein of Sendai virus were efficiently incorporated into a simian immunodeficiency virus (SIV) derived vector after truncation of the F protein cytoplasmic tail. Additionally, in this system the CT of the SIV envelope protein was added to the CT of the hemmagglutinin-neuraminidase (Kobayashi et al., 2003). Recently, another study was published where a chimeric glycoprotein was used to pseudotype a lentiviral vector. In this study, the cytoplasmic tail of rabies virus glycoprotein (RVG) was replaced with the cytoplasmic region of VSV-G. The resulting vectors showed an increase in transduction efficiency due to higher levels of glycoprotein incorporation and efficiently transduced the central nervous system of rats *in vivo* (Carpentier et al., 2011).

To date, glycoproteins of many virus families have been used for pseudotyping. But often, the specificities of the used glycoproteins are not sufficient to transduce only one certain cell type. To further narrow the tropism of viral vectors for gene therapy, additional modifications are necessary.

3.1.3. Targeting strategies for lentiviral vectors

Lentiviral vectors pseudotyped with heterologous glycoproteins allow transduction of a certain cell type or tissue, dependent on the tropism of the virus the glycoproteins are derived from. Since many viruses naturally infect more than one kind of tissue or cell type, different techniques to further increase efficiency and specificity of vectors were developed. One of the targeting strategies to restrict tropism is the use of specific promoters that are active only in target cells, a process called transcriptional targeting (Waehler, Russell, and Curiel, 2007). Examples for the use of LVs with cell type specific promoters are expression of a transgene in endothelial cells (De Palma, Venneri, and Naldini, 2003), in retinal cells (Miyoshi et al., 1997) or liver tissue (Oertel et al., 2003; VandenDriessche et al., 2002).

Another strategy is the development of protease-activatable viral vectors. For targeting, a polypeptide is fused to the envelope proteins that blocks attachment or fusion of the vector. Specific proteases on the cell surface are required that cleave the inhibitory peptide, resulting in gene transfer into the target cell (Szecsi et al., 2006).

One of the most desirable strategies remains transductional targeting, which means targeting on the entry level of the viral vector. This technique leaves other tissues or cells completely unaffected and can be achieved for example by the insertion of cell-type specific ligands or other molecules into the viral envelope, like growth factors (EGF), single chain antibodies (scAbs), cytokines or even receptors. The most common approach to alter the tropism of a lentiviral vector is the use of single chain antibodies. These molecules consist of the variable regions of one heavy and one light chain of an immunoglobulin that are connected with a serine-glycine linker and have the same specificity as the original molecule they were derived from. Lentiviral vectors pseudotyped with *Sindbis virus* glycoproteins are an example for targeting using antibodies. The envelope of *Sindbis virus* consists of the E1 and E2 protein, which are responsible for attachment and fusion. Targeting was achieved by a monoclonal antibody that was non-covalently bound to the glycoproteins of the viral envelope (Morizono et al., 2005). Since *Sindbis virus* uses more than one receptor for cell entry, in this approach the receptor binding sites of the E2 protein were mutated, resulting in an engineered virus, that is unable to recognize its natural receptor. One disadvantage of this system is the pH-dependent membrane fusion and the vector instability due to the non-covalent bond between the antibody and the glycoproteins. *Sindbis virus* enters the cell by receptor-mediated endocytosis which is required for E1-E2 activation. Subsequent fusion is induced by low pH in the endosomes.

Recently, efficient targeting of lentiviral vectors pseudotyped with glycoproteins of a paramyxovirus, the *measles virus* (MV) F (fusion) and H (attachment) proteins has been reported (Anliker et al., 2010; Frecha et al., 2008; Funke et al., 2008a; Funke et al., 2009; Munch et al., 2011). In contrast to *Sindbis virus*, the envelope proteins of MV mediate pH-independent membrane fusion and endocytosis for glycoprotein activation is not required. Targeting of MV is based on the covalently bond of specificity domains on the viral hemagglutinin (Cattaneo et al., 2008; Navaratnarajah, Leonard, and Cattaneo, 2009). However, to achieve full targeting, modifications of the H protein to avoid binding to its natural receptors CD46, SLAM and the recently identified nectin 4 are necessary (Nakamura et al., 2005; Noyce et al., 2011; Vongpunsawad et al., 2004). A potential disadvantage of the MV system is the presence of neutralizing antibodies in nearly all patients, either by previous infection with wild-type virus or by vaccination. In an animal colon carcinoma model, the

therapeutic effect of oncolytic MV vectors was abrogated in animals vaccinated against MV; however when the MV glycoproteins were replaced by those of the related animal paramyxovirus *Canine distemper virus* (CDV), the oncolytic effect was restored (Miest et al., 2011).

Nevertheless, glycoproteins of *measles virus* have great potential to pseudotype lentiviral vectors, since their fusion and attachment functions are separated into two proteins and retargeting can be achieved by displaying small molecules on the H protein. It has to be investigated, if other paramyxovirus envelope proteins, for example of non-human viruses that will not be neutralized by preexisting antibodies in patients, can also be targeted and used to pseudotype lentiviral vectors.

3.2.   Paramyxoviruses

The *Paramyxoviridae* are a family within the order *Mononegavirales.* They are enveloped negative-strand RNA viruses that are widespread among humans and animals. The family of *Paramyxoviridae* is further classified into the two subfamilies *Paramyxovirinae* and *Pneumovirinae*. Genera of *Paramyxovirinae* are *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Morbilivirus* and *Henipavirus*. *Pneumovirinae* contains two genera, *Pneumovirus* and *Metapneumovirus*. Each of the five genera of *Paramyxovirinae* includes species that are highly pathogenic for humans. Most prominent examples are the *measles virus*, which is a *Morbilivirus*, *mumps virus* (*Rubulavirus*) and *Sendai virus* (*Respirovirus*). In recent years, the *Hendra* and *Nipah viruses* (*Henipaviruses*) were identified which cause deadly diseases in animals and humans. The most famous member of *Pneumovirinae* is the *Human respiratory syncytial virus* (HRSV) which is often the causative agent for respiratory infections in young children (Counihan et al., 2001). There are several species of the paramyxoviruses that have not yet been classified into genera, for example *J virus, Mossmann virus* and the *Tupaia paramyxovirus* (Lamb, 2007).

All members of this virus family have a similar viral structure (Fig. 7). They are enveloped viruses with a diameter of 150 to 300 nm and in some cases even greater than 1 µm (Goldsmith et al., 2003). The envelope is a cell-derived lipid bilayer in which two kinds of virus-derived glycoproteins are embedded, the attachment protein and the fusion protein. The attachment glycoproteins are the HN (hemagglutinin-neuramidase) for *Respiroviruses*, *Rubulaviruses* and *Avulaviruses,* the H

(hemagglutinin) for *Morbilliviruses* and the G (glycoprotein) for *Henipaviruses* and members of *Pneumovirinae*. Dependent on the genera, the attachment proteins possess hemagglutination activity only (H), or hemagglutination and neuraminidase activity (HN) or none of these activities (G). The other glycoprotein is the F (fusion) protein that mediates fusion of the viral envelope with the cell membrane. Fusion occurs pH-independently. The glycoproteins are abundantly incorporated into the envelope and form spike-like complexes. The genome is enclosed by the envelope and consists of a non-segmented, negative RNA strand which is bound to the nucleocapsid to form helical structures called ribonucleoproteins (RNPs). The matrix (M) protein which organizes virus assembly and maintains viral structure is assembled between the envelope and the nucleocapsid.
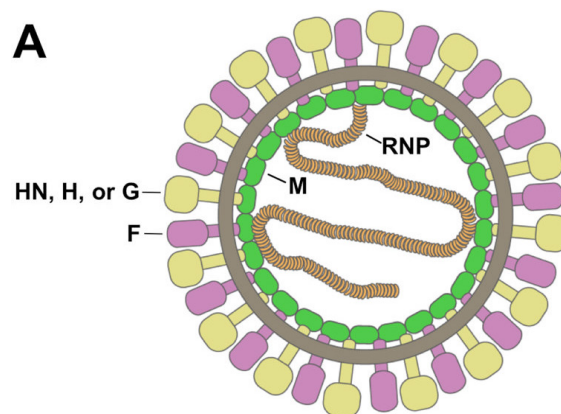
Figure 7: Schematic representation of a paramyxovirus
The M protein is underlying the inner surface of the lipid envelope in which the viral glycoproteins (HN, H and F or G) are embedded. The RNP is associated with the M protein and contains the negative-strand single-stranded RNA genome, the viral RNA-dependent RNA-polymerase and the N and P proteins. HN: hemagglutinin-neuraminidase; H: hemagglutinin; G: glycoprotein; F: fusion protein; M: matrix protein; RNP: ribonucleoproteins (Harrison, Sakaguchi, and Schmitt, 2010)

The life cycle of paramyxoviruses (Fig. 8) starts with binding of the virus to a receptor molecule on the surface of a target cell, followed by fusion of the viral membrane with the target cell membrane. Afterwards, the virion content including the RNP is released into the cytoplasma of the target cell and viral transcription starts. The viral RNA-dependent RNA polymerase complexes produce individual messenger RNAs from the negative-sense viral genomic RNA that are then translated into viral proteins.

Genes closest to the 3' end are transcribed more abundantly than those towards the 5' end, because the RNA polymerase often dissociates from the RNA genome and has to reenter it at the 3' end. As a result, there is a gradient of gene expression regulated by the position of each gene relative to the single promotor (Cattaneo et al., 1987a; Whelan, Barr, and Wertz, 2004). The negative-sense viral genomic RNA not only serves as a template for transcription but also for replication. First, positive-sense antigenomes are produced that are followed by production of negative-sense genomes from the antigenome template. The newly synthesized genomic RNA together with the newly produced viral proteins is transported to the cell membrane where assembly occurs and new viruses leave the infected cell by budding.
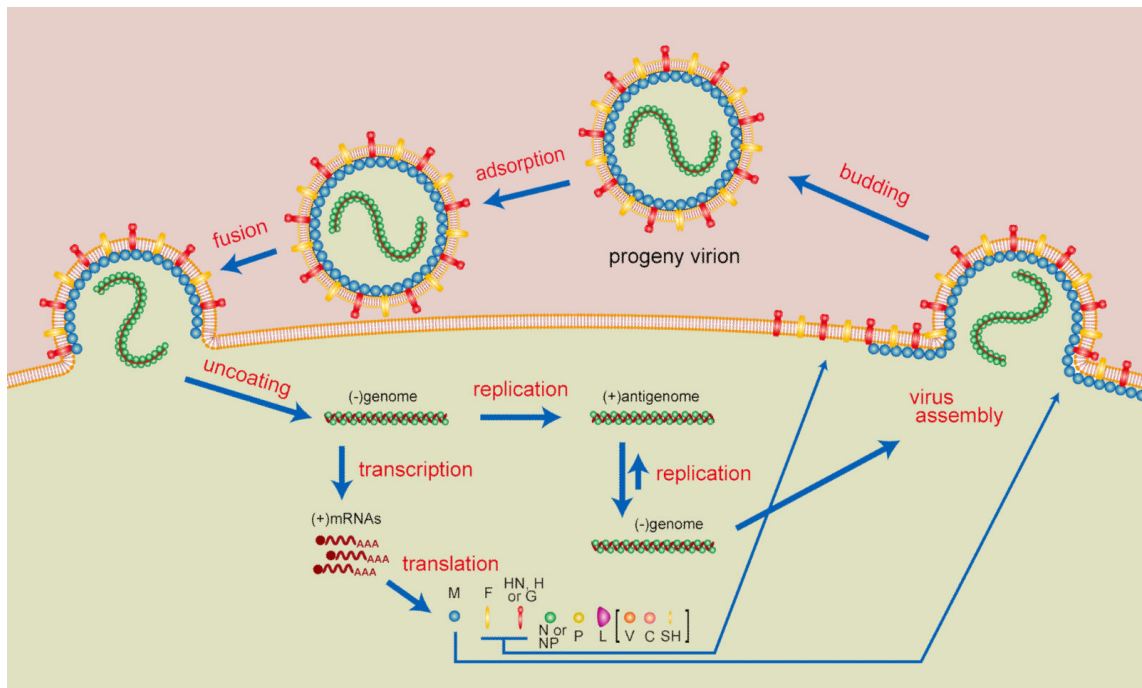
Figure 8: Life cycle of paramyxoviruses
After attachment and fusion of the virus with the cell membrane, the ss(-) RNA genome is released and transcribed into positive-stranded messenger RNA (mRNA) which is then translated into the viral proteins. For virus replication, a positive-stranded antigenome is produced that serves as a template for viral ss(-)RNA genome. Transcription and replication occur in the cytoplasm. Viral structure proteins and RNPs containing RNA genome assemble together and leave the infected cell by budding. M: matrix protein; F: fusion protein; HN: hemagglutinin-neuraminidase; H: hemagglutinin; G: glycoprotein; N or NP: nucleocapsid protein; P: phosphoprotein; L: large protein: V: protein V; C: cellular protein; SH: strongly hydrophobic protein (Harrison, Sakaguchi, and Schmitt, 2010)

3.2.1. The *Tupaia paramyxovirus*

The *Tupaia paramyxovirus* (TPMV) was first isolated in 1999 from spontaneously degenerating primary kidney cells of an apparently healthy Southeast Asian tree shrew (*Tupaia belangeri*) (Tidona et al., 1999). Tree shrews are related to insectivores and primates, but are classified as a separate mammalian order (Scandentia). These animals are phylogenetically more closely related to primates and humans than any other laboratory animal. This makes them very interesting as an animal model for human diseases caused by viral infections (Darai et al., 1978; Xie et al., 1998).

The host range of TPMV is restricted to *Tupaia* cells, it does not grow in any other cell line tested so far. The receptor for cell entry of the virus is not known. Infection of *Tupaia* cells with TPMV results in a cytopathic effect including multinucleated syncytia followed by cell lysis. Viruses released in the supernatant of infected cells reach titers around 10^5 PFU/ml. There is no serological cross-reactivity detected between TPMV and other paramyxoviruses including measles virus, suggesting that TPMV is antigenetically distinct from these viruses.

The TPMV genome shows the typical paramyxovirus gene order N-P/C//V-M-F-H-L (Fig. 9) whereas all genes are separated by the nontranscribed trinucleotide CUU. The complete genome consists of 17,904 nucleotides which is larger than those of most paramyxoviruses (Springfeld et al., 2005).
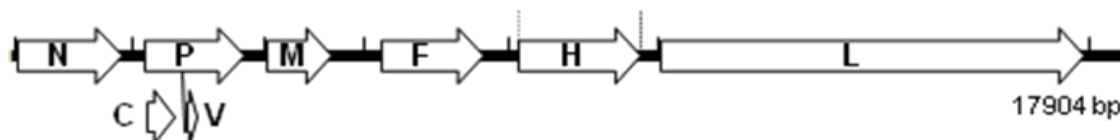
Figure 9: Gene order of the *Tupaia paramyxovirus*
The 17,904-nucleotide single-stranded TPMV antigenome is represented by the black bar. The arrows indicate the positions of the open reading frames (ORFs) of the N, P, C, V, M, F, H and L protein. The small vertical bars mark the positions of the intergenic sequences. (Springfeld et al., 2005)

The six genes of the TPMV genome are flanked by non-coding sequences, a control region called leader at the 3' end and a sequence known as trailer at the 5' end (Lamb, 2007).In general, for paramyxoviruses applies that the N gene encodes the nucleocapsid protein, which is the first gene transcribed from the viral genome and which interacts with viral RNA. The P gene contains overlapping open reading frames (ORFs) and encodes the proteins P (phosphoprotein), V and C. The phosphoprotein has an essential role in RNA-polymerase activity and interacts with

the L and N proteins. The V and C proteins are accessory proteins that probably counteract host antiviral responses (Goodbourn and Randall, 2009; Ramachandran and Horvath, 2009).

The L (large) gene encodes the viral RNA-dependent RNA-polymerase, which binds to the N and P proteins to from ribonucleoproteins (RNPs). The matrix (M) protein encoded by the M gene is associated with the nucleocapsid, interacts with the cytoplasmic tails of the glycoproteins F and H and initiates virus assembly and budding. The glycoproteins encoded by the genes F and H are part of the viral envelope and mediate attachment and fusion to the target cell. Because of their essential role in this thesis they are described in more detail later on.

Analysis of the nucleotide sequence of the N and P gene revealed typical characteristics of the subfamily *Paramyxovririnae* and provided sufficient evidence that TPMV is phylogenetically related to the *Henipaviridae* and the *Morbiliviridae*. But since there are only low amino acid sequence homologies among TPMV, *Henipaviridae* and other known paramyxoviruses, there is the assumption that TPMV represents a new genus within the *Paramyxovirinae (*Fig. 10) (Tidona et al., 1999).
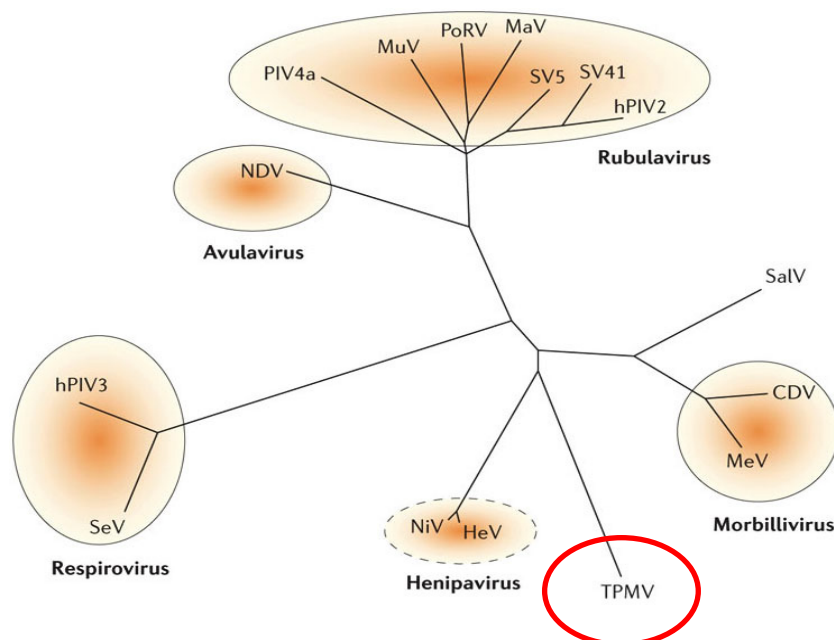
Figure 10: Phylogenetic tree of *Paramyxovirinae* subfamily members
Relationship of *Paramyxovirinae* subfamily members is based on the alignment of the amino-acid sequence of the N gene of selected *Paramyxovirinae* subfamily members. Morbillivirus genus: MeV (measles virus), CDV (canine distemper virus); Henipavirus genus: HeV (Hendra virus), NiV (Nipah virus); Respirovirus genus: SeV (Sendai virus), hPIV3 (human parainfluenza virus 3); Avulavirus genus: NDV (Newcastle disease virus); Rubulavirus genus: hPIV2 (human parainfluenza virus 2), MaV (Mapuera virus), MuV (mumps virus), PIV4a (parainfluenza virus 4a), PoRV (porcine rubulavirus), SV5 (simian parainfluenza virus 5), SV41 (simian parainfluenza virus 41); and unclassified viruses SalV (Salem virus) and TPMV (Tupaia paramyxovirus). (modified from (Eaton et al., 2006))

3.2.2. Glycoproteins of the *Tupaia paramyxovirus*

As already mentioned before, the glycoproteins of TPMV, namely F and H, are responsible for attachment and fusion of the virus with the target cell. The H protein is a type II transmembrane protein with a length of 665 amino acids. Due to the ability of TPMV virions to hemagglutinate *Tupaia* erythrocytes the protein is named hemagglutinin (H). It has no neuraminidase activity (Tidona, 1999). The protein is glycosylated at three N glycosylation sites and the corresponding molecular mass is ~ 80 kDa (Springfeld et al., 2005). Compared to other paramyxovirus attachment proteins, there is only low sequence identity (< 20%) and the cytoplasmic tail of TPMV H is unusually long (94 amino acids). The TPMV H protein mediates attachment of the virus to the cell surface through interactions with an unknown receptor.

A few years ago, it was shown that the TPMV H protein can be targeted to a designated cell type by displaying a single chain antibody (scAb) on the ectodomain (Springfeld et al., 2005), in a similar way as it had previously been shown for *measles virus* H protein (Schneider et al., 2000). Hence, it is possible to target TPMV H to non-*Tupaia* cells via respective ligands.

In contrast to TPMV H, the F protein is a trimeric type I transmembrane protein that mediates fusion of the virus with the host cell membrane. It is expressed as a F0 precursor protein with a length of 553 amino acids including a hydrophobic region, the signal peptide at the N-terminus. During virus maturation and after removal of the signal peptide, the F0 protein is cleaved by an unknown protease into an N-terminal F2 and a C-terminal F1 fragment. The last one contains two hydrophobic regions, a fusion peptide at the amino terminus and the transmembrane domain. Furthermore, the TPMV F protein contains four N glycosylation sites in the ectodomain, three on the F2 fragment and one on the F1 fragment. As mentioned for TPMV H, also for TPMV F the similarity to fusion proteins of other paramyxoviruses is low (33.4 % identity to MV, 31.8 % identity to Nipahvirus).

Fusion of paramyxoviruses occurs at the host cell membrane, it does not require the low pH of the endosome to trigger fusion (Lamb, 2007). In most paramyxoviruses, the attachment protein interacts with the receptor and this interaction initiates conformational changes in the F protein, resulting in activation of the membrane fusion (Lamb and Jardetzky, 2007). When *Tupaia* cells are transfected with plasmids encoding TPMV H and F, the cells fuse and form large syncytia, demonstrating that

no other virus proteins are necessary for attachment and fusion of target cells. This observation and the possibility to retarget the H protein in a similar way to MV H make the TPMV glycoproteins very interesting for pseudotyping lentiviral vectors. Additional advantage compared to MV glycoproteins (described in section 1.3) is that it is not necessary to mutate the contact residues of TPMV H since it does not recognize a receptor on human cells. Because TPMV glycoproteins are derived from an animal paramyxovirus, no neutralizing antibodies in the general population are expected.

3.3. Aim of the study

The aim of this thesis was to generate and characterize lentiviral vectors that are pseudotyped with the glycoproteins of the *Tupaia paramyxovirus* (TPMV).

Based on previously reported work for pseudotyping lentiviral vectors with truncated retargeted MV glycoproteins it was hypothesized that this system could be transferred to use TPMV glycoproteins for pseudotyping. It was previously shown that the TPMV glycoproteins can be targeted to normally non-permissive cells by displaying single-chain antibodies on the H protein that recognize cell surface antigens on target cells. Based on this work, the TPMV glycoproteins H and F had to be further characterized and cytoplasmic tail truncation mutants of H and F had to be screened. Additionally, in the same step, the retargeting possibility of the H protein had to be further investigated by displaying a single chain antibody against the B cell surface marker CD20 on the ectodomain. The contact residues responsible for attachment of the cell do not have to be mutated, since TPMV is restricted to *Tupaia* cells. After characterization, glycoprotein mutants that support incorporation into lentiviral vectors had to be identified for vector production. Furthermore, the produced TPMV-pseudotyped vectors had to be characterized in terms of glycoprotein incorporation, their ability to mediate gene transfer and gene expression and their stability. To increase viral titers, different vector concentration techniques had to be tested. In a next step, it was necessary to investigate, if the retargeted TPMV-pseudotyped vectors were able to discriminate between target and non-target cells, first on different separated receptor-positive and -negative cell lines, then in a mixed cell population. Finally, the efficiency of CD20-retargeted vector particles to transduce primary human B cells needed to be examined, both for activated and quiescent primary B cells.

Vectors pseudotyped with TPMV-glycoproteins would represent a major advance in gene therapy. Recently, retargeted vectors pseudotyped with MV glycoproteins were developed that allow specific cell entry to a certain cell type and transduction of quiescent primary human B cells. The presence of neutralizing antibodies in the general population due to MV vaccination or disease might limit the clinical use of these vectors. With TPMV-pseudotyped vectors, this problem can possibly be overcome as they might not be neutralized by pre-existing antibodies.

4. MATERIAL

4.1. Instruments

Instrument	Distributor
Analytical scales	Kern, Albstadt
Bacterial shaker	Infors AG, Böttmingen, CH
Blotting chamber	Bio-Rad, Munich
Camera *(Power Shot G6)*	Canon, Krefeld
Centrifuge *(Multifuge 3 SR+)*	Heraeus, Hanau
Centrifuge *(Biofuge fresco)*	Heraeus, Hanau
Centrifuge *(Biofuge pico)*	Heraeus, Hanau
Centrifuge *(XL 70 Ultracentrifuge)*	Beckmann, Munich
Centrifuge *(J2-HC)*	Beckmann, Munich
CO2 incubator	Heraeus, Hanau
Electrophoresis chamber	Steinbrenner, Wiesenbach
Electrophoresis power supply *(EPS 3500)*	Pharmacia Biotech, Freiburg
Electrophoresis power supply *(GPS 200/400)*	Pharmacia Biotech, Freiburg
Film cassette	Amersham, Freiburg
Film developer	Amersham, Freiburg
Fridge, 4°C	Liebherr, Biberach
Gel documentation station	Bio-Rad, Munich
Heat block	VWR, Darmstadt
Heat block *(Thermomixer Compact)*	Eppendorf, Hamburg
Horizontal shaker *(Duomax 1030)*	Heidolph, Kehlheim
Hotplate / stirrer	VWR, Darmstadt
Incubator *(Function Line)*	Heraeus, Hanau
Microscope *(Axiovert 40 CFL)*	Carl Zeiss, Jena
Microwave	Bosch, Stuttgart
Multistep pipette	Eppendorf, Hamburg
Neubauer counting chamber	Carl Roth GmbH, Karlsruhe
Nitrogen tank	Thermo Electron coorp., Erlangen

pH meter	VWR, Darmstadt
Photometer *(Nano Drop-1,000)*	Peqlab Biotechnology, Erlangen
Photometer	Amersham, Freiburg
Pipettes	Gilson, Middleton, USA
Scales *(BL 1500 S)*	Satorius, Göttingen
Scanner Epso	Epson, Meerbusch
SDS electrophoresis chamber	Bio-Rad, Munich
Sterile bench *(Class II Type A/B3)*	Sterilgard, Sanford, USA
Stand	Carl Roth GmbH, Karlsruhe
Tabletop centrifuge *(Mini Spin Plus)*	Eppendorf, Hamburg
Thermostatic water bath *(WBS)*	Fried Electric, Haifa, IL
Upright freezer, 20°C *(Comfort)*	Liebherr, Biberach
Vortex mixer	Neolab Heidelberg
Water bath	Neolab, Heidelberg

4.2. Consumables

Product	**Distributor**
Amicon 100-ultra centrifugal filter units	Millipore, Eschborn
Centrifuge tubes (35 ml)	Beckmann, Munich
Cover slips	Carl Roth GmbH, Karlsruhe
Cryo tubes (2 ml)	Greiner Bio-one, Frickenhausen
Cuvettes	Greiner, Frickenhausen
Disposable scalpels	Feather, Cuome, JP
DNase / RNase free water	Invitrogen, Karlsruhe
water	Sigma, Steinheim
Filters (0.45 µm)	Whatman, Dassel
Gloves *(Semper Guard Nitrile)*	Semperit, Vienna, Austria
Gloves *(Peha Soft, Latex)*	Paul Hartmann, Heidenheim
HyperfilmTM ECL	Amersham, Freiburg
Micro test tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg
Mouth protection	Meditrade, Kiefersfelden
Nitrocellulose membrane	Schleicher and Schüll, Dassel

Parafilm	Pechinery Inc., Wisconsin, USA
Pasteur pipettes	Brand, Wertheim
PCR tubes (200 µl)	Eppendorf, Hamburg
Pipette tips (10 µl, Filtertips)	Nerbe Plus, Winsen
Pipette tips (200 µl, Filtertips)	Nerbe Plus, Winsen
Pipette tips (1000 µl, Filtertips)	Nerbe Plus, Winsen
PVDF membrane	Bio-Rad, Munich
Safelock micro test tubes (1.5 ml and 2 ml)	Eppendorf, Hamburg
Serotological pipettes (5 ml, 10 ml, 25 ml, 50 ml)	BD Biosciences, San Jose, USA
Test tubes (15 ml and 50 ml)	Greiner Bio-one Frickenhausen
Tissue culture dishes (10 cm and 15 cm)	Falcon, Gräfeling-Lochham
Whatman paper	Whatman Int., Ltd., UK
6 well tissue culture dishes	Greiner Bio-one Frickenhausen
12 well tissue culture dishes	Corning Inc., NY, USA
24 well tissue culture dishes	Greiner Bio-one Frickenhausen
48 well tissue culture dishes	Corning Inc., NY, USA
96 well tissue culture dishes	Nunc, Wiesbaden
T75 cell culture flasks	Greiner Bio-one Frickenhausen
T75 cell culture flasks	Greiner Bio-one Frickenhausen

4.3. Chemicals and reagents

Chemicals	**Distributor**
Acetic acid (99%)	Sigma, Munich
Acrylamide bisacrylamide Solution (37.5 : 1)	Carl Roth GmbH, Karlsruhe
Adenosine triphosphate (ATP)	Sigma, Munich
Agarose	Carl Roth GmbH, Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH, Karlsruhe
Ampicillin	Sigma, Munich
Antipain	Roche, Penzberg
Aprotinin	Roche, Penzberg
Bestatin	Roche, Penzberg

Bovine serum albumin (BSA)	Sigma, Munich
Bromophenol blue	Sigma, Munich
Calcium chloride	Sigma, Munich
Chymostatin	Roche, Penzberg
Desoxynucleotides (dATP, dCTP, dGTP, dTTP)	Roche, Mannheim
Dipotassium phosphate	Applichem, Darmstadt
Disodium hydrogen phosphate	Sigma, Munich
DMSO (Dimethyl sulfoxide)	Sigma, Munich
DTT (Dithiothreitol)	Sigma, Munich
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen, Karlsruhe
E-64	Roche, Penzberg
EDTA (Ethylenediaminetetraacetic acid),	Sigma, Munich
EDTA-Na$_2$	Roche, Penzberg
Ethanol (99%)	University Hospital, Heidelberg
Ethidium bromide	Carl Roth GmbH, Karlsruhe
Fetal calf serum (FCS)	Invitrogen, Karsruhe
Glucose	Sigma, Munich
Glycerol	Baker, Deventer, NL
Hepes	Roth, Karlsruhe
Histopaque®-1077	Sigma, Munich
Hydrochloric acid (HCl) 37%	Acros Organics, New Jersey, USA
Igepal (Nonidet NP40)	Sigma, Munich
Isopropanol	Sigma, Munich
LB Agar	Carl Roth GmbH, Karlsruhe
LB Medium	Carl Roth GmbH, Karlsruhe
Leupeptin	Roche, Penzberg
L-glutamine	Biochrome AG, Berlin
Lipofectamine	Invitrogen, Karlsruhe
Pefabloc SC	Roche, Penzberg
Pepstatin	Roche, Penzberg
Phosphoramidon	Roche, Penzberg

Loading dye solution (6*x*)	Fermentas, St. Leon Rot
Magnesium chloride (MgCl2)	Merck, Darmstadt
β Mercaptoethanol (98%)	Sigma, Munich
Methanol (100%)	Merck, Darmstadt
Milk powder	Carl Roth GmbH, Karlsruhe
Penicillin / Streptomycin (P/S)	Invitrogen, Karlsruhe
Pepstatin	Sigma, Munich
Potassium chloride (KCl)	Sigma, Munich
Protamine sulfate	Sigma, Steinbach
Proteinase inhibitor cocktail	Roche, Penzberg
Puromycin	Sigma, Munich
Trypsin-EDTA	Invitrogen, Karlsruhe
Sodium chloride (NaCl)	AppliChem, Darmstadt
Sodium dodecyl sulfate (SDS)	Gerbu, Biotechnik GmbH, Gaiberg
Sodium hydroxide (NaOH)	Sigma, Munich
N,N,N',N' Tetramethylenediamine (TEMED)	Carl Roth GmbH, Karlsruhe
Tris base	Sigma, Munich
Trypsin-EDTA solution	Invitrogen, Karlsruhe
Tween-20	Sigma, Munich
Urea	Sigma, Munich

4.4. Comercial Kits

Kit	Distributor
Dynal® B cell negative isolation Kit	Invitrogen, Karlsruhe
EndoFree Plasmid Maxiprep Kit	Qiagen, Hilden
Enhanced Chemiluminescence (ECL) Kit	Amersham Biosciences, Freiburg
Expand High Fidelity PCR System	Roche, Mannheim
QiaPrep Plasmid Maxiprep Kit	Qiagen, Hilden
QiaPrep Plasmid Miniprep Kit	Qiagen, Hilden
QiaQuick Gel Extraction	Qiagen, Hilden
QiaQuick PCR Purification Kit	Qiagen, Hilden

4.5. Solutions and buffers

Blocking buffer (for Western blotting)   1x PBS

0.1% Tween 20

5% Milk powder

PBS (10x)   1.4 M NaCl

27 mM KCl

100 mM Na2HPO4

8 mM KH2PO4

pH 6.8

PBS-T   1x PBS

0.1% Tween-20

RIPA lysis buffer   150 mM NaCl

50 mM Tris-HCl, pH 7.5

1 % Nonidet P40

0.5 % Sodiumdeoxycholate

1 % sodium dodecyl sulfate

SDS running buffer (10x)   0.25 M Tris-base

1.92 M Glycine

1% SDS

TAE buffer   40mM Tris

20mM acetic acid

1mM EDTA.

TBS 10x   175.3 g NaCl/2l

24.2 g Tris/2l

TBS-T	48.5 g Tris/2l, pH 7.5
	584.4 g NaCl/2l
	0.05 % Tween-20
	a.d. H_2O

Transfer buffer SDS-PAGE	25 mM Tris-base
	192 mM Glycine
	20% Methanol
	0.01% SDS

6x SDS sample buffer	0.35 M Tris-Cl/
	0.28 % SDS pH 6.8
	30 % Glycerol
	10 % sodium dodecyl sulfate
	9.3 % DTT
	0.012 % Bromophenol Blue

All buffers were diluted in H_2O, unless stated otherwise

4.6. Molecular components (enzymes, cytokines, oligonucleotides, plasmids, antibodies and sera, marker)

Enzmes

Enzyme	Distributor
restriction endonucleases	New England Biolabs, Ipswich, MA
T4-DNA-ligase	New England Biolabs, Ipswich, MA
High Fidelity Polymerase	Roche, Mannheim

Cytokines

Cytokines	Distributor
CD40 ligand	R&D Systems, Wiesbaden
IL-2	R&D Systems, Wiesbaden
IL-4	R&D Systems, Wiesbaden
IL-10	R&D Systems, Wiesbaden

Oligonucleotides

All oligonucleotides were synthesized from the company Eurofins MWG Operon.

Primer	5' → 3' sequence
HαCD20 S	GGGGCGCGCAGGTTCAGCTGGTCCAG
TPMV-HΔ20 S	TTTGGGGGATCCATTATGTTGCAGAGCCAGAGTG
TPMV-HΔ40 S	TTTGGGGGATCCATTATGTATTCAAATCCTCCAC
TPMV-HΔ50 S	TTTGGGGGATCCATTATGGATCAAGGTATTGATG
TPMV-HΔ60 S	TTTGGGGGATCCATTATGCAACCATTATCAACTC
TPMV-HΔ70 S	TTTGGGGGATCCATTATGCGTTACTATGGAGTTAAC
TPMV-HΔ80 S	TTTGGGGGATCCATTATGAGGGTGTATAATCATTTAG
TPMV-HΔ84 S	TTTGGGGGATCCATTATGCATTTAGGGACAATC
TPMV-H AS	TTTGGGTCTAGATTACTTAGTATTAGGACATGTAC
TPMV-F S	TTTGGGGGATCCCCAAGGATGGCATCACTGCTA
TPMV-FΔ8 AS	CCCGGGTCTAGATCAATGATTCTGATGACCGTTGCTCCTGTGATG
TPMV-FΔ18 AS	CCCGGGTCTAGATCATGATGGATTACGGAGGGAGT
TPMV-FΔ25 AS	CCCGGGTCTAGATCAAACATAAGAATCCAGGAAATGGAGTTTGCT
TPMV-FΔ32 AS	CCCGGGTCTAGATCAGAGTTTGCTCCATATTTTGAATGCGTAATAGC
TPMV-FΔ33 AS	CCCGGGTCTAGATCATTTGCTCCATATTTTGAATGC
TPMV-FΔ34 AS	CCCGGGTCTAGATCAGCTCCATATTTTGAATGCGTA
TPMV-FΔ35 AS	CCCGGGTCTAGATCACCATATTTTGAATGCGTAATAGCTCACACC
TPMV-F MV3 AS	CCGGGGTCTAGATCAACGACCTCTGAATGCGTAATAGCTCACACC
TPMV-Fflag 483 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCCATTGA
TPMV-Fflag 491 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCCTTATC
TPMV-Fflag 492 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCTTTGAA
TPMV-Fflag 495 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCCAACTT
TPMV-Fflag 498 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCTGCTAT
TPMV-Fflag 499 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCAACAACAATCATTGCTAT
TPMV-Fflag 500 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCAAAAACAACAATCATTGC
TPMV-Fflag 501 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCAACAATCATTGCTATTAA

TPMV-Fflag 505 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCACCAAAAACAAC AATCAT
TPMV-Fflag 507 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCAAGTAGAGTACC AAAAAC
TPMV-Fflag 510 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCGAGCCAAAGAAG TAGAGT
TPMV-Fflag 513 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCCACACCAAAGAG CCAAAG
TPMV-Fflag 516 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCGTAATAGCTCAC ACCAAA
TPMV-Fflag 533 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCATTACGGAGGGA GTAAAC
TPMV-Fflag 553 AS	CCCGGGTCTAGATCACTTATCATCATCATCCTTATAATCTCCACTTATATCT GTACTG
Ala1 S	TTGTTAATAGCAATGATTGCTGCTGCTGGTACTCTACTTCTTTGG
Ala1 AS	CCAAAGAAGTAGAGTACCAGCAGCAGCAATCATTGCTATTAACAA
Ala2 S	TTAATAGCAATGATTGTTGCTGCTGCTACTCTACTTCTTTGGCTC
Ala2 AS	GAGCCAAAGAAGTAGAGTAGCAGCAGCAACAATCATTGCTATTAA

Plasmids

name	characterization	source
pCG-TPMV-H	TPMV H protein under control of CMV promotor	Group Dr. Springfeld
pCG-TPMV-HαCD20	TPMV H protein with αCD20-scAb at the C-terminus, under control of CMV promotor	Group Dr. Springfeld
pCG-TPMV-HΔ20αCD20	HαCD20 with truncated cytoplasmic tail and with αCD20-scAb at the C-terminus, under control of CMV promotor	this thesis
pCG-TPMV-HΔ40αCD20	HαCD20 with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-HΔ50αCD20	HαCD20 with truncated cytoplasmic tail and with αCD20-scAb at the C-terminus, under control of CMV promotor	this thesis
pCG-TPMV-HΔ60αCD20	HαCD20 with truncated cytoplasmic tail and with αCD20-scAb at the C-terminus,under control of CMV promotor	this thesis
pCG-TPMV-HΔ70αCD20	HαCD20 with truncated cytoplasmic tail and with αCD20-scAb at the C-terminus,under control of CMV promotor	this thesis
pCG-TPMV-HΔ80αCD20	HαCD20 with truncated cytoplasmic and with αCD20-scAb at the C-terminus, tail under control of CMV promotor	this thesis
pCG-TPMV-HΔ84αCD20	HαCD20 with truncated cytoplasmic tail and with αCD20-scAb at the C-terminus, under	this thesis

	control of CMV promotor	
pCG-TPMV-F	TPMV-F protein under control of CMV promotor	Group Dr. Springfeld
pCG-TPMV-FΔ8	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ18	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ25	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ32	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ33	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ34	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-FΔ35	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-F MV3	TPMV F with truncated cytoplasmic tail under control of CMV promotor	this thesis
pCG-TPMV-F$_{483}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{491}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{492}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{495}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{498}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{499}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{500}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{501}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{505}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{507}$	TPMV F protein with Flag-tag at	this thesis

	indicated amino acid position, under control of CMV promotor	
pCG-TPMV-F$_{510}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{513}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{516}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{533}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-TPMV-F$_{553}$	TPMV F protein with Flag-tag at indicated amino acid position, under control of CMV promotor	this thesis
pCG-F Ala1	TPMV F protein, amino acids at position 499-502 replaced by alanins; under control of CMV promotor	this thesis
pCG-F Ala2	TPMV F protein, amino acids at position 498-501 replaced by alanins; under control of CMV promotor	this thesis
pCG-MV$_{NSe}$FΔ30	MV$_{NSe}$ strain F protein with a truncated cytoplasmic tail; under control of CMV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pCG-MV$_{NSe}$HΔ18αCD20	MV$_{NSe}$ strain H protein with a truncated cytoplasmic tail and with the four point mutations Y481A, R533A, S548L, F549S and αCD20-scAb at the C-terminus; under control of CMV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pCG-MV$_{NSe}$HΔ18α	MV$_{NSe}$ strain H protein with a truncated cytoplasmic tail; under control of CMV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pCG-MV$_{Wt}$FΔ30	MV Wildtype strain F protein with a truncated cytoplasmic tail; under control of CMV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pCG-MV$_{WT}$HΔ18	MV Wildtype strain F protein with a truncated cytoplasmic tail; under control of CMV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pMD.G2	encodes VSV-G	D. Trono, Tronolab, Switzerland
pCMVΔR8.9	HIV-1 packaging plasmid	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen

pSEW	HIV-1 packagable vector encoding GFP under control of the SFFV promotor	kindly provided by the group of C.J. Buchholz, Paul-Ehrlich-Institut, Langen
pcDI-EGFP	encodes eGFP	kindly provided by Dr. Sascha Bossow, NCT, Heidelberg

Antibodies and sera

name	dilution	source of supply
$\alpha F_{ecto,}$ rabbit anti-TPMV F polyclonal peptide serum	1:1000 western blot	PSL, Heidelberg
αH_{ecto}, rabbit anti-TPMV-H polyclonal peptide serum	1:1000 western blot	R. Cattaneo, Mayo Clinic, Rochester
mouse anti-HIV-1 p24 mAb	1:1000 western blot	ZeptoMetrix
Monoclonal Anti-Flag antibody produced in mouse	1:1000	Sigma-Aldrich
HRP-conjugated swine-anti-rabbit antibody	1:2000 western blot	DakoCytomation, Glostrup/Denmark
R-Phycoerythrin (PE) conjugated anti-mouse IgG (whole molecule) $F(ab')_2$ fragment	1:50 FACS	Sigma-Aldrich
PE conjugated mouse anti-human CD19 mAb	1:10 FACS	DakoCytomation, Glostrup/Denmark
PE-Cy5, FITC or PE conjugated mouse anti-human CD20 mAb	1:10 FACS	BD Pharmingen
FITC-conjugated mouse anti-human CD69 mAb	1:10 FACS	DakoCytomation, Glostrup/Denmark
PE-Cy5, FITC or PE conjugated mouse $IgG_{2b,\kappa}$ isotype control	1:10 FACS	BD Pharmingen
FITC or PE conjugated mouse $IgG_{1,\kappa}$ isotype control	1:10 FACS	BD Pharmingen
PE conjugated mouse $IgG_{2a,\kappa}$ isotype control	1:10 FACS	BD Pharmingen
Serum 1, 2, 3	Neutralization-Assay	kindly provided by the group 'Virotherapy', NCT, Heidelberg
Serum MV-negative	Neutralization-Assay	kindly provided by the Universitiy Hospital, Heidelberg

Molecular weight markers

DNA marker	
100 bp DNA ladder	NEB, Ipswich, MA, USA
1 kb DNA ladder	NEB, Ipswich, MA, USA
Protein marker	
Precision Plus Protein™ Standards, Dual color, 10 – 250 kDa	Bio-Rad, Munich

4.7. Bacterial strains and culture media

Strain	Distributor
E.coli BL21 Chemically Competent Cells	Stratagene, La Jolla, USA
E.coli TOP 10 Chemically Competent Cells	Invitrogen, Karlsruhe
Luria-Bertani (LB) Bacto-Trypton 1.0 % (w/v) yeast extract 0.5 % (w/v) NaCl 1.0 % (w/v) pH 7.0	

4.8. Cell lines and culture media

name	characterization	Distributor
HEK-293T	human embryonic kidney cell line genetically engineered to express the large T antigen	ICLC HTL04001
HT1080	human fibrosarcoma cell line	ATCC CCL-121
HT1080-CD02	human fibrosarcoma cell line genetically engineered to express CD20	R. Cattaneo, Mayo Clinic, USA
Raji	human Burkitt's lymphoma cell line	ATCC CCL-86
K-562	human chronic myelogenous leukemia cell line	ATCC CCL-243
TBF	primary *Tupaia* baby fibroblasts	R. Cattaneo, Mayo Clinic, USA

Culture medium for HEK-293T, HT1080, TBF

Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FCS and 2 mM L-glutamine.

Culture medium for Raji, K-562

RPMI 1640 supplemented with 10 % FCS and 2 mM L-glutamine.

Culture medium for HT1080-CD20

RPMI 1640 supplemented with 10 % FCS, 2 mM L-glutamine and 3 µg/ml puromycin.

4.9. Software

Software	Distributor
Endnote	Thomson, Carlsbad, USA
Microsoft Office	Microsoft, Unterschleißheim
Photoshop	Adobe, San Jose, USA
Pubmed	NIH
Vector NTI Advance™ *Software*	Invitrogen, Karlsruhe
FCS-Express V3	DeNovoSoftware, Los Angeles, USA

5. METHODS

5.1. Molecular Biology

5.1.1. Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze DNA molecules such as PCR products and digested plasmids, since it separates the fragments by their size. For production of gels, 1 % agarose was dissolved in TAE buffer by heating the emulsion in a microwave until it boiled. Upon cooling, 5 µg/ml ethidium bromide was added. DNA samples were mixed with 8 µl sample buffer and applied to the gel. Gels were run at 100 V for approximately 60 min. Subsequently, DNA fragments were photographed under a UV lamp or isolated from the gel.

5.1.2. Isolation of DNA from agarose gels

DNA fragments were cut out of the gel under a UV lamp using a scalpel, transferred into a 1.5 ml reaction tube and purified using the QIAquick Gel Extraction Kit, according to the manufacturer's instructions.

5.1.3. Restriction analysis of DNA fragments or plasmids

For restriction analysis, restriction enzymes from New England Biolabs (NEB) were used according to the manufacturer's instructions. DNA was incubated with 10-20 units/µg of the respective enzyme, the corresponding buffers and, if necessary, with 0.1 volume of BSA. Reactions were incubated at the temperature optimum of the enzyme for 2-4 hours. Restriction samples were then applied to agarose gel electrophoresis.
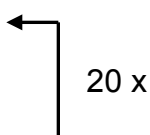
5.1.4. Polymerase chain reaction (PCR)

PCR was performed to amplify DNA fragments for further molecular biological methods using a thermally stable DNA-dependent DNA-polymerase. For a typical PCR, the following ingredients in their appropriate amounts are necessary:

Ingredients	Amount	Final concentration
10x polymerase buffer	5 µl	1 x
10 mM deoxynucleotide mix (dNTP)	1 µl	0.2 mM
Forward primer (100 µM)	1 µl	2 µM
Reverse primer (100 µM)	1 µl	2 µM
DNA template	~ 5 ng	
High Fidelity Polymerase	1 µl	3.5 U
Double distilled water	Ad 50 µl	

A typical PCR reaction was programmed as followed:

Step	Temperature	Time	
Denaturation	94°C	*2 min*	
Denaturation	94°C	15 sec	
Primer annealing	55°C	30 sec	20 x
Extension	72°C	90 sec	
Extension	72°C	7 min	
Hold	4°C		

Depending on the length and G-C content of the used primers, different annealing temperatures were chosen using the following formula: (T_{An}=69°C + 0.41 x (%GC) – 650 / bp).

Cloning of the unmodified TPMV H and F protein has been described previously (Springfeld et al., 2005). The expression plasmid pCG-TPMV-HαCD20 that encodes a fusion protein of TPMV H and a single-chain antibody (scAb) to human CD20 was generated by amplification of the open reading frame of the αCD20-scAb from the plasmid pCGHαCD20 (Bucheit et al., 2003) using the forward primer 5'-GGGGCGCGCAGGTTCAGCTGGTCCAG-3' that inserts a BssHII site to the 3′ end of the H open reading frame and a reverse primer in the backbone of the pCG vector. After cutting with *Bss*II and *Xba*I, the fragment was cloned into the plasmid pCG-TPMV-HXαCEA (Springfeld et al., 2005), thereby exchanging the αCEA-scAb against the αCD20-scAb. TPMV H cytoplasmic tail (CT) variants were generated by PCR-cloning using primers that deleted the desired amino acids and introduced a new start codon. The TPMV F CT variants and F_{Flag} variants were constructed using primers that introduced a stop codon and a flag-tag at the desired position,

respectively, and deleted the following amino acids. The TPMV-F MV3 expression plasmid was generated using a primer that introduced the three cytoplasmic tail amino acids (arginine - glycine - arginine) followed by a stop codon.

All PCR reactions were performed using an PCR Express, Therm Hybai cycler. Subsequently, they were analyzed by agarose gel electrophoresis or frozen at -20°C.

5.1.5. Ligation

For Ligation, 50-200 ng of vector DNA was used for each reaction. The ratio of insert to vector DNA was 3:1. Vector and insert DNA were mixed in a volume of 10 µl and incubated at 45°C for 5 min. Afterwards ligation buffer and T4 DNA ligase were added and the reaction was incubated at room temperature for 2-5 hours. The ligated DNA was used immediately for transformation of bacteria or stored at -20°C.

5.1.6. Transformation of bacteria

For transformation, chemically competent *E. Coli* bacteria were thawed on ice and 1 µl DNA or for ligations, the whole ligation reaction were added and incubated on ice for 20 min. Afterwards, a heat shock of 42°C was performed for 45 sec in a water bath following 5 min further incubation on ice. Then, 500 µl LB medium (RT) were added, followed by incubation at 600 rpm, 37°C for 60 min in a thermoblock. The suspension was added on LB plates containing ampicillin and incubated over night at 4°C.

5.1.7. Cultivation of bacteria

Culture plates: Bacteria were cultured on LB plates containing ampicillin (50µg/ml, LB_{Amp}) by adding the bacterial suspension onto the plate and incubation overnight at 37°C until bacterial colonies were visible. Plates with bacterial colonies were stored up to 6 weeks at 4°C.

Liquid culture: Bacteria were cultured in liquid LB medium containing ampicillin (0.1 mg/ml, LB_{Amp}) overnight at 37°C on a shaker (~ 200 rpm).

5.1.8. Plasmid preparation

For plasmid preparation from the transformed bacteria, either the QIAprep® Spin Miniprep kit or the EndoFree® Plasmid Maxi kit was used according to the instructions of the manufacturer (Quiagen).

For extractions of small amounts of DNA (Miniprep), 5 ml LB_{Amp} were inoculated with bacteria and incubated overnight at 37°C. The next day, 1.5 ml of the bacterial suspension were applied to a 1.5 ml reaction tube and centrifuged in for 3 min at 5000 rpm in a table centrifuge (RT). This step was repeated three times in the same reaction tube. DNA extraction out of the resulting bacterial pellet was performed with the the QIAprep® Spin Miniprep kit.

For extraction of large amounts of DNA (Maxiprep), 1.0 l LB_{Amp} were inoculated with bacteria and cultivated overnight at 37°C. The bacteria suspension was centrifuged for 15min, 4°C and 6000 rpm (J2-HC, Beckman) and the resulting pellet was treated with the EndoFree® Plasmid Maxi kit according to the manufacturer's instructions.

The concentrations of the isolated plasmid DNA was determined photometrically (absorption 260 nm).

5.1.9. DNA sequencing

Plasmids were sequenced by the company GATC. Samples were prepared in a volume of 30 µl containing 50 ng/µl DNA and send together with 30 µl of appropriate primers (10 pmol/µl) via mail to the company.

5.2. Cell Biology and virology

5.2.1. Cultivation of cell lines

Cells were cultivated in an incubator at 37°C, 5 % CO_2 and saturated water atmosphere. They were passaged twice a week by removing the medium, washing with PBS and adding trypsin. After 1-2 minutes, fresh medium was added to the cells and trypsin. Out of this solution, an appropriate fraction was seeded into a new culture flask with fresh medium.

5.2.2. Freezing and thawing of cells

For freezing, cells were trypsinised and resuspended in medium as described in section 2.2.1. This cell solution was then centrifuged at 900 rpm for 4 min at 4°C (Multifuge 3SR+), the pellet resuspended in cold freezing medium (4°C, 70 % FCS, 15 % DMSO, 15 % DMEM or RPMI) and aliquoted in 1 ml fractions containing approximately 1×10^6 cells. Afterwards, the aliquots were stored for 24 h at -20°C, then for 24 h at -80°C and finally transferred into liquid nitrogen.

Cells were thawed by putting the cryotube into a waterbath (37°C) until the cell suspension is liquid. Then, the cells were immediately transferred into a 15 ml reaction tube containing pre-warmed (37°) fresh medium. To exclude the toxic DMSO, the cells were centrifuged at 900 rpm for 3 min at RT (Multifuge 3SR+) and the pellet resuspended in 10 ml fresh medium which was then transferred into a cell culture flask.

5.2.3. Transient transfection of cells

293T cells, HT1080-CD20 cells and TBF cells were transient transfected using Lipofectamine. Twenty-four hours before transfection, cells were seeded as suggested by the manufacturer. One hour before transfection, medium was replaced by fresh medium. The plasmids were mixed with Lipofectamine and added to the cells. Fourty-eight hours after transfection, the cells were analyzed.

5.2.4. Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from human blood from Buffy coats (Blutspendedienst Hessen, Frankfurt a.M.) using the method of density centrifugation with Histopaque®-1077. 15 ml of cold Histopaque (4°C) were overlaid with 25 ml of a 1 :1 mixture of human blood and PBS in a 50 ml Falcon tube. The mixture was centrifuged at 1,800 rpm for 30 min (RT, no break, Multifuge 3SR+) to pellet the red blood cells and granulocytes. After centrifugation, a small white fraction is visible above the Histopaque that contains lymphocytes, monocytes and macrophages. Above this fraction, the plasma is present. The white lymphocyte-containing fraction was collected into a 50 ml Falcon (5 ml per Falcon). Then, 50 ml PBS were added to each Flacon tube followed by centrifugation at 1,500 rpm for 10 min at RT. The pellets were combined in one single Falcon tube and washed against with PBS as described before. Remaining

erythrocytes were lysed by adding 10 ml 0.86 % ammonium chloride and incubation for 20 min at 37°C. Again, the cells were washed twice with PBS as described before and resuspended in 50 ml PBS for determination of the cell number. Subsequent, the PBMCs were applied for B cell isolation.

5.2.5. Isolation and activation of primary human B cells

Primary human B cells were isolated from fresh isolated PBMCs using the Dynal B cell negative isolation kit (Invitrogen) as suggested by the manufacturer instructions. Isolated B cells were either used immediately for transduction or activated for 48 hours in medium together with 300 ng/ml CD40 ligand, 50 ng/ml interleukin-2, 10 ng/ml interleukin-4 and 10 ng/ml interleukin-10. Stimulated B cells were detected by an antibody against the activation marker CD69.

5.2.6. Generation and concentration of lentiviral vectors

For screening of all F- and H-combinations, vector particles were generated by transfection of 293T cells using polyethylene-imine (PEI) with 1.73 μg of the lentiviral transfer vector plasmid pSEW (Miyoshi et al., 1997), 1.65 μg of the packaging plasmid pCMVΔR8.9 (Zufferey et al., 1997) and 0.27 μg each of the TPMV-H- and F-protein variant expression plasmids. Twenty-four hours before transfection, 2.0×10^6 cells/well were seeded into a single well of a six-well plate. On the day of transfection, the medium was replaced by 0.5 ml DMEM (+1 % glutamine). The plasmids were mixed with 17.7 μl of 5 % (wt/vol) glucose. In a different tube, 17.7μl of 5 % glucose was mixed with 4 μl of 18 mM PEI. Both solutions were incubated for 10 minutes at room temperature, then combined, mixed and incubated again at room temperature. After 10 minutes, the volume of the mixture was increased with 1 ml of DMEM (+1 % glutamine) and then added to the cells. Finally, 100 μl of FCS per well was added to the cells 5-6 hours after transfection. Forty-eight hours afterwards, the cell supernatant containing the viral particles was collected and 150 μl were directly used for transduction. The resulting vectors were titrated on Raji cells. For measles virus pseudotyped vectors, 0.15 μg H-plasmid and 0.46 μg F-plasmid were used, for VSV-G-pseudotyped vectors, 0.6 μg of pMD.G2 (Didier Trono, Tronolab, Lausanne, Switzerland).

For all other experiments, vector stocks were produced by transfection using 70 µl PEI and 310 µl glucose for a T175 flask. One day before transfection, 1.5 x 10^7 293T cells were seeded and transfected with 28.7 µg of packaging plasmid, 30.2 µg of transfer vector plasmid and 5.3 µg each of TPMV-H and F-expression plasmids. The measles virus H protein expression plasmid [4] was used in an amount of 2.6µg, the measles virus F protein plasmid in an amount of 8 µg. For VSV-G-pseudotyped vectors, 10.6 µg pMD.G2 plasmid was used. To the transfection mixture 2.2 ml DMEM (+1% glutamine) was added. For concentrating the vector particles, the supernatant was collected after 48 hours, filtered (0.45 µm filter) and centrifuged by low-speed centrifugation over a sucrose-cushion (20 % wt/vol) (4000 g for 24 hours at 4 °C). The supernatant was discarded and the pellet was resuspended in 60 µl Opti-MEM (200 µl for VSV-G-pseudotyped vectors).

For stability assays, vectors were concentrated by ultracentrifugation or ultrafiltration. Ultracentrifugation was performed at 28,000 rpm for 3 hours at 4°C over a sucrose-cushion (20 % wt/vol). For ultrafiltration, the vector-containing cell supernatant was applied to Amicon 100-ultra centrifugal filter units (Millipore) and centrifuged at 4000 g for 90 min at 4°C.

5.2.7. Titration of lentiviral vectors

Vector titration was done using Raji cells. For the initial screening of all TPMV-F-and H-protein combinations, 2.0 x 10^4 Raji cells were seeded on the day of transduction into a single well of a 96-well plate in a total amount of 50 µl. The vector stocks were serially diluted in steps of 1:10 in a total volume of 200 µl. Of these dilutions, 150 µl were added to the cells together with 4 ng/ml protamine sulfate and incubated for 3 hours at 37°C. Then, 100 µl fresh medium was added to the cells. After 72 hours, GFP-positive cells were determined by FACS analysis and the titer was calculated. For titration of large scale productions, 1.0 x 10^5 cells (Raji) were seeded into a single well of a 48 well plate. Serial dilutions were performed in a total volume of 300 µl and 250 µl were added to the cells together with 4 ng/ml protamine sulfate. After 3 hours, 0.7 ml fresh medium was added to the cells and FACS analysis was performed 72 hours after transduction. For titer calculation, dilutions were chosen in which 2 – 20 % of the cells showed GFP expression. Titers (in transducing units (t.u.)/ml) were calculated using the following formular:

$$\text{Titer [t.u./ml]} = \frac{\text{Number of cells at time of transduction}}{\text{Volume of vectors}} \times \frac{\text{GFP positive cells [\%]}}{100}$$

5.2.8. Transduction of adherent and suspension cells

One day before transduction of adherent cell lines, 5.0×10^4 cells were seeded into a single well of a 24 well plate. Before transduction, the medium was removed. Depending on vector titer and MOI (multiplicity of infection), vectors were diluted with medium in an appropriate ratio in a total volume of 300 µl which was then added to the cells together with 4 ng/ml protamine sulfate and incubated for 3 hours at 37°C. Then, 700 µl of fresh medium was added.

For transduction of suspension cells, 1.0×10^5 cells were seeded on the day of transduction into a single well of a 48 well plate in a total amount of 50 µl. As described above, vector and medium was mixed in an appropriate ratio depending on vector titer and MOI and added to the cells together with 4 ng/ml protamine sulfate and incubated for 3 hours at 37°C. Then, 700 µl of fresh medium was added.

For the transduction of the mixed cell population, 5.0×10^4 Raji cells together with 5.0×10^4 K-562 cells were seeded into a single well of a 48-well plate in a total amount of 50 ml and transduced as described above. FACS analysis was performed 72 hours after transduction.

5.2.9. Transduction of primary human B cells

For transduction, the isolated B cells were seeded into a single well of a 48 well plate (5.0×10^4 cells/well) together with the virus particles in a total volume of 300 µl and with 4 ng/ml protamine sulfate. This was followed by centrifugation at 430 g for 30 min at 32°C. Subsequently, the cells were incubated at 37°C. Next day, 500 µl/well medium (as described above) was added to the cells and 72 hours after transduction, the fraction of GFP-positive cells was determined by FACS analysis.

5.2.10.   Neutralization assay

To examine whether TPMV-pseudotyped LVs by MV-antibodies, human sera were heat-inactivated for 30 min at 56°C and stepwise diluted (1:20) in medium without FCS. 100 µl each of these dilutions were incubated for 20 min at 4°C with the appropriate amount of vector stock using a MOI of 0.4. Afterwards, the vector-

containing serum dilutions were incubated for 5 min at 37°C and $2.0x10^4$ Raji cells in an amount of 20 µl medium without FCS were added. Three hours later, 100 µl fresh medium (+ FCS) was added to the cells and 72 hours after transduction, GFP expression was analyzed by FACS.

5.2.11. Cultivation and concentration of the *Tupaia paramyxovirus*

TPMV was propagated in TBF cells (*Tupaia* baby fibroblasts). TBFs were infected when they were 80 % confluent with 1 ml frozen virus-containing cell supernatant by adding the virus to the medium. After 5-6 days, the supernatants of two T175 flasks were combined and ultra-centrifuged at 28,000 rpm for 3 hours at 4°C over a 20% sucrose-cushion. The virus-containing pellet was resuspended in 200 µl PBS.

5.2.12. Fluorescent activated cell sorting (FACS)

FACS analysis was performed on a BD LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany). Raji and K-562 cells were washed twice in 500 µl FACS washing buffer (phosphate-buffered saline, 1% FCS, 0.1% NaN_3) and finally resuspended in 100 µl phosphate-buffered saline/1% paraformaldehyde. To detect CD20-positive cells, the cells were incubated with a 1:10 dilution of the mouse anti-human CD20/PE-Cy5 antibody (BD Pharmingen, Heidelberg, Germany) after the first washing step. After 20 minutes at 4°C, the antibody solution was removed and the cells were washed twice and fixed as described before. For detection of CD19-positive cells, the mouse anti-human CD19/PE antibody (DakoCytomation, Hamburg, Germany) was used. Stimulated B cells were detected by an antibody against the activation marker CD69 (mouse anti-human CD69/FITC, DakoCytomation, Hamburg, Germany). The data were analyzed with the FCS-Express V3 program.

5.3. Biochemistry

5.3.1. SDS-Polyacrylamide gelelectrophoresis

For analysis of protein expression levels and for separation of proteins according to their molecular weight, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was applied (Laemmli, 1970). In this method, proteins get denatured and negatively charged in the presence of sodium dodecyl sulphate (SDS). Thus,

they can be applied to electrophoresis and run towards the positive pole under current conduction. Additionally, exposed disulfide bonds are reduced by the presence of dithiothreitol (DTT) resulting in prevention of intra- and intermolecular interactions by disulfide bond formation.

For analysis, proteins were resuspended in a defined volume of PBS or RIPA-Lysis buffer and 5 volumes of 6 x SDS sample buffer. Samples were denatured by incubation for 5 minutes at 95 °C. Afterwards, the protein samples were applied to 10 % sodium dodecyl sulfate-polyacrylamide electrophoresis gels within a Bio-Rad-Mini Protean II chamber filled with SDS-running buffer. As protein ladder, Precision Plus Protein Standards Dual Color (Bio-Rad) was used. For separation of proteins inside the stacking gel a voltage of 80 V was used, whereas the voltage was raised to 120 V inside the separating gel until the dye front had left the separating gel.

5.3.2. Western Blot analysis

This technique allows the transfer of proteins onto a Polyvinylidene fluoride (PVDF) membrane. Based on this transfer, proteins can be visualized by immunostaining with specific antibodies. In general, proteins are transferred onto membranes after SDS-Polyacrylamide gelelectrophoresis, a process also called blotted. In this thesis, proteins were blotted using a wet blot. To set up the blot, the membrane was shortly incubated in methanol for activation and subsequently soaked in Western Blot transfer buffer together with four Whatman filter papers and two Western Blot sponges. Blotting was performed at 100 V for 1 hour or at 30 V overnight in transfer buffer. Subsequently, the blot was incubated in 10% milk powder/TBS-T for one hour (RT) or overnight (4°C) to block unspecific protein binding sites. Specific primary antibodies were diluted in 5% milk powder/TBS-T, respectively, and incubated with the membranes for 1 hour at room temperature or overnight at 4°C. Then, the membrane was washed once with 1 x TBS-T and at least three times with 1 x TBS for 10 min followed by an one hour incubation with the secondary horse radish peroxidase (HRP)-coupled antibody directed against the first antibody at a dilution of 1 : 2000. Again, the membrane was washed as mentioned above. Detection of specific bands was carried out using the enhanced chemiluminescence system (ECLTM) Western Blotting Detection Reagent. Hyperfilm ECL films were exposed to the resulting chemiluminescent signal. Different exposure times were chosen, depending on the quality of specific antibodies.

5.3.3. Preparation of cell lysates

Cells were grown in 6 wells and washed with 2 ml ice-cold PBS. Subsequently, 200 µl RIPA-lysis buffer were added and the plate was incubated for 5 min on ice. After incubation, the lysate was carefully transferred into a 1.5 ml reaction tube and centrifuged at 13,000 rpm for 10 min at 4°C in a table centrifuge to remove the cell debris. The resulting supernatant was then transferred into a fresh 1.5 ml reaction tube and either used immediately for Bradford assay or stored at -20°C.

5.3.4. Protease inhibitor assays

To identify the protease involved in TPMV F protein processing different protease inhibitors were tested. The inhibitors were diluted as recommended by the manufacturer (Roche). Cells were transiently transfected in a 6 well plate as described before. Five hours after transfection, the medium was replaced by 2 ml fresh medium supplemented with protease inhibitors. Cell lysates were harvested 48 hours after transfection as described above.

5.3.5. Bradford assay

For determination of protein concentration of cell lysates the Bradford Dye reagent from Bio-Rad was used according to the manufacturer's instructions. Absorption of protein samples was measured in a spectrophotometer at a wavelength of 595 nm. A standard curve was determined and protein concentrations were calculated.

6. RESULTS

6.1. Generation and characterization of modified TPMV glycoproteins

The aim of this thesis is to develop and characterize targeted lentiviral vectors (LVs) that are pseudotyped with the *Tupaia paramyxovirus* (TPMV) F and H glycoproteins. Recently, it was reported that lentiviral vectors can be pseudotyped with the glycoproteins of *measles virus* (MV) when their cytoplasmic tails are truncated (Frecha et al., 2008; Funke et al., 2008a; Funke et al., 2009). Accordingly, a panel of TPMV H and F proteins with truncated cytoplasmic tails was generated and biochemically characterized. Since it was previously shown that the TPMV attachment protein (H) can be retargeted to cell surface molecules similar as the MV H protein (Springfeld et al., 2005), a single-chain antibody (scAb) against CD20 was displayed on the ectodomain. To identify TPMV cytoplasmic tail variants that have the potential to form functional pseudotyped LVs, surface expression of the H protein variants and syncytia assays were performed.

6.1.1. Modification of the TPMV H protein

The TPMV H protein is a type II membrane protein with a length of 665 amino acids and has an unusually long cytoplasmic tail of 94 amino acids. It can be retargeted to cell surface molecules by displaying single-chain antibodies on the ectodomain (Springfeld et al., 2005). Since the retargeted MV-pseudotyped LVs were established with a scAb against the B cell surface marker CD20 displayed on the ectodomain of the MV H protein, this molecule was also chosen as a target to establish the TPMV-pseudotyped vectors.

To retarget the TPMV H protein to CD20, the open reading frame of the corresponding scAb was amplified from the plasmid pCGHαCD20 (Bucheit et al., 2003) and after cutting with *Bss*II and *Xba*I, the fragment was cloned into the plasmid pCG-TPMV-HXαCEA (Springfeld et al., 2005), thereby exchanging the CEA scAb against the CD20 scAb on the carboxyterminus of the H protein, resulting in the plasmid pCG-TPMV-HαCD20 (Fig. 11A).
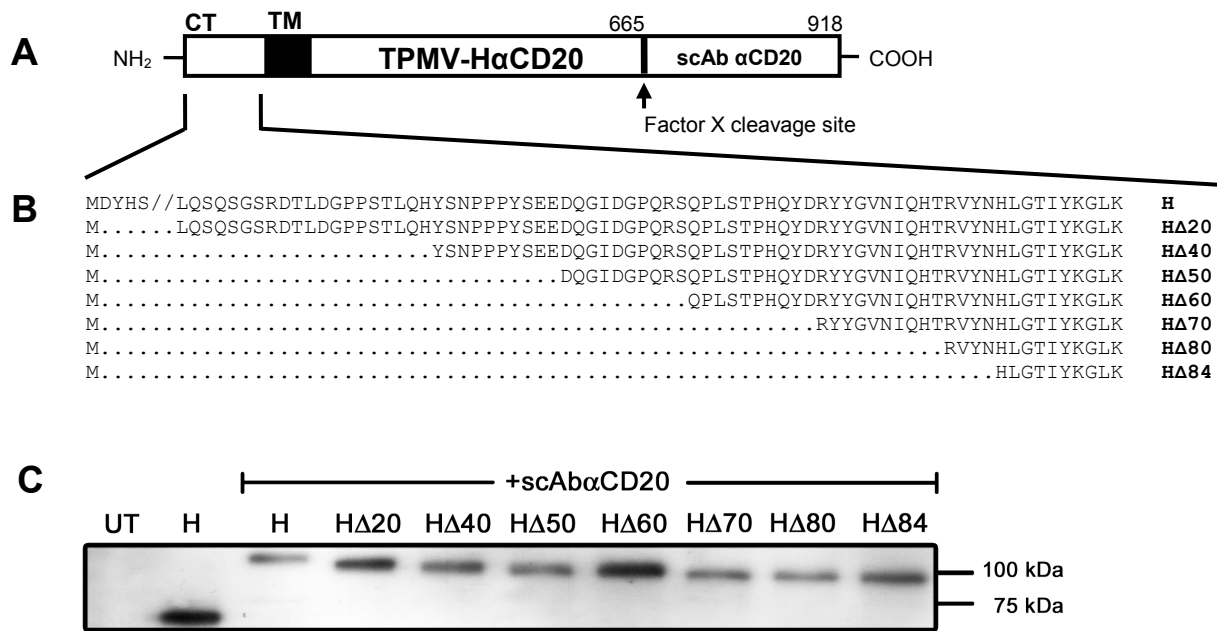
Figure 11: Schematic drawing of the TPMV H protein, amino acid sequences of the truncated cytoplasmic tails and Western blot analysis of the different H protein variants
For retargeting, a single chain antibody (scAb) against CD20 was fused to the carboxyterminus of the H protein together with a Factor X cleavage site (A). The glycoprotein's cytoplasmic tail (CT) was truncated as indicated (B). All proteins were expressed in 293T cells, fractionated by SDS-10% PAGE, and detected with a peptide antiserum against the ectodomain of the H protein (αH_{ecto}, C)
TM: transmembrane domain; UT: untransfected cells

Measles virus-pseudotyped LVs can only been generated when cytoplasmic tails of the glycoproteins are truncated. For the MV H protein, a deletion of 18 amino acids results in highest vector titers. Accordingly, a panel of TPMV H proteins with truncated cytoplasmic tails was generated by mutation PCR, where the numbers of deleted amino acids were randomly chosen. For the different deletions forward primers were used that are complementary until the start codon followed by an interruption and continue exactly after the deleting sequence. Additionally, the forward primers introduced a BamHI cleavage site. The reverse primer was the same for all TPMV H variants and introduced an XbaI cleavage site after the stop codon. As initial plasmid pCG-TPMV-HαCD20 was used. The resulting PCR fragments contained a BamHI cleavage site at the 5' end, a XbaI cleavage site at the 3' and were deleted by 20, 40, 50, 60, 70, 80 or 84 amino acids at the aminoterminus (Fig. 11, B), leaving only 10 aa on the shortest variant (HΔ84αCD20). Subsequently, the TPMV HαCD20-coding region in the original plasmid pCG-TPMV-HαCD20 was replaced upon BamHI/XbaI digestion by the PCR fragments.

For biochemical analysis of the different TPMV H protein variants, 293T cells were transfected with the respective plasmids and after 48 hours cell lysates were harvested, applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the H_{ecto} antibody. In repeated experiments, no relevant differences in the expression levels of the H protein were observed (representative Blot is shown in Fig. 11, C). Fusion of the αCD20 scAb to the carboxyterminus of the H protein resulted in a larger molecular mass of approximately 110 kDa compared to the unmodified H protein. As expected, H protein size was reduced in the proteins with truncated cytoplasmic tail.

6.1.2. Modification of the TPMV F protein

The TPMV F protein is a 553 amino acid type I membrane protein. It is expressed as a F0 precursor protein and, in addition to the removal of the signal peptide, cleaved during activation, resulting in the fragments F1 and F2 (Fig. 12, A).
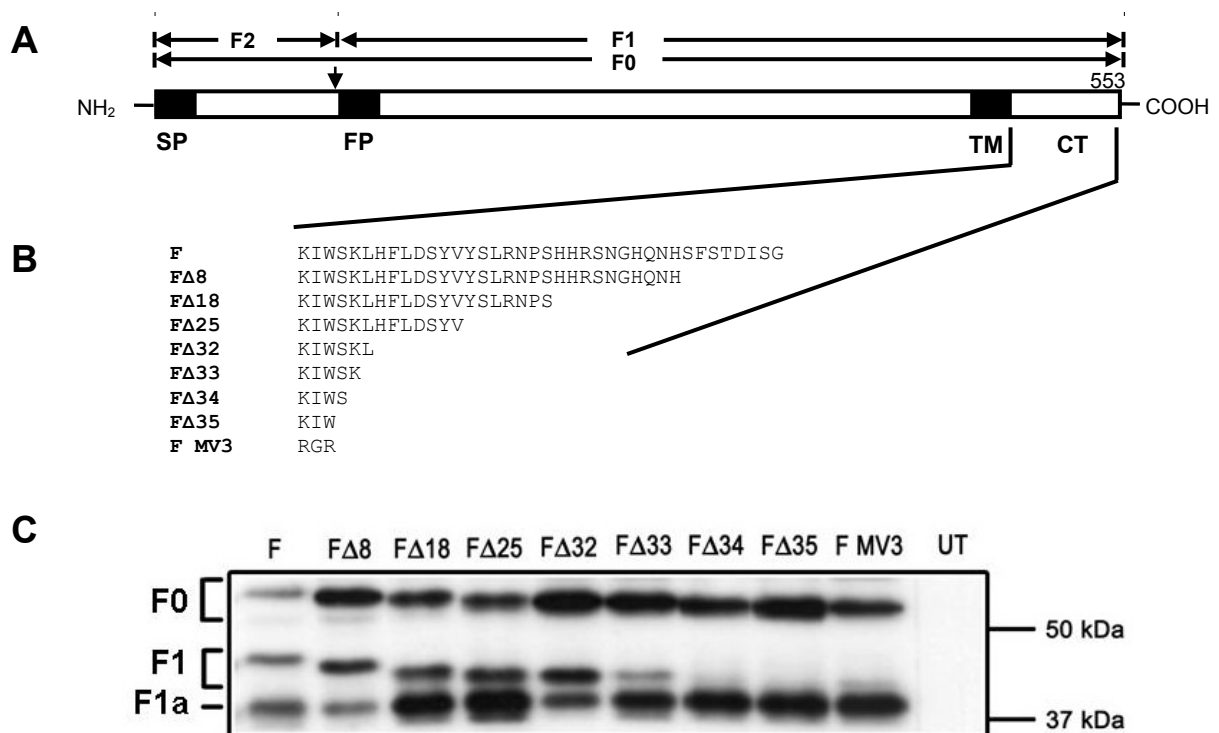
Figure 12: Scheme of the TPMV F protein and sequences of the truncated cytoplasmic tails and Western blot analysis of different F protein variants

TPMV F is expressed as F0 precursor protein and cleaved during activation, resulting in the fragments F1 and F2 (A). For pseudotyping, the cytoplasmic tail (CT) was truncated as indicated (B) or replaced by the truncated cytoplasmic tail of *Measles virus* F protein (F MV3). All proteins were expressed in 293T cells, fractionated by SDS-10% PAGE, and detected with a peptide antiserum against the ectodomain of the F protein (αF$_{ecto}$, C)

TM: transmembrane domain; SP: signal peptide; FP: fusion peptide, UT: untransfected cells

The production of the plasmid encoding the unmodified TPMV F protein was already described (Springfeld et al., 2005). Corresponding to the TPMV H protein, the cytoplasmic tail of the F protein was truncated at the carboxyterminus by 8, 18, 25, 32, 33, 34, and 35 amino acids, leaving only three residues on the shortest variant (Fig. 12, B). This was done by PCR using a reverse primer that introduced a stop codon and an XbaI cleavage side behind the respective amino acid in the plasmid pCG-TPMV-F at the 3' end of the protein coding sequence. The forward primer introduced a BamHI at the 5' end of the PCR product. Additionally, a protein was generated that has the same three-amino acid cytoplasmic tail (arginine - glycine - arginine) as the recently described MV protein that was used successfully to pseudotype lentiviruses (Funke et al., 2008a). In Western blots, all F protein variants were expressed at a similar level and according to their predicted sizes (Fig. 12, C). The proteins were detected with a novel antibody generated against a peptide in the ectodomain of the F protein (αF_{ecto}). Surprisingly, with this antibody an additional fragment with a molecular mass of about 45 kDa was detected, suggesting that the F protein is cleaved twice in addition to the removal of the signal peptide, resulting in the fragments F1a, F1b and F2. However, in the proteins with the shortest cytoplasmic tails (FΔ34, FΔ35 and F MV3), the F1 fragment was barely detectable, suggesting that in these proteins the F1a/F1b cleavage was more efficient than in the unmodified protein. The F1a/F1b cleavage is further characterized in section 6.2.

6.1.3. Surface expression of the modified TPMV H proteins

For further investigation of the modified TPMV glycoproteins the level of protein expression of the H protein on the cell surface was tested. Since surface expression is a critical step in vector formation (Sakalian and Hunter, 1998; Sandrin et al., 2004) it is important to investigate if the proteins are processed correctly after their synthesis and displayed on the cell surface. The H protein was heavily modified by fusing the scAb to the carboxyterminus and truncating the CT and might therefore not be properly processed. For this purpose, 293T cells were transfected with the respective TPMV H-plasmids and 48 hours after transfection the cells were harvested and incubated with the primary antibody H_{ecto} and the FITC-labeled secondary antibody goat-α-rabbit-IgG. Surface expression was then analyzed by fluorescence-activated cell-sorting (FACS) and the geometric mean levels of

fluorescence intensity, representing the average amount of surface-expressed H protein, were compared. Surface expression levels were normalized to that obtained after expression of the unmodified TPMV H protein (100 %).

Surface expression of almost all modified protein variants was not relevantly inhibited by the αCD20-scAb (80 – 100 %) (Fig. 13), in contrast, for two variants it was slightly increased (112.3 % for HΔ40αCD20 and 116.9 % for HΔ84αCD20). Only for the variants HΔ70αCD20 and HΔ60αCD20 the expression level is significantly reduced (56.2 % and 66.9 %, respectively).
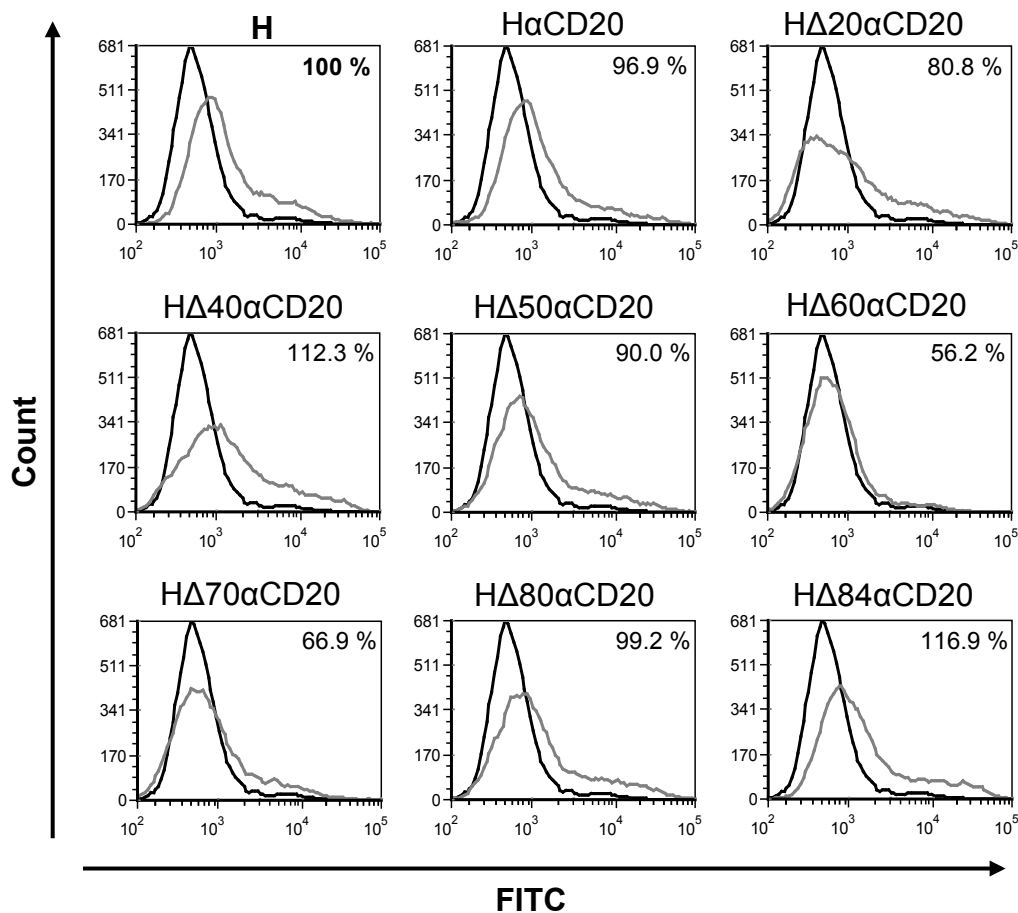
Figure 13: Cell surface expression levels of the different TPMV H protein variants
293T cells were mock-transfected (black line) or transfected with the different TPMV H expression plasmids (grey line), respectively. After 48 hours, mock- and H-transfected cells were stained for surface expression using the antibody αH$_{ecto}$ and the fluorescence-labeled secondary antibody α-rabbit-FITC and analyzed by FACS. The geometric mean levels of fluorescence intensity, representing the average amount of surface-expressed H variants, were normalized to that obtained after expression of the unmodified TPMV H protein (100 %, upper left).

For the TPMV F variants, no antibody is available that allows FACS analysis of surface expression.

6.1.4. Functional analysis of the truncated TPMV protein variants

Since surface expression of the modified TPMV H proteins is not the only critical step, next the functionality of the truncated protein variants was investigated. To test, if the TPMV H proteins with shortened cytoplasmic tail were still able to fulfill their fusion helper function and if the truncated TPMV F proteins still mediate membrane fusion, HT1080 cells that stable express CD20 were co-transfected with the wildtype F protein together with all HαCD20 protein variants and vice versa. Are the proteins functional, the cells fuse and form syncytia. For transfection control and for a better visualization of syncytia formation, a plasmid encoding GFP was transfected together with the TPMV-encoding plasmids. Fourty-eight hours after transfection, the cells were monitored for the formation of syncytia.

Unexpectedly, F proteins with a cytoplasmic tail shorter than 5 amino acids (FΔ34, FΔ35 and F MV3) together with HαCD20 did not support fusion at all, whereas all proteins with longer CTs supported fusion similar to the wildtype F protein (Fig. 14 A). This indicates that at least 5 amino acids of the cytoplasmic tail of the F protein are necessary to mediate membrane fusion. These results correlate with the lack of the fusion-triggering F1 fragment in these proteins in Western blots (Fig. 12 C). As a control, cells were transfected with a GFP-encoding plasmid only and as expected, no syncytia formation was observed.

A

F (+++)   FΔ8 (+++)   FΔ18 (+++)   FΔ25 (+++)   FΔ32 (+++)   HαCD20

FΔ33 (++)   FΔ34 (-)   FΔ35 (-)   F MV3 (-)   control (-)

B

H (-)   HαCD20 (+++)   HΔ20αCD20 (+++)   HΔ40αCD20 (+++)   HΔ50αCD20 (+++)   F

HΔ60αCD20 (++)   HΔ70αCD20 (++)   HΔ80αCD20 (+)   HΔ84αCD20 (++)   control (-)
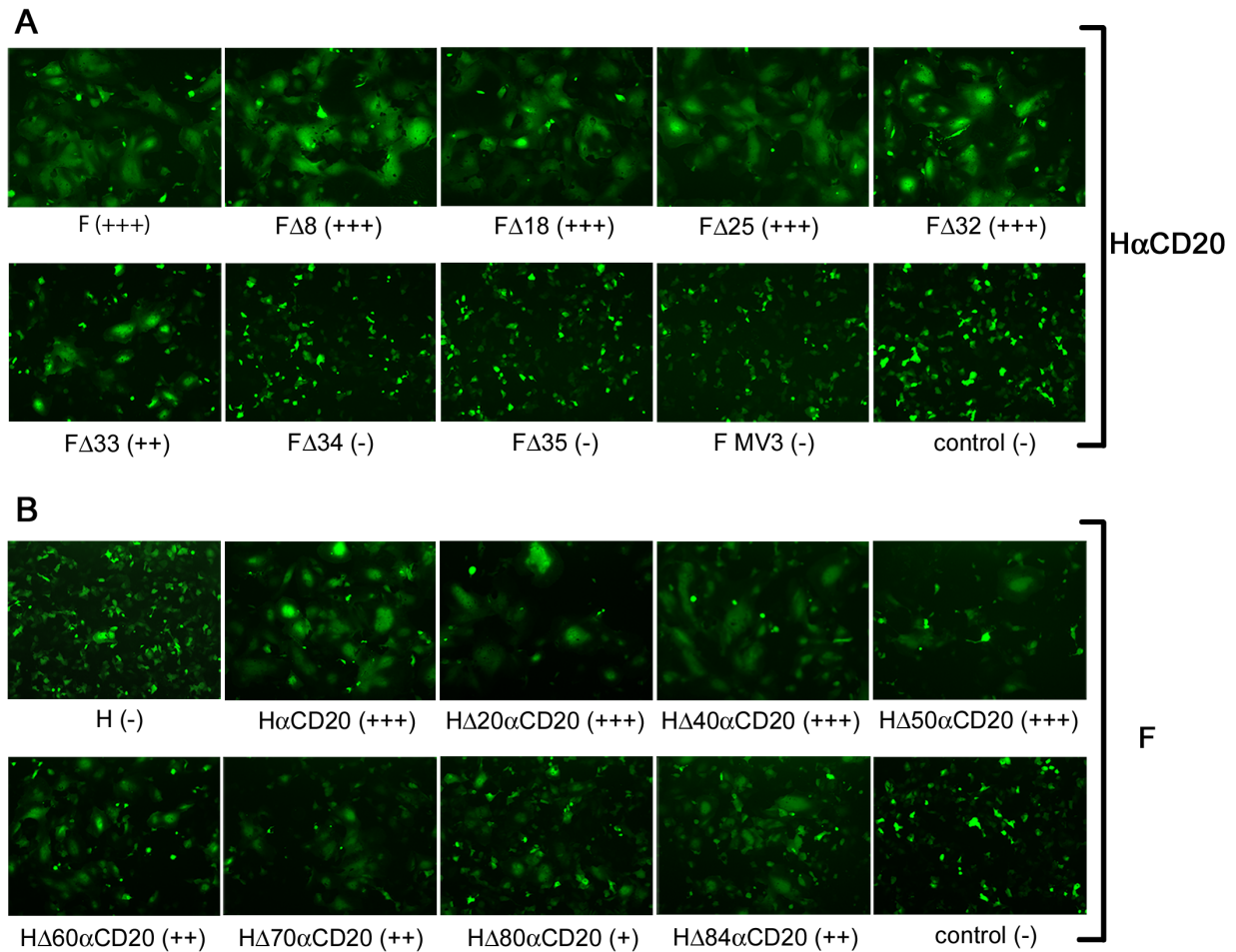
Figure 14: Functional analysis of the modified TPMV glycoproteins in syncytia assays
HT1080-CD20 cells were transfected with plasmids encoding the modified proteins and a plasmid encoding eGFP to test their ability to induce syncytia formation. The untruncated TPMV HαCD20 together with all F protein variants (A) and unmodified TPMV F with all TPMV H variants (B) were tested. Fourty-eight hours after transfection, GFP expression was analyzed by fluorescence microscopy (100x magnification). Control: cells transfected with GFP-encoding plasmid only; GFP: green fluorescent protein; (+) and (–) indicates the size of syncytia formation

In contrast to the results with the F proteins, there was no similar cut-off with a complete loss of function observed when the truncated H proteins were tested together with TPMV F (Fig. 14 B). However, the degree of fusion was slightly reduced in proteins with larger truncation at the cytoplasmatic tail when compared to the wildtype. As expected, the untruncated TPMV H protein without the scAb (H) does not form syncytia because of the lack of a receptor for TPMV on HT1080 cells.

6.2. Identification of a novel TPMV fusion protein fragment

As already mentioned, the TPMV F protein is expressed as a F0 precursor protein and cleaved during activation. To characterize the TPMV F protein mutants with truncated modified cytoplasmic tail for pseudotyping lentiviral vectors, a novel antibody against a peptide in the ectodomain of the F protein was generated. Surprisingly, with this antibody a novel fragment with a molecular mass of about 48 kDa was detected, suggesting that the F protein is cleaved twice in addition to the removal of the signal peptide, resulting in the fragments F1a, F1b and F2 (Fig. 12 A, C). In this part of the result section, the additional cleavage of the TPMV F protein is further described.

6.2.1. Further characterization of the TPMV F protein with the novel antibody F_{ecto}

The TPMV F gene is located between the gene for the M protein and the attachment (H) protein, showing the typical paramyxovirus gene order (Fig. 15, A). The 553 amino acid protein (61 kDa) has three hydrophobic regions, the signal peptide (SP), the fusion peptide (FP), and the transmembrane domain (TM) (as predicted by TMHMM, http://www.cbs.dtu.dk/services/TMHMM) (Fig. 15, B). In the ectodomain there are four possible glycosylation sites, three on the F2 fragment (11 kDa) and one on the F1 fragment (50 kDa), which are all glycosylated (Fig. 15, B, triangles) (Springfeld et al., 2005). The novel antibody was generated against the stalk region of the protein's ectodomain (αF_{ecto}, amino acids 462 to 480) (Fig. 15, B), corresponding to the peptide ELEMDKTQKALDRSNKIL (Fig.15, C, written in bold) which is located in the heptad repeat region B (HRB) (von Messling et al., 2004). Since a novel fragment around 45 kDa can be detected with αF_{ecto}, an additional F protein cleavage has to occur downstream of the αF_{ecto} binding peptide. Alignment of this part of the ectodomain and the transmembrane region of selected paramyxoviruses reveals a certain degree of sequence conservation with a characteristic pattern of hydrophobic amino acids (Fig. 15, C).
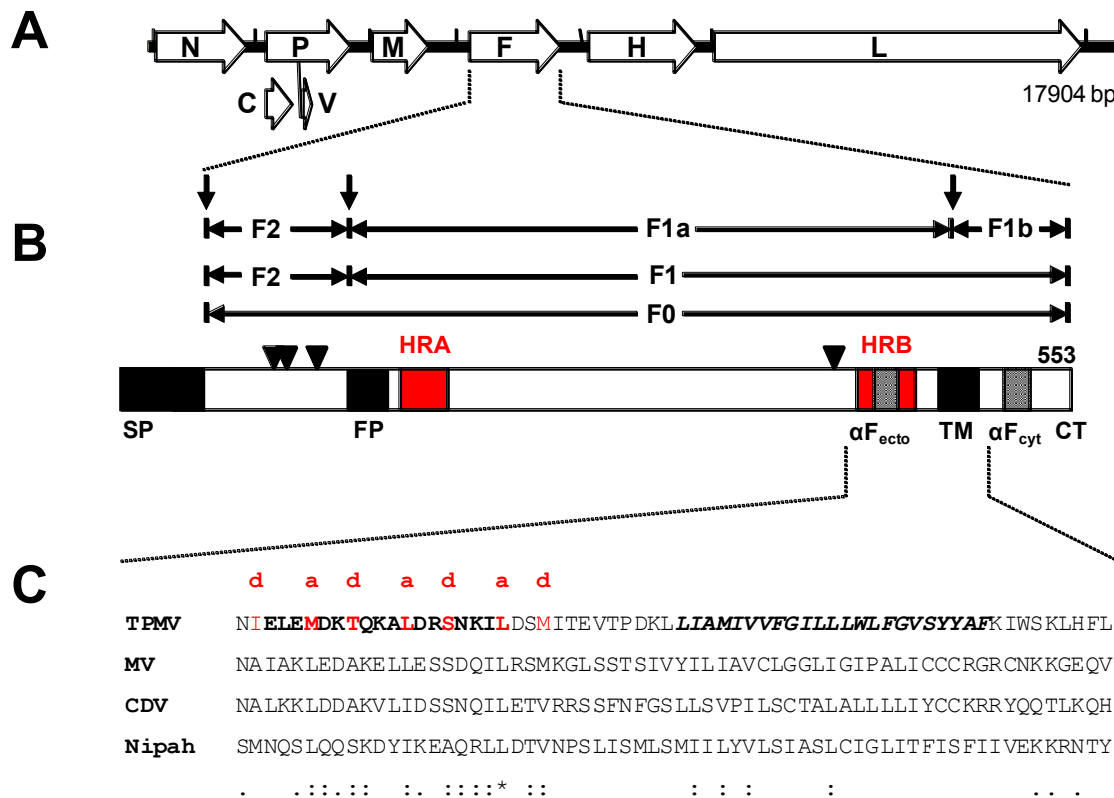
Figure 15: Genome organization of TPMV and structure and sequence of the F protein
The map of the TPMV genome shows typical paramyxovirus gene order (A). In the linear drawing of the TPMV F protein (B), the black triangles (▼) indicate the glycosylation sites and the arrows the cleavage sites of the F0 precursor protein. Cleavage activation results in the fragments F1a, F1b and F2. The grey-colored boxes mark the peptide binding side of the antibodies αF$_{ecto}$ and αF$_{cyt}$. The red-colored boxes indicate the positions of the heptad repeat regions HRA and HRB. Alignment of the F1a/F1b junction, the αF$_{ecto}$ binding site (in boldface) and the predicted transmembrane domain (in boldface and italic) of TPMV, MV, CDV and Nipah virus shows only low similarities (C). Identical amino acids are indicated by an asterisk, highly conserved amino acids are indicated by a colon, and well conserved amino acids are indicated by a period. The hydrophobic residues of the first and fourth (a and d) positions of heptad repeat region B (HRB) are in red color. HRA: heptad repeat region A; SP: signal peptide; FP: fusion peptide; TM: transmembrane domain; CT: cytoplasmic tail; MV: measles virus; CDV: canine distemper virus

6.2.2. The novel F1a fragment is transported along the secretory pathway and cleaved at the carboxy-terminus

For further characterization of the novel TPMV F1a fragment, the glycosylation pattern was investigated. For this purpose, 293T cells were transfected with a plasmid encoding TPMV F and the protein content of the lysate was determined by Bradford assay. Subsequently, the lysate was incubated either with the deglycosylation enzyme PNGase F or Endoglycosidase H (Endo H), applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the novel

F_{ecto} antibody and an antibody against the C terminus of the TPMV F protein (αF_{cyt}, Fig. 15, B).

In lysate analyzed by Western Blot using the F_{ecto} antibody the novel fragment F1a with a molecular mass of about 45 kDA was detected, but not with an antibody against the cytoplasmic tail (Fig. 16, A) (Springfeld et al., 2005). The lysate was incubated with PNGase F, a deglycosylation enzyme that catalyzes the complete removal of N-linked oligosaccharide chains from glycoproteins. Digestion with PNGase F resulted in fragments with smaller molecular weight, confirming that the glycosylation residues were removed. The shift of the F0 fragment was larger compared to the F1 and F1a fragment, accordingly to the removal of four glycosylation residues on the F0 and only one on the F1 and F1a fragment. To investigate whether the new F1a fragment is expressed on the cell surface, the lysate was incubated with Endo H. This enzyme is used to monitor posttranslational modifications in the Golgi apparatus. It removes only high mannose and some hybrid oligosaccharides from N-linked glycoproteins which are added before the protein passes the medial Golgi region, but it does not remove complex oligosaccharides. Proteins, that are resistant to Endo H, are correctly processed through the Golgi, containing complex oligosaccharides added in the trans-Golgi and rapidly transported to the cell surface. Endo H-sensitive proteins have not been processed beyond the medial Golgi region. As expected, the precursor protein F0 was mostly sensitive towards Endo H (Fig. 16 A) resulting in a shift of the signal, respectively. The F1 and F1a fragment were almost completely resistant towards the enzyme. These data demonstrate that the F0 fragment was mainly located intracellular whereas the F1 and also the F1a fragment were transported to the cell surface. Two additional bands around 60 and 70 kDa were detectable in lysate of TPMV F incubated with Endo H. Since these signals were also detectable in lysate of untransfected cells together with Endo H, they were probably due to unspecific binding of the αF_{cyt} antibody to the enzyme.
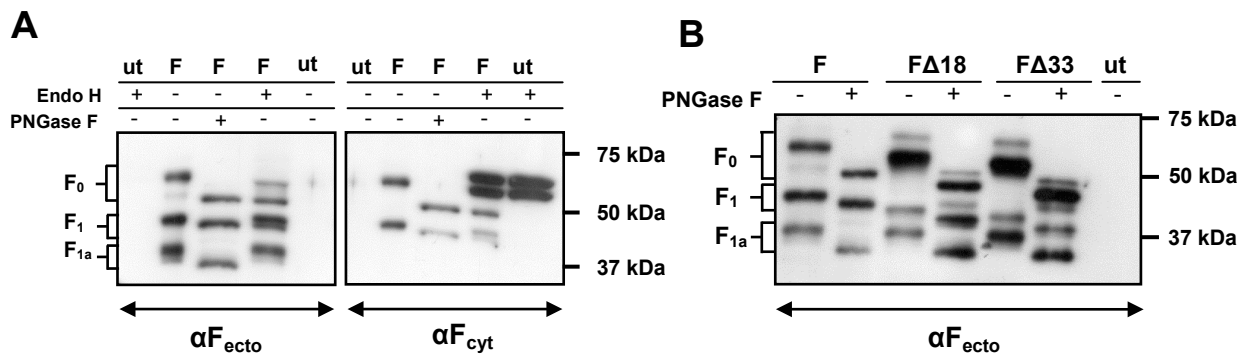
Figure 16: Deglycosylation of TPMV F variants and Western Blot analysis using αF$_{ecto}$ and αF$_{cyt}$
The TPMV F protein variants were expressed in 293T cells, the lysate incubated with Endo H or PNGase F and fractioned by SDS-10% PAGE followed by detection with αF$_{ecto}$ and αF$_{cyt}$.
Deglycosylation with PNGase F resulted in a shift of F0, F1 and F1a fragment whereas the F1a fragment is resistant to Endo H and could not be detected with αF$_{cyt}$ (A). After PNGase F incubation of F variants truncated at their cytoplasmic tails, same deglycosylation pattern of F1a was detected as for the unmodified F protein (B).The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells

Next, it was investigated, whether the F1a fragment was truncated at the N- or C-terminus with regard to the F1 fragment. Two selected proteins with truncated cytoplasmic tail (FΔ18, FΔ33) were compared to the native TPMV F protein. 293T cells were transfected with the respective expression plasmids and the protein content of the lysate was determined by Bradford assay. Afterwards, the lysate was digested with PNGase F, applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the novel F$_{ecto}$ antibody.

The F0 and the F1 fragment of the truncated proteins migrated faster according to their molecular weight, while the F1a fragment stayed at the same height (Fig. 16, B) compared to the unmodified TPMV F protein. This observation demonstrates that the F1 fragments were cleaved at the carboxy-terminus, since the molecular weight of the F1a fragment was not affected by truncation of the cytoplasmic tail. Hence, the cleavage site was estimated to be located closely proximal to the transmembrane domain of the protein (amino acids 493 to 515, Fig. 15, C). Since αF$_{ecto}$ is directed against amino acids 462 to 480, cleavage has to occur downstream of these amino acids. It is noteworthy, that a strong F1 signal is detectable in all lysates, demonstrating that the second cleavage occurred only in a fraction of the F protein.

To demonstrate that the second cleavage occurs also in cells naturally infected by the *Tupaia paramyxovirus*, Tupaia baby fibroblasts (TBFs) were transfected with expression plasmids for TPMV F, FΔ18 and FΔ33. The lysate was harvested 48 hours after transfection and protein content determined by Bradford assay.

Subsequently, the lysate was applied to SDS-polyacrylamide-gelelectrophoresis and Western Blot analysis was performed using F_{ecto} antibody.
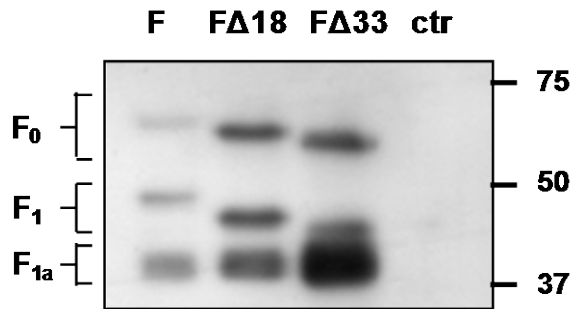
Figure 17: F0, F1a and F1a fragment detection of TPMV F variants expressed in TBF cells
TBF cells were transfected with TPMV F protein variants and the proteins separated by SDS-10% PAGE followed by Western Blot analysis using αF_{ecto}. F0, F1 and F1a were detected after expression in TBF cells. The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells

All three TPMV F protein fragments (F0, F1, F1a) were detected in cell lysate of Tupaia baby fibroblasts after transient transfection with different TPMV F expression plasmids (Fig. 17), confirming, that the second F1a/F1b cleavage occurs also in cells naturally infected by TPMV. Since TBFs are primary cells and difficult to transfect, all further experiments were performed in 293T cells if not indicated different.

6.2.3. The TPMV F protein ectodomain is shed into the supernatant of cells

Next, it was tested whether the F1a fragment was shed into the supernatant of cells expressing TPMV F and the modified variants FΔ18 and FΔ33. For this purpose, 293T cells were transiently transfected with plasmids expressing TPMV F, FΔ18 and FΔ33. Fourty-eight hours after transfection, the supernatant of the cells and the lysate was harvested. Protein content of the lysate was determined by Bradford assay and applied to SDS-polyacrylamide-gelelectrophoresis. Of the supernatant, the highest possible volume was applied with subsequent Western Blot analysis using F_{ecto} antibody.

Indeed, the F1a fragment was detected in all supernatants of 293T cells transfected with the different plasmids expressing TPMV F protein variants (Fig. 18) and almost no amounts of F0 and F1, suggesting, that F1a was secreted and released in the supernatant of the cells. Since it was predicted that the membrane anchor is cleaved of, the presence of the F1a fragment was expected.
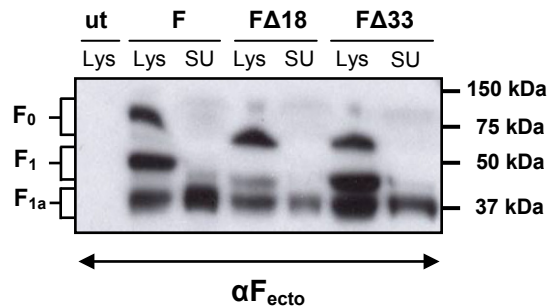
Figure 18: TPMV F1a fragment is shed into the supernatant of cells
Transfection of 293T cells with TPMV F protein variants. The lysate and supernatant were harvested and proteins were separated by SDS-10% PAGE followed by Western Blot analysis using αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells; Lys: lysate; SU: supernatant; inf. Infected TBF cells

6.2.4. The TPMV F1a fragment is present in TPMV virions

Next, it was investigated if the F1a fragment is also present in TPMV virions. Although the F1a fragment has lost its membrane anchor, it could possibly be present in F protein trimeric complexes as described previously for *measles virus* (von Messling et al., 2004). For this purpose, TBFs were infected with TPMV and after 5-6 days, the supernatant (SU) and lysate (Lys) of infected and non-infected cells were harvested and analyzed as described before. To exclude detection of F0, F1 and F1a in the supernatant due to cell lysis after syncytia formation or shedding, the TPMV-virion-containing supernatant was concentrated and purified by ultra centrifugation (UC) over a 20 % sucrose cushion. Only 2.5 µg of total protein content of concentrated virions was analyzed compared to lysate, whereat a total protein concentration of 10 µg was applied.

Indeed, the F1a fragment was clearly detectable in high amounts in purified TPMV virions (Fig. 19). Surprisingly, an additional fragment around 47 kDa was detected in virions, which was not present in lysate of infected TBF cells, suggesting that F protein processing is even more complex. As expected, no F0 fragment was detectable in TPMV virions.
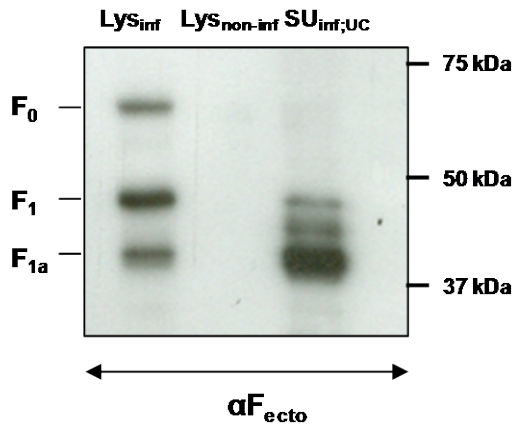
Figure 19: The F1a fragment is present in TPMV virions
Tupaia baby fibroblasts (TBF cells) were infected with TPMV and lysate and supernatant were harvested. TPMV-virion-containing supernatant was concentrated and purified by ultra centrifugation (UC) over a 20 % sucrose cushion. Proteins were separated by SDS-10% PAGE followed by Western Blot analysis using αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left. Lys_{inf}: lysate of infected TBFs; $SU_{inf;UC}$: concentrated supernatant of infected TBFs; $Lys_{non-inf}$: lysate of non-infected TBFs

6.3. Characterization of the newly identified cleavage site

Having demonstrated that the novel F1a fragment is not only secreted but cleaved at the carboxy-terminus, now the cleavage site between the F1a and F1b-fragment was characterized.

6.3.1. Mapping of the F1a-F1b cleavage site

To determine the cleavage site between the F1a and F1b-fragment, eleven c-terminally truncated proteins were generated with a Flag-tag added at positions 483, 491, 492, 495, 498, 501, 505, 507, 510, 513 and 516 (Fig. 20, modified from (von Messling et al., 2004)) (Fig. 21, A) and detected with an αFlag-antibody and the αF_{ecto}-antibody. It was assumed that the F1a-F1b cleavage may result in loss of protein detection, as the αFlag-antibody does not recognize the cleaved protein anymore.
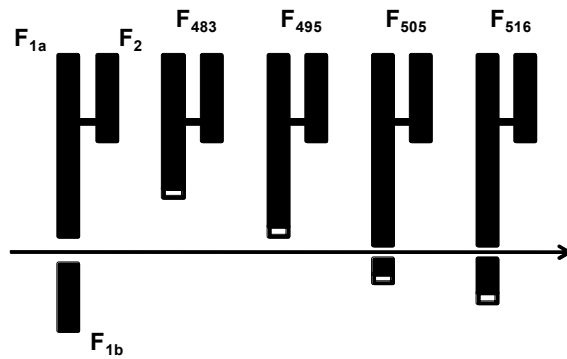
Figure 20: Schematic drawing of truncated Flag-tagged TPMV F variants
The TPMV F protein was truncated at indicated amino acid positions and a Flag-tag (white box) was fused at the carboxy-terminus, resulting in shorter F1a and F1b fragments compared to the native F protein (left).The arrow indicates the expected position of F1a/F1b cleavage. The line connecting F1a and F2 fragments symbolizes the disulfide bond. Only four representative Flag variants are shown here, F_{438}, F_{495}, F_{505} and F_{516}.

The different truncated TPMV F-Flag-variants were generated by PCR using primers that introduced a Flag-tag at defined amino acid positions, followed by a stop codon. Afterwards, 293T cells were transfected with the respective expression plasmids and 48 hours later, the lysate was harvested and protein content determined by Bradford assay. The lysate was then applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the antibodies αFlag and $αF_{ecto}$.

All F0 fragments were detected using $αF_{ecto}$ (Fig. 21, B). However, when the same cell lysates were tested in Western Blots with αFlag antibody, TPMV F Flag variants longer than 498 amino acids were not detected anymore, suggesting that cleavage of F1a/F1B occurred between amino acid position 498 and 501. Surprisingly, there was no F1 fragment detectable with αFlag-antibody. Regarding Western Blot analysis with $αF_{ecto,}$ there was the F0 fragment and a strong signal around 40 kDa, suggesting that this was shortened F1 together with F1a. In F_{Flag} variants longer than 498 amino acids, the F1 together with F1a signal was strongly decreased. Currently, these observations cannot be explained and further investigations are necessary. Nevertheless, cleavage site of F1a/F1b was mapped between amino acids 498 and 501. To map the cleavage site in detail, two further protein variants were generated with a flag-tag after amino acid position 499 and 500 and analyzed by Western Blot as described before (Fig. 21, C). The variant TPMV F_{500} was not detected anymore by αFlag-antibody, demonstrating that cleavage occurred exactly between amino acid 499 and 500. Surprisingly, this position was located in the predicted transmembrane domain. Again, no F1 fragments were visible using αFlag antibody. In contrast, F1 was detected when the Flag-tag was added after amino acid position

533, which is located in the cytoplasmic tail, or at the C-terminal end of the protein (amino acid position 553). However, some unexpected results were obtained that cannot be explained and might be due to the heavy modifications of the protein.
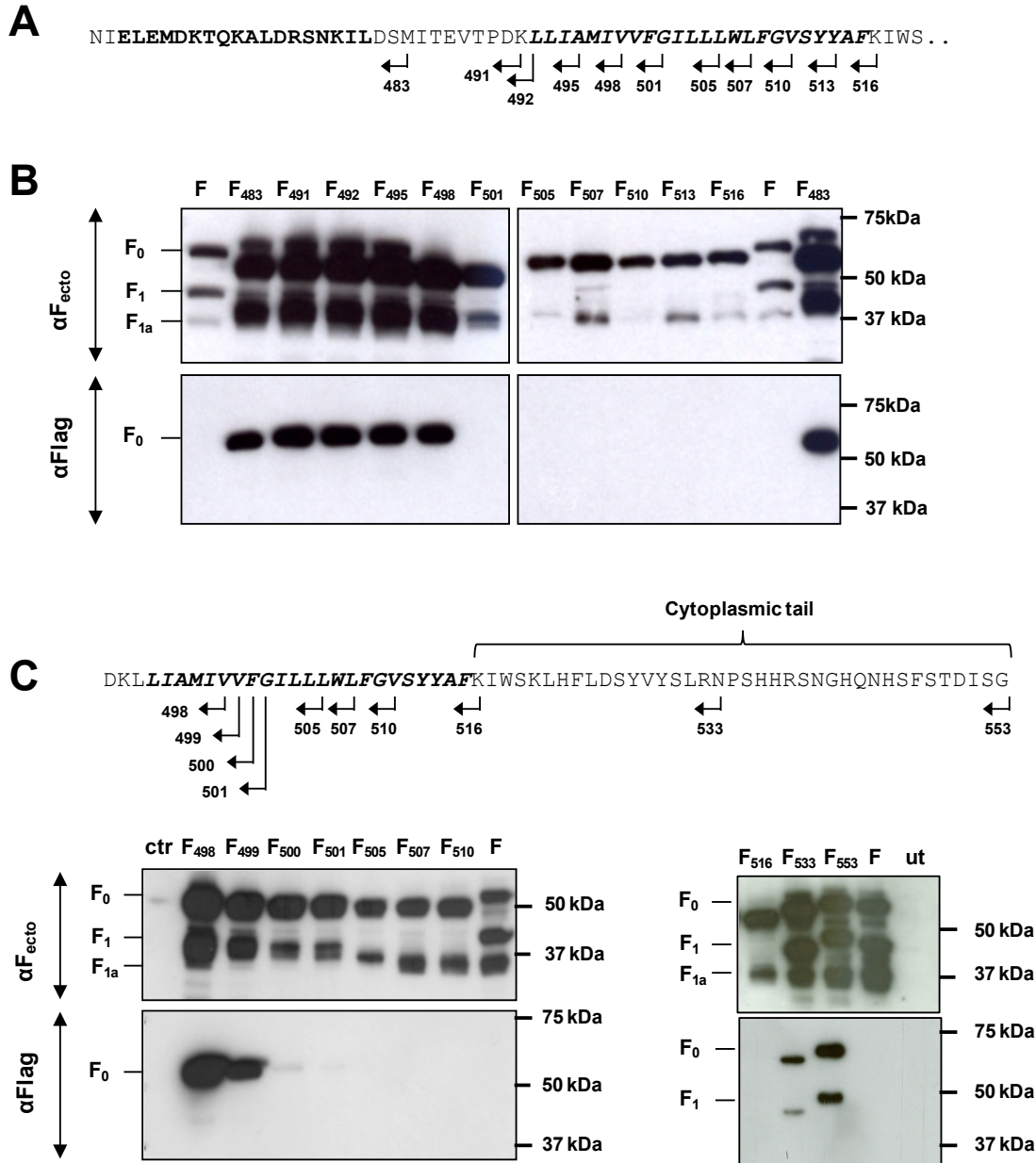
Figure 21: Mapping of F1a/F1b cleavage site by Western Blot analysis of TPMV F Flag-variants
Scheme of TPMV F transmembrane region (A) and mapping of the F1a/F1b cleavage site (B and C). (A) The TPMV F protein was truncated at the indicated amino acid (aa) positions (number and arrow) and a Flag-tag was added at the truncation site. The binding peptide of the antibody αFecto is in bold, the predicted transmenbrane domain in bold and italics. (B) Western Blot analysis of truncated Flag-tagged F protein variants. 293T cells were transfected with TPMV F Flag-tagged variants and the lysate was harvested. Proteins were separated by SDS-10% PAGE followed by Western Blot analysis using the antibodies αF_{ecto} and αFlag. (C) Detailed mapping of the cleavage site. A Flag-tag was added at single aa positions between aa 498, 501 and aa 533, 553 of TPMV F. Western Blot analysis was performed as described before. The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells

6.3.2. Mutation of the F1a-F1b cleavage site

After identification of the exact amino acid positions of the F1a/F1b cleavage site, it was investigated whether cleavage was inhibited by mutation of the amino acids, respectively. For this purpose, two different cleavage mutants were generated by mutagenesis PCR replacing the three amino acids at position 498-500 (F_{Ala1}) or at position 499-501 (F_{Ala2}) by alanin (Fig. 22, A). 293T cells were transfected with the respective expression plasmids and 48 hours later, the lysate was harvested and protein content determined by Bradford assay. The lysate was then applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using αF_{ecto}-antibody.
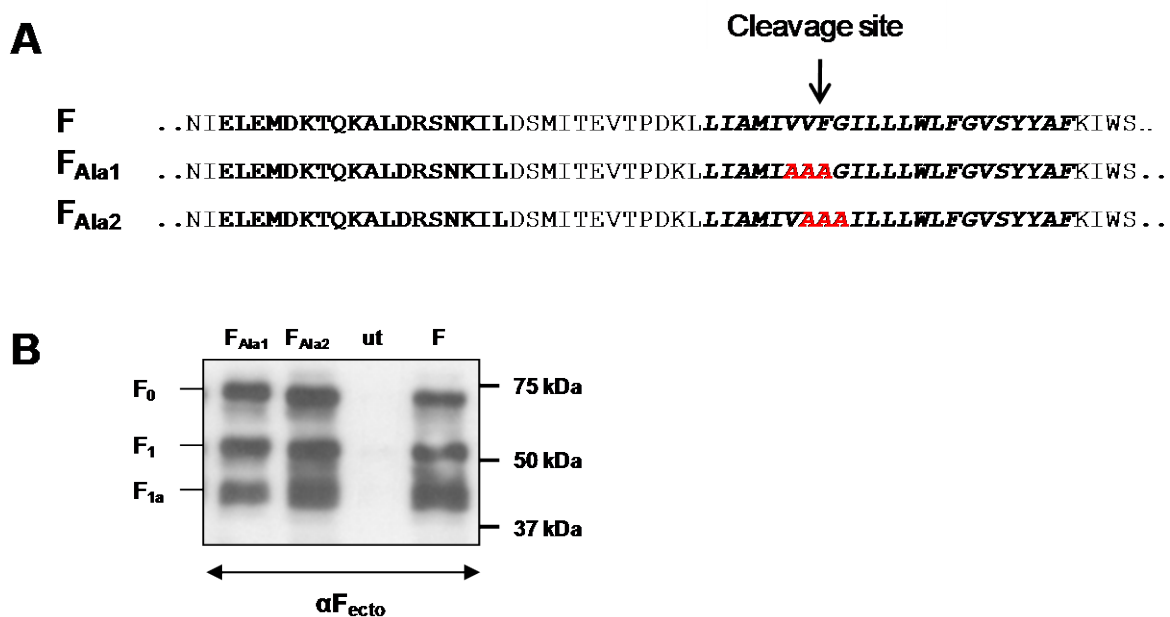
Figure 22: Mutation of the identified F1a-F1b cleavage site does not prevent F1a-F1b cleavage
Amino acids surrounding the new identified F1a-F1b cleavage site were replaced by alanins resulting in the TPMV F mutants F_{Ala1} and F_{Ala2} (A). The mutated amino acids are in red, the binding peptide of the antibody αFecto is in bold, the predicted transmenbrane domain in bold and italics. The identified cleavage site between valine and phenylalanine is indicated by an arrow. For Western Blot analysis of the F mutants (B), 293T cells were transfected with the respective expression plasmids and the lysate was harvested. Proteins were separated by SDS-10% PAGE followed by Western Blot analysis using the antibody αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells

In both F protein mutants, F_{Ala1} and F_{Ala2}, the fragments F0, F1 and F1a were detected (Fig. 22, B). Compared to the unmodified TPMV protein, there was no difference in molecular size of the fragments. These data demonstrate that the F1/F2 and the F1a/F1b cleavage still occurred, assuming that the mutated amino acids are not necessary for F protein cleavage.

6.4. Characterization of the cleavage protease

After mapping the F1a/F1b cleavage site, next step is to characterize the cleavage protease. As mentioned before, the F protein is cleaved twice during activation. Although the first cleavage (F1/F2) is already described (Springfeld et al., 2005), the responsible protease is not yet identified. To get a first overview about the involved protease the commercially available protease inhibitor cocktail 'Complete' (Roche) was tested. One tablet of 'Complete' was dissolved in DMEM medium according to the manufactures instructions. 293T cells were transfected with pCG-TPMV-F expression plasmid and five hours after transfection, the medium was changed against medium supplemented with stepwise diluted concentrations of 'Complete'. After 48 hours, the lysate was harvested and protein content determined by Bradford assay. The lysate was then applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the αF_{ecto}-antibody.

In the presence of high concentrated protease inhibitor, F0 was detected in very low amount, but no F1 and F1a fragment (Fig. 23). In lysates of transfected cells incubated with lower 'Complete' concentrations small amounts of F1 and F1a were presented in addition to the F0 fragment.
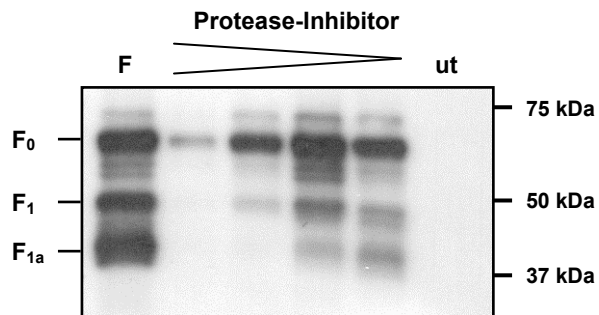
Figure 23: Protease inhibitor prevents F1-F2 and F1a-F1b cleavage
293T cells were transfected with TPMV F and five hours after transfection the medium was replaced by medium supplemented with 'Roche Complete' protease inhibitor cocktail in different concentrations. 48 hours later, the lysate was harvested and proteins were separated by SDS-10% PAGE followed by Western Blot analysis using the antibody αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left. ut: untransfected cells

In conclusion, it has been shown that the amount of F1 and F1a increased with lower concentrations of 'Complete', demonstrating that both cleavages, F1/F2 and F1a/F1b, can be inhibited by protease inhibitor added to the medium. Since the exact

composition of this inhibitor is confidential, no conclusions about the protease can be drawn and further experiments have to be performed.

6.4.1. Screening of different protease inhibitors

Since it was shown that cleavage of the TPMV protein was prevented by incubation with the protease inhibitor cocktail 'Complete', the question raised if it is possible to identify the cleavage protease by analyzing different protease inhibitors separately.

For this reason, 293T cells were transfected with TPMV F expression plasmid and treated with ten different protease inhibitors as described before. The inhibitors and their specificities are summarized in table 1. They were added to the cells in concentrations suggested by the manufacturer (Roche, Mannheim).

Table 1: Protease inhibitors and their specificities

Inhibitor	Specificity of Inhibitor
Antipain-dihydrochloride	papain, trypsin is inhibited to a small extent
Bestatin	amino peptidase, including aminopeptidase B, leucine aminopeptidase, tripeptide aminopeptidase
Chymostatin	α-, β-, γ-, δ-chymotrypsin
E-64	cysteine proteases
Leupeptin	serine and cysteine proteases such as plasmin, trypsin, papain, cathepsin B
Pepstatin	aspartate proteases like pepsin, renin, cathepsin D, chymosin
Phosphoramidon	metallo endopeptidases, specifically thermolysine, collagenase, metallo endoproteinases
Pefabloc SC	serine proteases, e.g. trypsin, chymotrypsin, plasmin, thrombin
EDTA-Na$_2$	metalloproteases
Aprotinin	serine proteases

After incubation of the cells for 48 hours with the inhibitors, the lysate was harvested and protein content determined by Bradford assay. Afterwards, the lysate was applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot using the αF_{ecto}-antibody.

No cleavage inhibition was achieved with the inhibitors phosphoramidon, EDTA-Na$_2$, bestatin, pepstatin and aprotinin, the F1 and the F1a fragment were still detectable in high amounts and in addition to the F0 fragment (Fig. 24). In contrast, reduced amounts of F1 and F1a were observed when transfected cells were incubated with the inhibitors antipain, E-64 and leupeptin and possibly chymostatin. Incubation with Pefabloc SC resulted in cell death, thus, no analysis of the effect of this inhibitor on protease cleavage was possible.
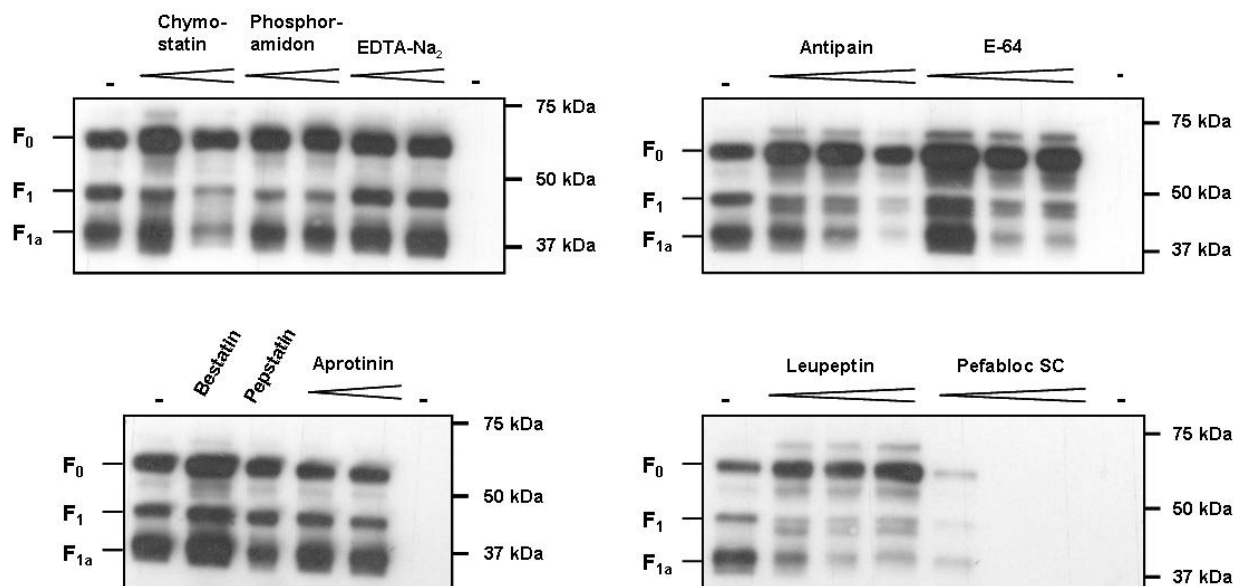
Figure 24: Screening of ten different protease inhibitors
293T cells were transfected with TPMV F and five hours after transfection the medium was replaced by medium supplemented with the respective protease inhibitors in different concentrations suggested by the manufacturer. 48 hours later, the lysate was harvested and proteins were separated by SDS-10% PAGE followed by Western Blot analysis using the antibody αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left.

Having demonstrated the effects of different protease inhibitors in this first screening, now the potentially effective inhibitors chymostatin, antipain, E-64 and leupeptin were selected and tested in several concentrations, starting with lowest concentration suggested by the manufacturer. Lysate samples were prepared as described before followed by Western Blot analysis.

In the presence of leupeptin, no cleavage occurred, neither for F1/F2 nor F1a/F1b, even with the lowest concentration of 1 µg/ml only the F0 fragment was detectable (Fig. 25, A). Antipain inhibited protease cleavage with increasing concentrations (Fig. 25, B). With higher amounts of inhibitor higher amounts of the F0 fragment were detected. The F1 fragment is less, but independently of the inhibitor concentration expressed, whereat there was almost no F1a expressed after incubation with 200 and 400 µg/ml. A similar effect was observed for E-64 (Fig. 25, D). Incubation with chymostatin with concentrations of 25 and 50 µg/ml resulted in detection of F0, F1 and F1a fragments (Fig. 25, C) demonstrating that cleavage is not inhibited.
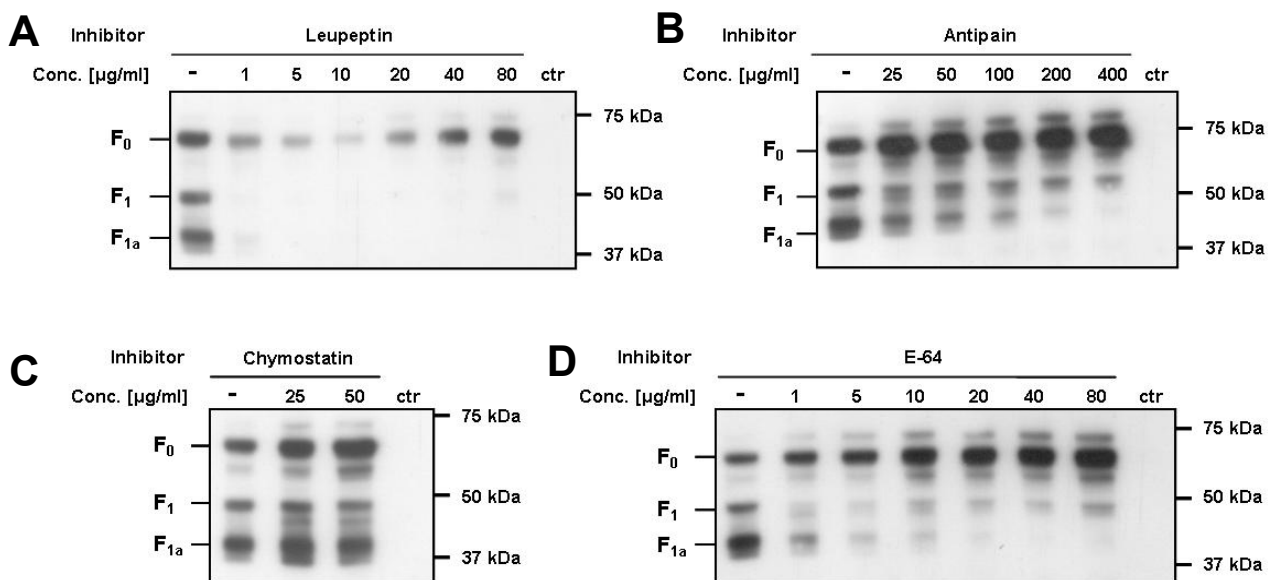
Figure 25: F protein cleavage is inhibited by leupeptin, antipain and E-64
To further characterize the promising protease inhibitors leupeptin (A), antipain (B), chymostatin (C) and E-64 (D), 293T cells were transfected with TPMV F and five hours after transfection the medium was replaced by medium supplemented with stepwise diluted protease inhibitor, respectively. 48 hours later, the lysate was harvested and proteins were separated by SDS-10% PAGE followed by Western Blot analysis using the antibody αF_{ecto}. The positions of F0, F1 and F1a are indicated on the left.

The inhibitors and the respective protease families are summarized in Table 2. Taken together, all effective substances (Leupeptin, Antipain, E-64) inhibited the family of cysteine proteases. These data suggest that a cysteine protease might be involved in F protein cleavage and activation.

Table 2: Protease families and their inhibitors

	Aspartic	Cysteine	Metallo	Serine	Threonine	Inhibition of TPMV F cleavage
Antipain		x		x		+
Bestatin			x			-
Chymostatin		x		x		-
E-64		x				+
Leupeptin		x		x	x	+
Pepstatin	x					-
Phosphoramidon			x			-
Pefabloc SC				x		-
EDTA-Na$_2$			x			-
Aprotinin				x		-

6.4.2. F protein cleavage is necessary for cell fusion

To answer the question if F1/F2 and F1a/F1b cleavage is necessary for cell fusion the effect of the three identified protease inhibitors leupeptin, antipain and E-64 were examined in fusion assays. TBF cells were transiently transfected with TPMV-H- and –F-expression plasmids. Five hours after transfection the medium was replaced by medium supplemented with the protease inhibitors at different concentrations, respectively, and as control, medium without inhibitor was added. Fourty-eight hours later, the cells were observed under the microscope and the lysate was harvested. Afterwards, the protein content was determined by Bradford assay and the lysate applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot.

As expected, TBF cells transfected with pCG-TPMV-H and pCG-TPMV-F fused and form large syncytia after incubation with non-supplemented medium (Fig. 26).
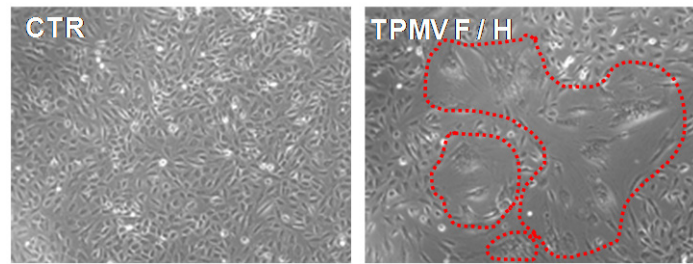
Figure 26: TBF cells form large syncytia after transfection with TPMV H and F
TBF cells were transfected with expression plasmids for TPMV H and F. Fourty-eight hours after, cells were analyzed by microscopy using 10x magnification and representative pictures were taken. Left: ut, untransfected cells; right: cells transfected with TPMV F and H.

In TPMV-H and –F-transfected TBFs incubated with antipain-supplemented medium (100 µg/ml and 400 µg/ml) fusion of cells was completely inhibited (Fig. 27). For E-64 and Leupeptin, a complete inhibition of cell fusion was only observed when cells were incubated with higher inhibitor concentrations [80 µg/ml].
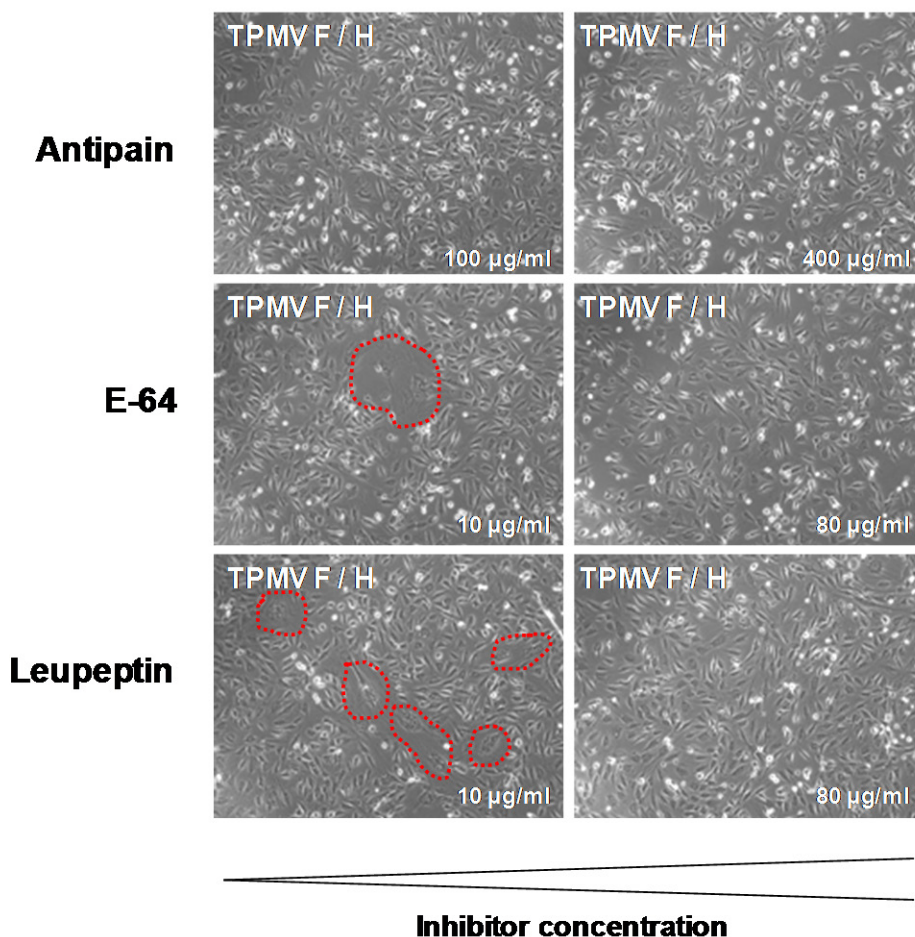
Figure 27: Protease inhibitors prevent syncytia formation of TPMV F/H-transfected TBF cells
TBF cells were transfected with expression plasmids for TPMV H and F. Five hours after transfection, the medium was replaced by medium supplemented with the protease inhibitors antipain, E-64 or leupeptin in two different concentrations. 48 hours later, cells were analyzed by microscopy using 10x magnification and representative pictures were taken.

6.5. Pseudotyping of lentiviral vectors with the modified TPMV proteins

Characterization of the modified TPMV glycoproteins showed that all variants were expressed after transient transfection. Additionally, modified TPMV H proteins that are expressed on the cell surface and have fusion helper function were identified, as well as F proteins with truncated cytoplasmic tails, that still support fusion of the cell membrane. The next step for generating retargeted lentiviral vectors was to pseudotype them with the modified TPMV glycoproteins.

6.5.1. Generation of TPMV-pseudotyped vectors

In a first screening, the untruncated TPMV HαCD20 protein was tested together with all F protein variants for the ability to pseudotype lentiviral vectors. For the production, 293 T cells served as packging cells and were transiently transfected in a T75 flask with the packaging plasmid pCMVΔR8.9, the green fluorescent protein (GFP) encoding transfer vector plasmid pSEW, the TPMV HαCD20 plasmid and one of the TPMV F variant-encoding plasmids, respectively. The TPMV-plasmids were used in equal amounts. As positive control, VSV-G-pseudotyped vectors were generated by transfection of the VSV-G encoding plasmid pMD.G2 along with the packaging and transfer plasmids. Additionally, vector particles that are pseudotyped with MV_{NSe} HΔ18αCD20 and FΔ30 (MV vaccine strain Edmonston B) or MV_{WT} HΔ18 and FΔ30 (MV wildtype strain) (Funke et al., 2008a) were produced. Fourty-eight hours after transfection, the supernatants of vector-producing 293T cells were harvested, concentrated by low-speed centrifugation and used for transduction of the CD20-positive cell line Raji (Human Burkitt's lymphoma cell line). After 72 hours, the transduced cells were analyzed by FACS (fluorescent activated cell sorting) and the titers were calculated.

Efficiency of transfection was analyzed by fluorescence microscopy. The packaging cells showed high GFP expression due to the pSEW plasmid (Fig. 28). For both MV and TPMV vector producing cells, syncytia formation was observed that was possibly due to the high transfection levels and αCD20-scAb interactions. In MV-vector producing cells, syncytia formation was increased, probably because of incomplete mutation of the MV H contact residues to the MV receptors.
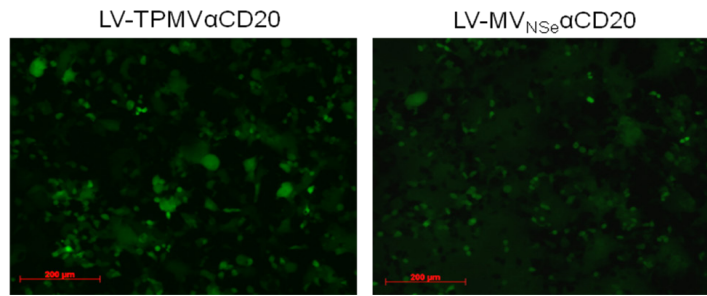
Figure 28: GFP expression of packaging cells after plasmid transfection for LV generation
293T packaging cells were co-transfected with lentiviral expression plasmids and plasmids encoding TPMV HαCD20 and F (LV-TPMVαCD20) or MV_{NSe} HΔ18αCD20 and FΔ30 (LV-MV_{NSe}αCD20), respectively. Fourty-eight hours after transfection, GFP expression was analyzed by fluorescence microscopy (100x magnification). Representative pictures are shown. Scale bar: 200 µm

Seventy-two hours after transduction of Raji cells, highest titers of the TPMV-pseudotypes were achieved with HαCD20 together with the unmodified F, FΔ18 and FΔ32 ($9x10^5$ – $3x10^6$ t.u./ml) (Fig. 29). These values are almost in the same range as the MV_{NSe}-control ($3.5x10^6$ t.u./ml). Higher titers were only reached with MV_{WT}-pseudotypes ($5x10^7$ t.u./ml) and vectors pseudotyped with VSV-G ($4x10^{10}$ t.u./ml).
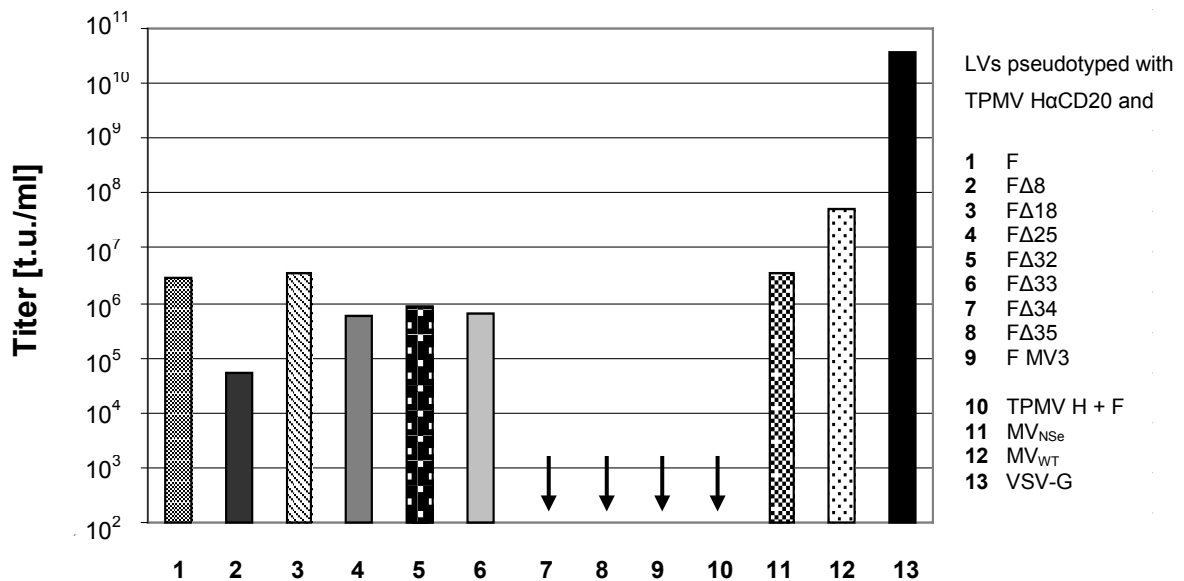
Figure 29: Initial screening titers of TPMV HαCD20 and F protein variants to pseudotype LVs
293T cells were co-transfected with lentiviral expression plasmids and plasmids encoding TPMV HαCD20 and different truncated F variants, respectively. After 48 hours, vector-containing supernatant was harvested and concentrated by low-speed centrifugation for 24 hours at 4°C. The pellet was then used for transduction of Raji cells. Seventy-hours later, GFP expression was analyzed by FACS and titers [t.u./ml, titration units/ml] were determined. The arrows indicate titers below the detection limit. MV_{NSe}: glycoproteins of measles virus vaccine strain Edmonston B, HΔ18αCD20 and FΔ30; MV_{WT}: glycoproteins of measles virus wild type strain, HΔ18 and FΔ30; VSV-G: glycoprotein of vesicular stomatitis virus; GFP: green fluorescent protein; LVs: lentiviral vectors

Surprisingly, TPMV HαCD20 together with untruncated F allowed efficient pseudotype formation ($3x10^6$ t.u.ml). This is in contrast to the MV-pseudotyped vectors, where only truncated MV glycoproteins could be incorporated (Funke et al., 2008a). In accordance with the results obtained in syncytia assays, F proteins with a cytoplasmic tail shorter than 5 amino acids are not able to mediate gene transfer, as well as the TPMV H protein without the αCD20-scAb.

In further experiments, MV_{NSe} truncated and retargeted glycoproteins (HΔ18αCD20 and FΔ30) are used as a control and will be referred to as LV-MVαCD20.

6.5.2. Screening of all TPMV H and F protein variants

Next, all TPMV H and F protein variants were screened in all combinations for their ability to mediate gene transfer into CD20-positive cells. Since it is associated with high effort to test all combinations in T75 flasks, this screening was performed in a 6 well format.

Vectors were produced by transfection of 293T cells with lentiviral expression plasmids and plasmids encoding the TPMV glycoproteins in equal amounts as described above, but with corresponding less plasmid DNA and in a 6 well plate. Two days post transfection, the supernatant of the packaging cells was directly used for transduction of CD20-positive Raji cells in a 96 well plate. After 48-72 hours, transduced cells showed GFP expression and were analyzed by FACS. Based on the percentage of GFP-expressing cells the titer (transducing units (t.u.)/ml) was calculated.

Titers obtained by screening all F and H protein combinations are shown in Table 3.

Table 3: Screening titers of TPMV H and F combinations for LV-pseudotyping

TPMV[a] [t.u./ml]	HαCD20	HΔ20αCD20	HΔ40αCD20	HΔ50αCD20	HΔ60αCD20	HΔ70αCD20	HΔ80αCD20	HΔ84αCD20	TPMV-H
F	4.0E+04	2.9E+03	4.9E+03	4.6E+03	2.3E+04	6.3E+04	5.2E+04	1.4E+04	< 1.3E+03
FΔ8	8.9E+03	< 1.3E+03	< 1.3E+03	1.8E+04	1.9E+04	4.6E+03	5.4E+04	1.8E+04	< 1.3E+03
FΔ18	4.2E+04	2.9E+03	1.3E+04	1.8E+04	3.4E+04	1.1E+04	8.9E+04	4.9E+04	< 1.3E+03
FΔ25	6.1E+04	7.4E+03	6.0E+04	1.9E+03	2.1E+04	2.6E+04	8.4E+04	5.9E+04	< 1.3E+03
FΔ32	3.1E+04	5.4E+03	8.4E+04	1.7E+03	6.9E+04	2.9E+04	1.3E+05	9.9E+04	< 1.3E+03
FΔ33	2.0E+04	< 1.3E+03	1.5E+04	3.4E+03	1.3E+04	3.5E+03	2.3E+04	6.2E+03	< 1.3E+03
FΔ34	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03
FΔ35	< 1.3E+03	4.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03
F MV3	2.6E+03	< 1.3E+03	3.4E+03	8.1E+03	1.7E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03	< 1.3E+03

detection limit[b] 1.3E+03
< 1.3E+03
1.3E+03 - 1.00E+04
1.0E+04 – 1.0E+05
> 1.0E+05

[a] screening titers in transducing units/ml [t.u./ml];
  screening was performed in duplicate
[b] detection limit: detection of > 1 % GFP expression in FACS analysis

Highest titers (10^5 t.u./ml) were reached with the truncated F variant FΔ32, where only six amino acids of the cytoplasmic tail were left, together with HΔ80αCD20 that has a cytoplasmic tail length of 14 aa (Table 3). High titers were also obtained with the combinations HΔ80αCD20 / FΔ18 and HΔ84αCD20 / FΔ32 (9.8 x 10^4 and 9.9 x 10^4 t.u./ml). Concentration of pseudotyped vectors produced in large scale (T75 or T175 flask) by low speed centrifugation increased titers up to about 10^6 t.u./ml. Altogether, there was a trend to higher titers when glycoproteins with shorter cytoplasmic tails were used. However, F proteins with cytoplasmic tails shorter than five amino acids did not lead to infectious vectors. Considering the loss of fusogenicity of these proteins in fusion assays, this result was expected and already observed in a first screening (Fig. 18). In this screening it was also shown that in contrast to MV, it was possible to pseudotype lentiviral vectors with unmodified TPMV H and F proteins although titers were low. Untargeted TPMV H protein was used as a negative control, truncated MV glycoproteins (Funke et al., 2008a) were used as a positive control and led on average to vector titers in the range of 2-3 x 10^4 t.u./ml (data not shown). VSV-G-pseudotyped vectors reached titers much higher than 10^6 t.u./ml that were above the detection limit in this experiment. The optimal TPMV F and H combination identified in this screening was used for all further experiments (HΔ80αCD20 together with FΔ32) and will be referred to as LV-TPMVαCD20.

6.5.3. Determination of the optimal H to F ratio

In natural occurring MV infections, it was shown, that more F mRNA than H mRNA is produced (Cattaneo et al., 1987a; Plumet, Duprex, and Gerlier, 2005). Following this observation, the H to F ratio of MV-pseudotyped vectors was optimized, which resulted in a more than ten-fold increase in titer. The optimal ratio of H to F identified for MV was 1 : 7 (Funke et al., 2008a). Since there is a gradient of mRNA in all paramyxoviruses, it was tried to enhance titers of TPMV-pseudotyped by optimization the TPMV HΔ80αCD20 to FΔ32 plasmid ratio.

For this purpose, vectors were produced by co-transfection of 293T cells in T75 flasks with the lentiviral expression plasmids and different ratios of pCG-TPMV-HΔ80αCD20 and pCG-TPMV-FΔ32, respectively. After 48 hours, the cell supernatants were harvested and concentrated for 24 hours by low-speed centrifugation. The concentrated particles were then used for transduction of Raji cells. Seventy-two hours later, GFP expression was analyzed by FACS and titers were calculated. The titers were normalized to that obtained after transfection of equal amounts of pCG-TPMV-HΔ80αCD20 and pCG-TPMV-FΔ32.
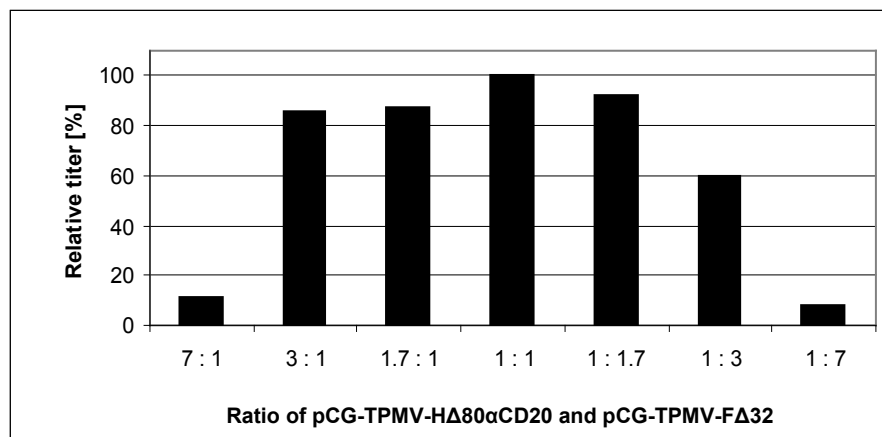
Figure 30: Identification of optimal H : F ratio for pseudotyping lentiviral vectors
During vector production, the plasmid ratio of HΔ84αCD20 to FΔ32 was altered as indicated. Vectors were titrated on Raji cells and titers determined by FACS analysis. Titers were normalized to that obtained after transfection with equal amounts of TPMV HΔ84αCD20 and FΔ32 expression plasmids (100%). This figure shows the average of 3 independently experiments.

In contrast to MV-pseudotypes, there was no significant enhancement of titers by altering the ratio of HΔ80αCD20 and FΔ32 plasmids during vector particle production (Fig. 30). The data show the average of 3 independent experiments. Titers obtained

with equal amounts of H and F plasmids were slightly higher than that obtained with other H : F ratios. Using lesser amounts of H or F plasmids, respectively, resulted in a decrease of titers. Compared to MV-pseudotypes, where a ratio of 1 : 7 is optimal for production, in this case, in a ratio of 1 : 7 and vice versa the titers were decreased to less than 15 %. Further experiments were performed in a HΔ80αCD20 and FΔ32 plasmid ratio of 1 :1.

6.5.4. Confirmation of incorporation of the modified TPMV proteins into LVs

Having demonstrated that lentiviral vectors pseudotyped with truncated TPMV glycoproteins mediated efficient gene transfer, now the incorporation level of the gylcoproteins was investigated.

For this purpose, TPMVαCD20 vectors were generated as described before, concentrated and purified by low speed centrifugation over a 20 % sucrose cushion for 24 h at 4°C. The vectors were resuspended in PBS, inactivated for 5 minutes at 95°C and their protein content determined by Bradford assay. As a control, 293T cells were transfected with HΔ80αCD20- or FΔ32-plasmids. After 48 hours, the cells were lysed and the protein content determined. The purified particles and the cell lysates were applied to SDS-polyacrylamide-gelelectrophoresis and analyzed by Western Blot.

Both glycoproteins were detectable in the particles and migrated according to their molecular size albeit HΔ80αCD20 was barely detected (Fig. 31, lane 1). A stronger signal was observed for FΔ32 and the fragments F0 and F1 were detectable whereat F1a seemed not to be present. This observation was very different from the F protein fragments present in TPMV virions (section 6.2.4).

In particles containing only HΔ80αCD20 but no FΔ32, stronger signals for the H protein were observed (lane 2). The p24 protein, that is part of the HIV-1 capsid, could be detected in all four vector productions, demonstrating, that particles are also produced in the absence of any glycoproteins (lane 4). Analysis of p24 suggested an increased protein expression level compared to TPMV glycoproteins.
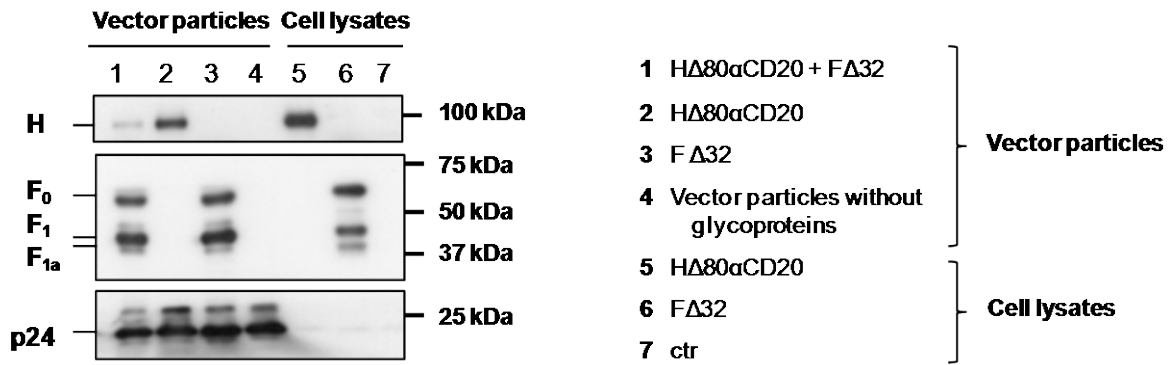
Figure 31: Biochemical confirmation of assembly of TPMV-pseudotyped lentiviral vectors
Concentrated vector particles were analyzed by Western Blot using αHecto, αFecto and αp24-antibody, respectively. As control, cell lysates of HΔ84αCD20, FΔ32 or untransfected (ut) cells were used. The positions of TPMV H, F0, F1, F1a and p24 are indicated on the left.

6.5.5. Targeting of different CD20-positive and negative cell lines

After characterization of the TPMV-pseudotyped vectors, their targeting potential was investigated. For this purpose, the cell lines 293T, HT1080, HT1080 that stable express CD20 (HT1080-CD20) and *Tupaia* baby fibroblasts (TBFs) were transduced immediately after production of the respective vector types with LV-TPMVαCD20, a TPMV-pseudotyped vector (TPMV HΔ80, FΔ32) that did not carry a scAb on the H protein (TPMV (w/o scAb)) and as controls, with LV-MVαCD20 and LV-VSV-G. After 72 hours, GFP expression was analyzed by fluorescence microscopy and representative pictures were taken.

Remarkably, LV-TPMVαCD20 showed no background transduction of the receptor negative cell lines 293T and HT1080 but as expected, TBF cells were transduced (Fig. 32). HT1080 cells that stable express CD20 (HT1080-CD20) were also transduced, demonstrating that cell entry occurs via the αCD20-scAb. The TPMV-pseudotyped vector without a scAb only transduced the TPMV-native cell line TBF, indicating the lack of a receptor for TPMV on human cells. GFP expression in TBF cells is lower compared to GFP expression of HT1080-CD20 cells transduced by LV-TPMVαCD20, for example.

Some background transduction could be observed for LV-MVαCD20, suggesting that mutation of contact residues was incomplete or that vectors entered cells via the recently identified new MV receptor nectin-4 (Noyce et al., 2011) for which the H protein is not blinded yet. Apart from that, this vector type also entered cells primary

via αCD20-scAb, demonstrated by transduction of HT1080-CD20 cells. As expected, VSV-G-pseudotyped vectors entered all cell lines tested with high transduction efficiencies.
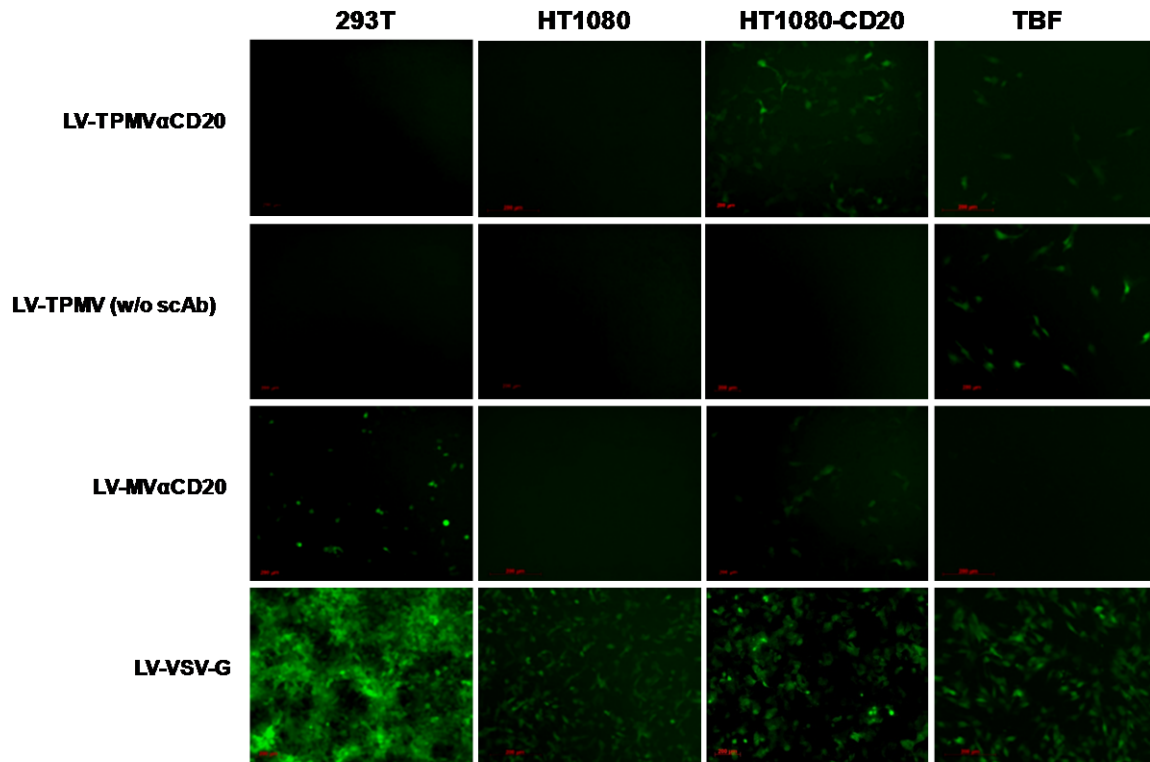
Figure 32: GFP expression of different cell lines transduced with the indicated vector types
293T cells, HT1080 cells, HT1080 cells stable expressing CD20 (HT1080-CD20) and *Tupaia* baby fibroblasts were transduced with LV-TPMVαCD20, LV-TPMV(w/o scAb) (TPMV FΔ32 and HΔ80 without scAb) and LV-VSV-G. After 72 hours, GFP expression was analyzed by fluorescence microscopy (100x magnification). Representative pictures are shown. Scale bar: 200 µm

6.6. Stability assays of TPMV-pseudotyped lentiviral vectors

Up to now, the TPMV-pseudotyped vectors were concentrated by low-speed centrifugation and used for experiments immediately after production. But for some experiments it might be necessary to produce high amounts of vectors and to store them until needed. To investigate the stability of the new vectors and how to maintain their functionality, different production and storage conditions were tested.

6.6.1. Concentration of vector particles

In a first step, different concentration methods of vector particles were investigated and the recovery calculated. The recovery indicates how many functional vector particles get lost during concentration. Since low speed centrifugation for 24 hours is very time-consuming, two more concentrating techniques were tested. The vectors were produced as described before and 15 ml cell supernatant, respectively, concentrated by low speed centrifugation (LS) for 24 h at 4000 g, ultra centrifugation (UC) for 3 hours at 28000 g over a 20 % sucrose cushion or by ultra filtration (UF) for 2 hours at 4000 g. Pellets containing viral vectors were resuspended in 120 µl RPMI for concentrating by LS and UC, by ultra filtration the volume achieved was 240 µl.

For LV-TPMVαCD20 and LV-MVαCD20, highest recovery was achieved by low speed centrifugation (48.5 % and 82.0 %, Table 4) although there was a loss of more than 50 % of infectious vector particles for TPMV-pseudotypes. Concentration by ultra centrifugation resulted in a recovery of only 6.6 % for LV-TPMVαCD20 and 39.2 % for LV-MVαCD20. Regarding the concentrating technique of ultra filtration, there was a very low recovery observed for LV-TPMVαCD20 (5.4 %) but for LV-MVαCD20, the recovery was 73.5 % which was almost as high as for low speed centrifugation, respectively.

Table 4: Titer and recovery of vectors after different concentration methods

LV-	Concentrated by	Titer Supernatant [t.u./ml][a]	Volume before concentration [ml]	Volume after concentration [ml]	Titer after concentration [t.u./ml][a]	Recovery [%]
TPMV-αCD20	Low speed centrifugation	6.9×10^3	15	0.12	4.2×10^5	**48.5**
	Ultra centrifugation	4.0×10^3	15	0.12	3.3×10^4	**6.6**
	Ultra filtration	1.1×10^4	15	0.24	3.7×10^4	**5.4**
MV-αCD20	Low speed centrifugation	4.2×10^3	15	0.12	4.3×10^5	**82.0**
	Ultra centrifugation	5.1×10^3	15	0.12	2.5×10^5	**39.2**
	Ultra filtration	2.4×10^4	15	0.24	1.1×10^6	**73.5**

[a] Titer in titration units/ml [t.u./ml]

Taken together, of the three tested concentration methods low speed centrifugation seemed to be best suited for the TPMV-pseudotyped vectors, although the recovery was only about 50 %. Ultra centrifugation resulted probably in damage of the

particles since there was only low transduction efficiency after titration of the concentrated vectors. Thus, concentration by ultra filtration was unsuitable for LV-TPMVαCD20. Since there was no transduction efficiency observed for the flow through (data not shown), the particles seemed to attach at the membrane of the filter or were damaged. For LV-MVαCD20, highest recovery was achieved by low speed centrifugation, too, but even for UC, there was a much higher recovery than for LV-TPMVαCD20. Concentrating by ultra filtration resulted in high transduction efficiencies for the MV-pseudotypes, indicating that the vectors are still functional and retained by the membrane filter. As described for LV-TPMVαCD20, there was also no transduction observed for MV-pseudotyped vectors by titration of the flow through (data not shown).

6.6.2. Storage of vector particles

As mentioned before, for some experiments it is necessary to produce high amounts of vector particles and to store them until needed. It is also important to know the titers of the used vector stocks for some transduction experiments. In general, it takes around 72 hours to observe gene expression and to determine titers after titration of concentrated vectors.

In this experiment it was investigated, if the particles could be stored in the fridge at 4°C for 72 hours until the titer of the appropriate vector stock is determined, so that they could be used at the same day for further experiments. Since there is the assumption that the functionality of the vectors decreases when they are frozen and thawed, storage at 4°C for 72 hours might be an alternative.

To investigate if there is a loss of function when vectors are frozen and thawed or stored at 4°C for 72 hours, TPMV- and MV-pseudotyped particles were produced as described before and concentrated by low speed centrifugation. Afterwards, the pellet was resuspended in 90 µl RPMI (+ Glutamine) of which 30 µl were used immediately for transduction, 30 µl were frozen at -80°C for at least two hours and 30 µl were stored in a fridge for 72 hours (4°C) and subsequent transduction.

Freezing and thawing of LV-TPMVαCD20 resulted in a decrease of titers of 50 % (Fig. 33). After storage of viral particles for 72 hours at 4°C, almost no transduction efficiency was observed (< 5 %), suggesting that the TPMV-pseudotypes lost their functionality. For LV-MVαCD20, there was a decrease of relative titer of only 2 % after freezing and thawing of the vector stock and after storage at 4°C for 72 hours,

still 25 % transduction efficiency could be achieved. Compared to TPMV-pseudotypes, LV-MVαCD20 also lost some functionality, but was not sensitive towards freezing and still retained functional after a three-day-storage at 4°C.
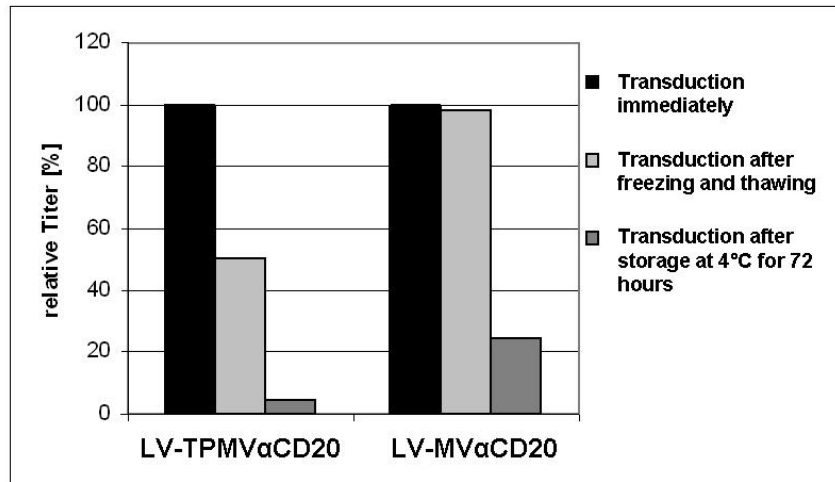
Figure 33: Loss of function after freezing and storage at 4°C of TPMV-pseudotyped particles
Concentrated vector particles were frozen at -80°C or stored at 4°C for 72 hours and afterwards used for transduction of Raji cells. Titers were normalized to that obtained after transduction immediately after concentration by low-speed cetrifugation (100%).

Up to now all concentrated vector types were resuspended in RPMI$^{+ \text{ Glutamine}}$. Now that a titer decrease was observed for LV-TPMVαCD20 after freezing and thawing and after storage at 4°C for 72 h, the cell culture medium OptiMEM was used as an alternative to RPMI$^{+ \text{ Glutamine}}$ to resuspend the concentrated vector particles. This medium is buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements and growth factors (Invitrogen, complete formulation is confidential). For this experiment the vectors were generated as described before, but after concentration by low speed centrifugation they were resuspended either in RPMI$^{+ \text{ Glutamine}}$, OptiMEM$^{\text{without additives}}$ or OptiMEM supplemented with 1 % bovine serum albumin (BSA), which is often used as a cryoprotective agent.

As already shown before, again there was a decrease of titers for LV-TPMVαCD20 (60 %) when the vectors were frozen and thawed and resuspended in RPMI after concentration (Fig. 34). Using OptiMEM without any additives resulted in a decrease of titers of 40 % for TPMV-pseudotypes, which was also observed for OptiMEM$^{+1\%BSA}$, suggesting that OptiMEM retained functionality of vector particles but BSA had no additional cryoprotective effect. For LV-MVαCD20, there was an

increase of titers after freezing and thawing of the vectors (18 - 35 %), independently, which medium was used to resuspend the vectors after concentration, assuming, that a freeze cycle has an enhancing effect of transduction.
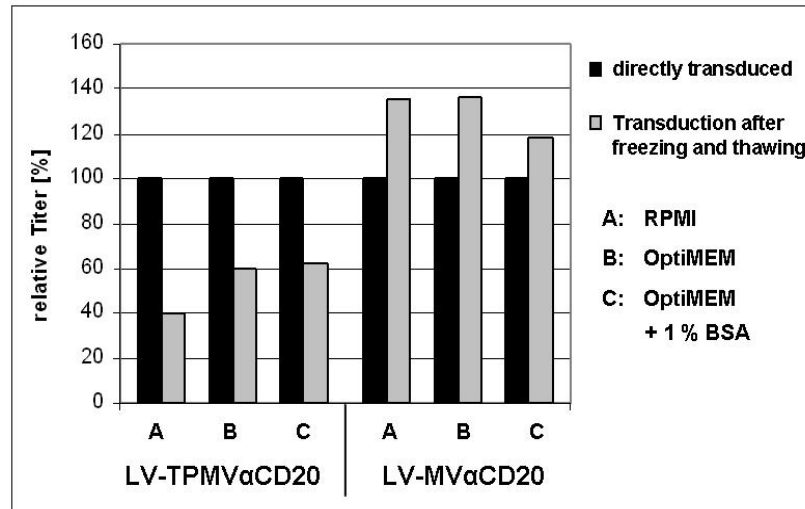
Figure 34: Loss of function of LV-TPMVαCD20 can partially be rescued after freezing in OptiMEM
Concentrated vector particles were resuspended either in RPMI, OptiMEM or OptiMEM supplemented with 1 % BSA (bovine serum albumin) and frozen for at least one hour at -80°C. Afterwards, vectors were thawed on ice and used for transduction of Raji cells. Titers were normalized to that obtained after transduction immediately after concentration by low-speed centrifugation (100%).

In conclusion, it was demonstrated that the highest recovery for TPMVαCD20- and MVαCD20-vector particles could be achieved by low speed centrifugation. However, the recovery was much less for LV-TPMVαCD20 compared to LV-MVαCD20. Freezing and thawing resulted in a third and storage at 4°C for three days in a nearly complete loss of functionality for TPMV-pseudotypes, which was not observed for LV-MVαCD20. Resuspending the vector pellet in OptiMEM after concentration had a cryoprotective effect, although there was still a loss of titers of 40 %. These data demonstrate that TPMV-pseudotypes are not as stable as MV-pseudotyped vectors based on these performed concentration techniques and storage conditions. For further experiments TPMV- and MV-pseudotyped vectors were concentrated by low speed centrifugation for 24 hours at 4°C and the vector pellet was resuspended in OptiMEM without additives.

6.7.   TPMV-pseudotyped vectors selectively transduce CD20-positive cells

Having shown that retargeted TPMV-pseudotyped lentiviral vectors could be generated to high titers and identified optimal production conditions, the next step was to test the targeting capability of the vectors. Main objective of gene therapy is the precise transgene expression in selected target cells, so that other cells or tissues remain unaffected. One of the aims of this thesis was to show selective transduction of CD20-positive cells.

6.7.1. Selective transduction of CD20-positive cells

For investigation of the selectivity of the retargeted TPMV-pseudotyped vectors, the CD20-positive cell line Raji was mixed in a ratio of 1:1 with CD20-negative myeloid cells (K-562) and incubated with different pseudotyped vectors at a multiplicity of infection (MOI) of 0.3. Vectors were generated as described above. Seventy-two hours post transduction, CD20 positive cells were stained with a PECy5-coupled antibody against CD20 and the amount of GFP-expressing cells in this population was analyzed by FACS.

At a MOI of 0.3, transduction of the mixed cell population with LV-TPMVαCD20 resulted in 15.7% GFP/CD20 double-positive cells, while CD20 negative cells remained almost unaffected (Fig. 35, left column, middle panel). There was virtually no transduction with untargeted TPMV vectors, confirming the assumption that there is no TPMV receptor on human cells (Fig. 35, right column, middle panel).

Mixed population of CD20+/CD20-
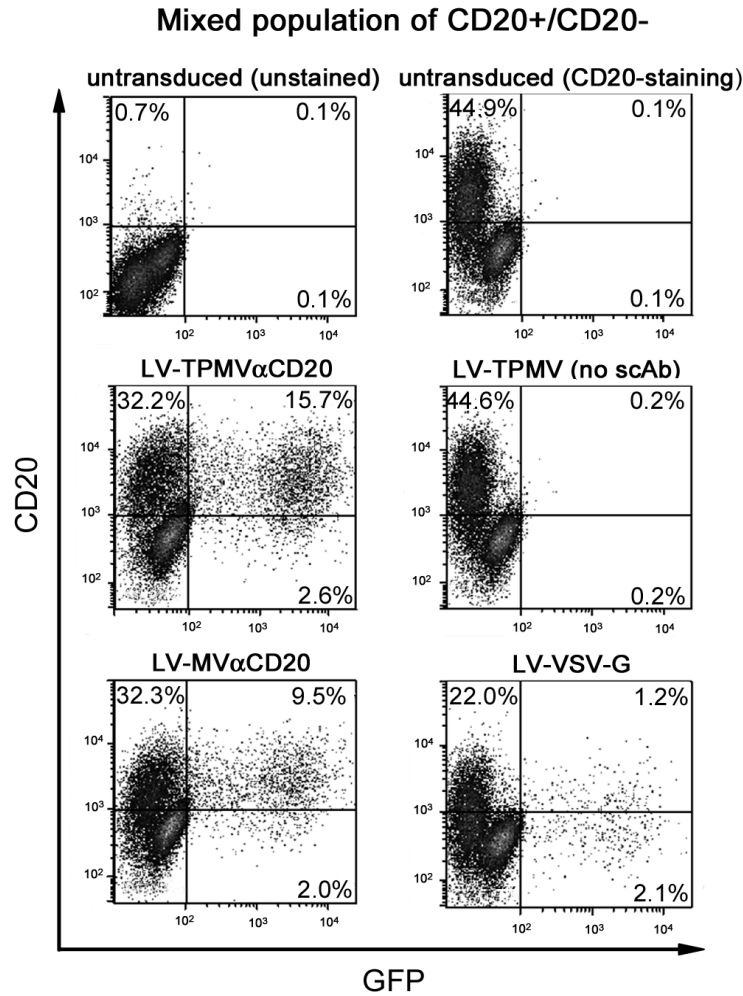
Figure 35: Selective transduction of CD20$^+$ cells in a mixed cell population
CD20-positive (Raji) and negative (K-562) cells were mixed (1:1) and transduced with different vector types (multiplicity of infection 0.3). 72 hours after transduction, the mixed population was stained against CD20 and GFP expression was analyzed by FACS. Untargeted TPMV-pseudotyped vectors were used as a negative control (LV-TPMV). GFP: green fluorescent protein; VSV-G: vesicular stomatitis virus G protein

Transduction efficiency of the LV-TPMVαCD20 was higher than that of MV-pseudotyped vectors that were used as a positive control (9.5% GFP/CD20 double-positive cells) (Fig. 35, left column, lowest panel). VSV-G-pseudotyped particles transduced both cell types, although with very low transduction efficiency (Fig. 35, right column, lowest panel), assuming a mistake in calculating the MOI.

6.8.   Transduction of primary human B cells

The previous experiments demonstrated that TPMVαCD20-pseudotyped vectors efficiently mediate gene transfer to different CD20-positive cells and transduce only target cells in a mixed cell population. The next step was to investigate whether the vectors are able to enter primary human B cells. These cells naturally express CD20, so the TPMVαCD20-pseudotyped vectors are a promising tool for gene therapy approaches for diseases related to this cell type, like inherited immunodeficiencies, cancer and autoimmune diseases (Frecha et al., 2010).

6.8.1. Transduction of activated primary human B cells

Up to date, gene transfer to primary human B cells by viral vectors was not very successful. Lentiviral vectors pseudotyped with the vesicular stomatitis virus G protein (VSV-G) do enter the cells but only with low efficiency (Serafini, Naldini, and Introna, 2004). Recently, it was shown that lentiviral vectors pseudotyped with the MV glycoproteins and targeted to CD20 are able to efficiently transduce primary B cells.

To test whether the novel TPMV vectors can also transduce this cell type, primary human B cells were isolated by negative depletion from peripheral blood mononuclear cells (PBMCs) and activated with a cytokine cocktail for 48 hours. The cocktail consisted of 50 ng/ml IL-2, 10 ng/ml IL-4, 10 ng/ml IL-10 and 300 ng/ml CD40-ligand. Incubation of primary B cells with these cytokines should result in expression of the activation marker CD69. To investigate whether CD69 could be detected on the cell surface, the B cells were stained with a fluorescence-coupled antibody and analyzed by FACS.

Stimulation of primary human B cells with the cytokine cocktail resulted in 58.3 % CD69-positive cells that is equivalent to 58.3 % activated cells (Fig. 36). This number is less than expected, but since there is a clear shift of the population compared to the isotype control it was assumed that almost all of the cells were activated and that the low percentage number is due to the high backdround of the isotype control. Control experiments with a different antibody showed an activation rate of about 90 % (data not shown).
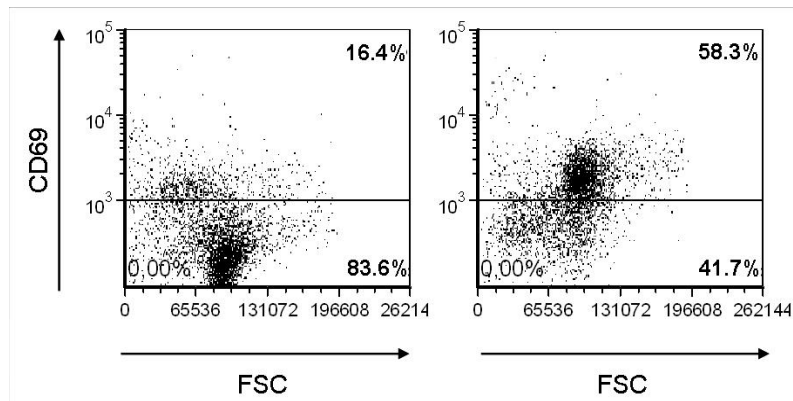
Figure 36: Activated primary human B cells express CD69
Primary human B cells were isolated and immediately activated for 48 hours with a cytokine cocktail containing 50 ng/ml IL-2, 10 ng/ml IL-4, 10 ng/ml IL-10 and 300 ng/ml CD40-ligand. Expression of the activation marker CD69 was then analyzed by FACS. left: B cells incubated with the FITC conjugated IgG$_{1,\kappa}$ isotype control; right: B cells incubated with FITC-conjugated αCD69-antibody

Next, the activated B cells were incubated at an MOI of 1 with concentrated LV-TPMVαCD20 and LV- MVαCD20. As mentioned above LV-VSV-G transduce primary human B cells only with low efficiency. In this experiment, concentrated LV-VSV-G was used for comparison at an MOI of 1 and 100. Vectors were produced as described before. After 72 hours, cells were stained for the B cell markers CD19 and CD20, and the percentage of GFP/CD19/CD20-triple positive cells was determined by FACS.

The analysis showed that most of the isolated and activated B cells were CD20/CD19-double positive (Fig. 37). Transduction with LV-TPMVαCD20 resulted in 65% GFP expression of CD19+/CD20+ cells (Fig. 37, A). With LV-MVαCD20, GFP expression was slightly lower (55.4%, Fig. 37 C). As expected, there was only low transduction efficiency with the VSV-G-pseudotyped vectors (10.0 %, Fig. 37, B) even with a MOI of 100 the transduction efficiency increased only up to 13.2 % (Fig. 37, D).

In conclusion, the results showed that it is possible to transduce activated primary human B cells with TPMV-pseudotyped vectors even with better efficiency than with MV-pseudotypes, compared to LV-VSV-G, where transduction efficiency is considerable lower. This result was confirmed with cells from a second donor (data not shown).
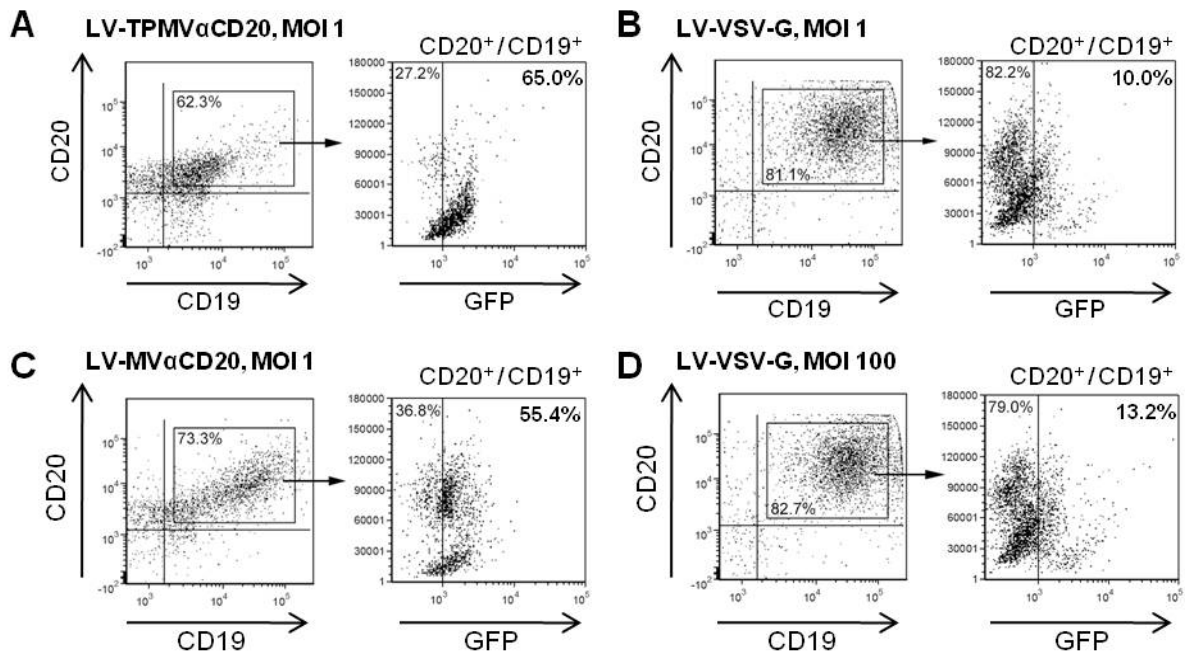
Figure 37: Transduction of activated primary human B cells
Primary human B cells (CD20^{+}/CD19^{+}) were isolated from human peripheral blood mononuclear cells and transduced after activation with a cytokine cocktail with LV-TPMVαCD20 (A), LV-VSV-G (B) or LV-MVαCD20 (C) a an MOI of 1. LV-VSV-G was used additionally with a MOI of 100 (D). 48 hours after transduction, cells were stained for the B cell markers CD20 and CD19 and GFP-expression of CD20/CD19 double-positive cells was analyzed by FACS. GFP: green fluorescent protein; VSV-G: vesicular stomatitis virus glycoprotein; MOI: multiplicity of infection

6.8.2. Transduction of quiescent primary human B cells

Surprisingly, it was demonstrated, that LV-MVαCD20 was able to efficiently transduce even quiescent primary human B cells (Funke et al., 2008a), a cell type, that was known to be resistant against transduction with lentiviral vectors (Serafini, Naldini, and Introna, 2004). As a next step, it was investigated if LV-TPMVαCD20 is also able to transduce quiescent primary human B cells or if this is due to the MV glycoproteins.

To answer this question, primary human B cells were isolated from human PBMCs by negative depletion and immediately transduced by LV-TPMVαCD20, LV-MVαCD20 (MOI 1) and LV-VSV-G (MOI 1 and MOI 100). Before transduction, the cells were stained against the activation marker CD69 to ensure that they were not activated. After 48 hours, the cells were incubated with antibodies against the B cell markers CD19 and CD20, following analysis of GFP expression in this population by FACS.

Antibody-staining of the isolated B cells shows no expression of CD69, confirming that they are not activated (Fig. 38).
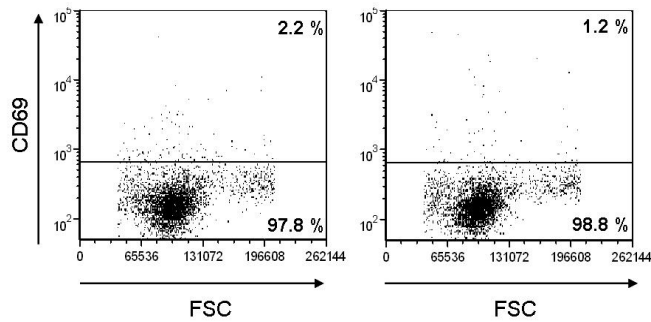
Figure 38: Quiescent primary human B cells do not express the activation marker CD69
Primary human B cells were isolated, immediately stained with an αCD69-antibody and analyzed by FACS. left: B cells incubated with the FITC conjugated IgG$_{1,κ}$ isotype control; right: B cells incubated with FITC-conjugated αCD69-antibody

Remarkably, 45.2 % of quiescent B cells were transduced by the TPMV-pseudotyped vector (Fig. 39, A) whereas transduction efficiency for LV-MVαCD20 was slightly less (Fig. 39, C, 35.5 %). This confirmed the observation already made for activated primary human B cells, namely that LV-TPMVαCD20 transduces this cell type more efficient than MV-pseudotyped vector. As expected, there was virtually no transduction with the VSV-vectors, even not with a MOI of 100 (< 1%, Fig. 39, B and D).
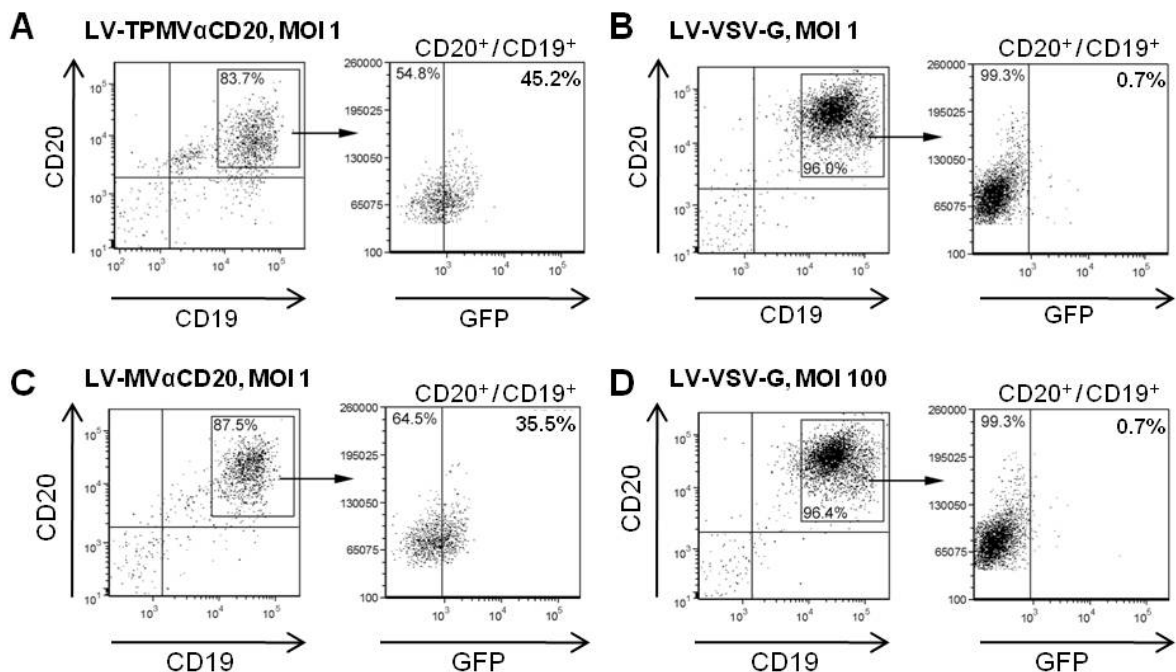
Figure 39: Transduction of quiescent primary human B cells
Primary human B cells (CD20$^+$/CD19$^+$) were isolated from human peripheral blood mononuclear cells and transduced immediately with LV-TPMVαCD20 (A), LV-VSV-G (B) or LV-MVαCD20 (C) a an MOI of 1. LV-VSV-G was used additionally with a MOI of 100 (D). 48 hours after transduction, cells were stained for the B cell markers CD20 and CD19 and GFP-expression of CD20/CD19 double-positive cells was analyzed by FACS. GFP: green fluorescent protein; VSV-G: vesicular stomatitis virus glycoprotein; MOI: multiplicity of infection

6.8.3. Neutralization assays with retargeted LV-pseudotypes

Viral vectors have to evade the immune system of the host to reach their target cells. This is problematic for MV-derived vectors for gene therapy because almost everybody in the general human population has *measles virus* antibodies, either due to MV vaccination or MV infection. At the beginning of this thesis it was hypothesized that TPMV-pseudotyped vectors would not be neutralized by human sera since no antibodies against TPMV are expected in the human population.

To confirm this hypothesis and to test whether TPMV is neutralized or whether there is any cross-reactivity with MV-antibodies, neutralization assays were performed with serum containing MV antibodies, either due to vaccination (serum 1, 2) or MV infection (serum 3). As control, MV antibody-negative serum was used (MV-negative serum). The sera were incubated for 30 minutes at 56°C to inactivate the complement system. LV-TPMVαCD20, LV-MVαCD20 or vectors pseudotyped with MV glycoproteins that do not contain a scAb (LV-MV(w/o scAb)) were pre-incubated with four different sera in several dilutions and added to CD20-positive Raji cells. After 72 hours, GFP expression of transduced cell was analyzed by FACS. The transduction efficiency of vectors that were incubated with medium only (RPMI) was set as 100 % and vectors incubated with different serum dilutions were normalized to this value.

Transduction efficiency of vectors pseudotyped with MV glycoproteins without scAb (LV-MV(w/o scAb)) dramatically decreased when vectors incubated with MV-antibody-containing sera (sera 1, 2, 3), demonstrating that this vector type was neutralized even with high serum dilutions (Fig. 40). Regarding LV-MVαCD20, this vector type was completely neutralized only by serum 3, although transduction efficiency also decreased by incubation with serum 1. Serum 2 had almost no neutralization effect since transduction efficiency was around 80 %, even at the lowest serum dilution. These data indicate that the αCD20-scAb had a shielding effect on neutralization of this vector and that higher MV-antibody titers were necessary for neutralization. Transduction efficiency of TPMV-pseudotyped vectors (LV-TPMVαCD20) was decreased after incubation with serum 1, 2 and 3 but did not drop under 35 %. No complete neutralization with any of the sera was observed. Remarkably, transduction efficiency of all three vector types (LV-TPMVαCD20, LV-MVαCD20, LV-MV(w/o scAb)) was decreased in the presence of serum that does not contain MV antibodies (MV-negative serum). For LV-TPMVαCD20, transduction

efficiency after incubation with MV-negative serum was similar to that obtained after incubation with serum 3. This observation might be due to residual activity of the complement system.

In summary, these data show that MV-pseudotypes without scAb were completely neutralized by serum containing MV antibodies. In contrast, neutralization of LV-MVαCD20 was less efficient, suggesting a shielding effect of the αCD20-single chain antibody. For this vector type, neutralization seemed to depend on MV-antibody titer of the serum since only with serum 3 a complete neutralization was observed. This serum had probably the highest MV-antibody titer because it was derived from a person that was infected with MV while serum 1 and 2 were from vaccinated individuals. For TPMV-pseudotypes, no complete neutralization was observed. Although the transduction efficiency decreased, it is not as significant as for the other vector types and since this effect was also observed for MV-negative serum, cross-reactivity with MV antibodies could be excluded.
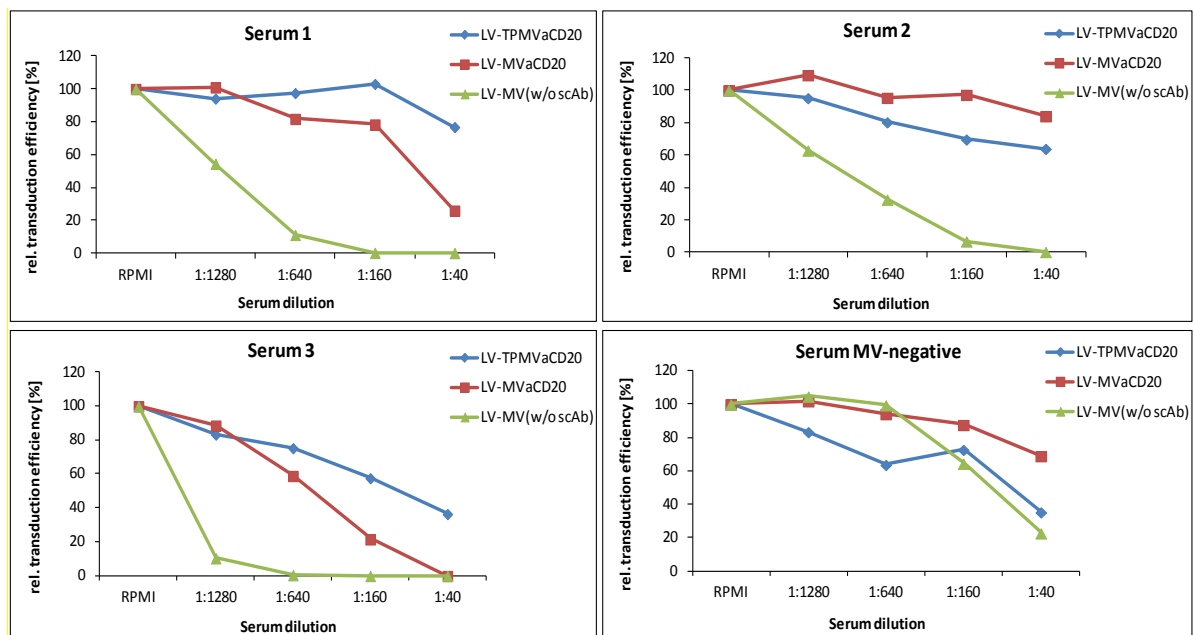
Figure 40: Neutralization assays of pseudotyped LVs with different sera containing MV antibodies
To analyze if MV-antibodies have neutralizing effect on TPMV or show any cross-reactivity, lentiviral vectors pseudotyped with TPMV glycoproteins were incubated with different sera in different dilutions and transduction efficiency was investigated. Vectors pseudotyped with retargeted MV glycoproteins (LV-MVαCD20) or MV glycoproteins without a scAb (LV-MV(w/o scAb)) were used as controls. Serum 1 and 2 were derived from a MV-vaccinated person, serum 3 from a person with MV infection. Serum MV-negative does not contain any MV antibodies. The transduction efficiency of vectors that were incubated with medium only (RPMI) was set as 100 % and vectors incubated with different serum dilutions were normalized to this value. The relative transduction efficiency shown here in percent [%] is the average of two independent experiments. LV: lentiviral vector; MV: *measles virus*; TPMV: *Tupaia paramyxovirus*; scAb: single chain antibody

7. DISCUSSION

Gene therapy is defined as the treatment of a disease or correction of a genetic disorder by introducing therapeutic genes into target cells, respectively (Escors and Breckpot, 2010). To treat a disease or correct a genetic dysfunction, the delivery of therapeutic genes has to be specific to a certain cell type and gene expression has to be efficient. Lentiviral vectors provide a promising tool for gene therapy, since they allow transduction of dividing and non-dividing cells and mediate long-term gene expression by integration of the transgene into the target cell's genome (Matrai, Chuah, and VandenDriessche, 2010). To increase specificity of gene delivery, different approaches have been developed, for example the use of tissue specific promoters so that the gene delivered by the vector is only expressed in target cells (transcriptional targeting) (Waehler, Russell, and Curiel, 2007). However, this method also affects non-target tissues since vectors can enter cells in a non-specific way. Therefore, an important point is to restrict cell entry of vectors to the desired cell type, a process called transductional targeting. This is the most desirable approach since it leaves other tissues or cells completely unaffected (Waehler, Russell, and Curiel, 2007). Although there has been great success in the development of lentiviral vector technology, safety and efficiency of gene transfer have to be further improved (Bouard, Alazard-Dany, and Cosset, 2009; Waehler, Russell, and Curiel, 2007).

Significant progress has been made in the development of MV-based vectors, but they are obviously unsuitable for long-term replacement gene therapy since infected cells are killed and there is no integration of the transgene into the genomes of the infected cells. MV can be targeted to many different cell types by displaying a variety of specificity domains on the H protein (Cattaneo, 2010; Schneider et al., 2000). Recently, it has been shown that the MV glyoproteins can be used to pseudotype lentiviral vectors (Anliker et al., 2010; Frecha et al., 2009; Frecha et al., 2008; Funke et al., 2008a; Funke et al., 2009). These vectors combine the versatile MV targeting system with the advantages of lentiviral vectors (reviewed in (Buchholz, Mühlebach, and Cichutek, 2009)). But since MV antibodies are widespread in the general population, either due to vaccination or infection with MV, neutralization of these vectors might be an important problem to overcome.

Therefore, in this thesis vectors pseudotyped with glycoproteins of an alternative paramyxovirus were developed, namely the glycoproteins of the *Tupaia*

paramyxovirus (TPMV). They can be retargeted in a similar way to MV glycoproteins and since TPMV is an animal paramyxovirus that does not infect humans, no pre-existing neutralizing antibodies are expected (Springfeld et al., 2005; Tidona et al., 1999). Furthermore, while the MV glycoproteins have to be modified to ablate receptor binding of the H protein to natural MV receptors (Nakamura et al., 2005; Vongpunsawad et al., 2004), this is not necessary for the TPMV H protein, resulting in increased safety of these vectors. Hence, in this study, retargeted lentiviral vectors were generated that combine the advantages of MV-pseudotyped vectors with the advantages of TPMV glycoproteins.

7.1. Characterization of TPMV glycoproteins

The first step in the development of TPMV-pseudotyped lentiviral vectors was the generation and characterization of modified TPMV F and H glycoproteins. Following the observation that unmodified MV glycoproteins are not incorporated into LVs, eight TPMV H variants and seven TPMV F variants with different truncations of their cytoplasmic tails were generated. Additionally, a single chain antibody (scAb) against CD20 was displayed on all truncated H variants. Western Blot analysis showed that all modified proteins are expressed at levels similar to wild type.

The TPMV glycoproteins are related to the MV glycoproteins but have some uncommon properties. The cytoplasmic tail of the H protein is considerably longer than those of all other paramyxoviruses. Previous results showed that targeting of the TPMV glycoproteins with a CEA scAb in analogy to MV is possible (Springfeld et al., 2005). In this thesis, a scAb against CD20 was displayed at the ectodomain of TPMV H. Analysis of surface expression of the H protein variants revealed that the scAb does not inhibit protein processing within the cell. To test the proteins in regard to their function, fusion assays were performed. All truncated TPMV HαCD20 variants were functional. Remarkably, a truncation of 80 or 84 amino acids (aa), leaving only 14 or 10 aa, respectively, is well tolerated in terms of function. For truncated MV H protein, at least 14 aa are necessary to mediate fusion. Variants with shorter CTs lost their functionality (Moll, Klenk, and Maisner, 2002). Demonstrating that the αCD20-scAb on TPMV H can also be used for targeting, strengthens the evidence that the TPMV targeting system is as versatile as the MV system.

In contrast to TPMV H, truncation of the CT of the F protein by 34 or more amino acids is not tolerated. As demonstrated in fusion assays, F variants with a cytoplasmic tail of 3 or 4 amino acids lost their function since they are not able to mediate the formation of syncytia. Surprisingly, truncation of 33 amino acids did not affect syncytia formation at all, so a difference of only one amino acid determined functionality. This is in contrast to the truncated MV F protein where only three amino acids of the CT are sufficient to mediate fusion (Moll, Klenk, and Maisner, 2002).

Regarding the details of the TPMV F protein, the F1/F2 cleavage site is different compared to other paramyxovirus fusion proteins and activated by a yet unidentified protease (Springfeld et al., 2005). In general, the F1/F2 cleavage site of paramyxoviruses is preceded either by one or several arginines and lysines, respectively, it is mono- or oligobasic (Klenk and Garten, 1994). Oligobasic cleavage sites are recognized by furin-like proteases in the *medial-* and *trans*-Golgi, monobasic cleavage sites by trypsin-like proteases on the cell surface. Exceptions are *Nipah* (NiV) and *Hendra* (HeV) viruses, which have amonobasic cleavage site but are neither cleaved by trypsin nor furin. The F0 protein of NiV and HeV is transported to the cell surface and subsequently endocytosed. Afterwards, it is cleaved in the endosome by cathepsin L into activated F1 and F2 and transported back to the cell surface (Lee and Ataman, 2011). TPMV has neither a mono- nor an oligobasic cleavage site.

For characterization of the TPMV F protein, a new antibody (αF_{ecto}) was generated against a peptide located in the proteins ectodomain. Since the antibody that was previously used to characterize TPMV F was directed against the cytoplasmic tail (αF_{cyt}), this antibody could not be used to analyze the cytoplasmic tail-deleted proteins. With the new antibody αF_{ecto}, a novel fragment was detected, suggesting that a significant fraction of the F1 protein is further cleaved into an F1a and F1b fragment, similar to the MV and CDV F protein (von Messling et al., 2004). F proteins of *Morbiliviruses* are contranslationally inserted into the membrane of the endoplasmatic reticulum (ER), glycosylated and folded with subsequent trimer formation (Hernandez et al., 1996). These F protein trimers are arranged into a ring-like structure to form the fusion pore. For MV and CDV F proteins it was hypothesized that partial F1a/F1b cleavage induces a conformational change of the trimers that leads to fusion pore formation by breaking symmetry.

To further characterize the newly observed TPMV F1a/F1b cleavage a number of experiments were performed. The data revealed that the novel F1a fragment is transported along the secretory pathway and secreted into the supernatant of transfected cells. Furthermore, significant amounts were also detected in purified TPMV virions. The cleavage site was mapped and surprisingly, it is located within the outer half of the transmembrane domain of TPMV F. These results indicate that F1a is no longer anchored in the cell membrane and can therefore be released into the supernatant. The presence of F1a in virions can probably be explained by the formation of mixed trimer complexes that contain membrane-anchored F1 as well as F1a.

The cleavage site was mapped by insertion of a Flag-tag at different amino acid positions in the ectodomain expecting that F1a/F1b cleavage results in loss of protein detection (section 6.3.1). With an αFlag-antibody, the F0 fragment of F_{499} could be detected but not of F_{500}, indicating that the cleavage site is located between amino acid position 499 and 500. Surprisingly, no F1 fragment was found for F_{483}-F_{499} using the αFlag-antibody, but when the Flag-tag was added after aa position 533 or at the C-terminal end of the F protein (F_{553}), the F1 fragment was visible.

In the case of the MV and CDV F protein, it was not possible to completely ablate F protein function by mutating the F1a/F1b cleavage site. Mutation or substitution of the respective amino acid sequence only reduced cleavage efficiency. These data demonstrate that factors other than the primary amino acid sequence, for example an exposed structure, influence protease cleavage (von Messling et al., 2004). Similar results were obtained by mutating the F1a/F1b cleavage site of TPMV F (section 6.3.2). The respective amino acids were replaced by alanine residues. Replacement of the amino acids of the cleavage site by alanins did not result in cleavage inhibition.

Characterization of the cleavage protease using the inhibitors Antipain, E-64 and Leupeptin (section 6.4.1) revealed that both F protein cleavages (F1/F2 and F1a/F1b) did not occur, resulting in an inactive F protein as demonstrated in fusion assays. These three chemicals have in common that they are cysteine protease inhibitors, suggesting that a cysteine protease is involved in cleavage and subsequent activation of TPMV F. Antipain and leupeptin are peptidyl aldehydes and their inhibition is reversible. E-64 is an irreversible inhibitor and belongs to the group of epoxysuccinyl-based inhibitors which only inhibit cysteine proteases. All three substances contain a peptide segment for recognition by the enzyme which is

coupled to a nucleophilic group. This group can attack or substitute the cysteine residue of the active site of the enzyme (Otto and Schirmeister, 1997).

Interestingly, for NiV and HeV, the endosomal/lysosomal cysteine protease cathepsin L has been identified as an activating protease (Craft and Dutch, 2005; Meulendyke et al., 2005; Pager et al., 2006; Pager and Dutch, 2005). As mentioned before, NiV F0 is cleaved in the endosome by L cathepsin forming F1 and F2 after clathrin-mediated endocytosis. This is due to a tyrosine-based internalization signal in its cytoplasmic tail, the YSRL sequence (aa 525-528) (Diederich et al., 2005). Similar results were obtained for HeV (Pager et al., 2006; Pager and Dutch, 2005). The YSRL sequence, which is located in the CT of NiV and HeV F protein, fits in the YXXΦ-type endocytosis signal consensus motif, whereat Y is a tyrosine, X can be any amino acid (aa) and Φ is a large hydrophobic aa. Tyrosine-dependent motifs are often involved in protein sorting and targeting signals of transmembrane proteins (Bonifacino and Traub, 2003). Regarding the CT of TPMV F, two tyrosine residues can be found at aa positions 527 and 529. At aa position 529-532 of TPMV F the sequence YSLR is present which is similar to the endocytosis signal of NiV and HeV. But since it contains an arginine instead of a hydrophobic aa at position four, it does not match the YXXΦ-type endocytosis signal. Another tyr-containing motif can be found at aa position 527-530 (YVYS), but this also lacks the hydrophobic aa at position four of the YXXΦ- motif. However, there are some exceptions. The Influenza HA protein is known to not be endocytosed, but a cytoplasmic tail mutant carrying a tyrosine at aa position 453 and a serine at aa position 546 (HA-Y543/S546), resulting in the motif YXXS, was endocytosed efficiently (Naim and Roth, 1994). Regarding the CT of TPMV F, the sequence YVYS (aa 527-530) is equivalent to the YXXS motif of the Influenza HA-mutant. Consequentially, it might act as endocytosis signal for TPMV F. Furthermore, it indicates that the consensus motif is possibly variable.

Supporting this hypothesis is the detection of high amounts of F0 fragment in addition to F1 in Western Blot analysis of TPMV-pseudotyped vectors (section 6.5.4). This indicates that only a fraction of F protein was cleaved and activated on the cell surface which might have consequences for the generation of TPMV-pseudotyped LVs. Since glycoprotein expression on the cell surface is a critical step in vector formation, titers might be reduced due to the high amount of uncleaved F protein. In contrast to this observation, there was no F0 detected in TPMV virions (section 6.2.4). Possibly, the virus has a different mechanism of F protein processing that only

allows incorporation of cleaved protein, for example, assembly in specialized domains of the cell membrane. An alternative explanation is that the truncated variant FΔ32 is incorporated in LVs in contrast to the full-length F protein in virions assuming that an endocytosis signal sequence in the CT was removed by truncation. Thus, endocytosis and subsequent cleavage and activation of FΔ32 might not have been complete.

The experiments performed in this section revealed only preliminary results, for a better understanding of the mechanism behind TPMV F cleavage and activation and to verify the above described hypothesis, further investigation is necessary. To find out whether F cleavage occurs during transport along the secretory pathway or at the plasma membrane, protein expression of cells treated with inhibitors of the secretory pathway has to be analyzed. For analysis of the endocytosis hypothesis, the respective tyrosine residues of the TPMV F cytoplasmic tail could be mutated and endocytosis of the native and mutant protein could be investigated in presence or absence of endocytosis inhibitors. Characterization of the protease revealed that a cysteine protease is likely to be involved in cleavage. The most important cysteine proteases in mammals are calpains which can be found in the cytoplasm and cathepsins that are mainly located in the lysosomes (Otto and Schirmeister, 1997), but it has been shown that cathepsins can also be secreted when cells are stimulated with proinflammatory cytokines (Reddy, Zhang, and Weiss, 1995; Sukhova et al., 1998; Turk, Turk, and Turk, 2000). Other important members of the cysteine protease family are caspases which are involved in apoptosis (Amarante-Mendes and Green, 1999). Since it was shown that the F1a/1b cleavage site is located in the transmembrane domain, the responsible protease could also be an extracellular protease. To further restrict the cysteine protease that is responsible for TPMV F cleavage and activation, investigation of protein expression in the presence of calpain inhibitors (calpeptin, PD150606), caspase inhibitors (caspase inhibitor III) or cathepsin inhibitors (Cath I, Cath LIII) has to be examined.

Identification of the cleavage protease might be important since it has been reported that it can act as target for viral therapy. For NiV and HeV it has been shown that treatment of virus-infected cells with chloroquine prevents virus spread *in vitro* (Freiberg et al., 2010). Unfortunately, this effect could not be observed in a golden hamster model *in vivo*. It has been proposed that chloroquine prevents the proteolytic cleavage of Henipavirus F protein by cathepsin L (Porotto et al., 2009). Although

TPMV does not infect humans and therefore no treatment is necessary, the virus might be used as a model to further investigate the characteristics of the emerging and highly pathogenic *Henipaviruses*.

The data raise the question of the biological function of the partial F1a/F1b cleavage. As mentioned before, one hypothesis is that proteolytic processing enhances fusion pore formation (von Messling et al., 2004). Another function of this cleavage could be that a fragment of TPMV F acts as modulator of the host's antiviral immune response. Importantly, there is an example from a paramyxovirus F protein, the *bovine respiratory syncytial virus* (BRSV). Cleavage of the fusion protein results in a small fragment that is converted into a biologically active molecule by additional posttranslational modifications (Zimmer, Rohn et al. 2003). This molecule is called virokinin and can interact with tachikinin receptors of the host that are involved in local inflammatory and immune processes.

Although there are still open questions about the mechanism of F protein activation, the TPMV glycoproteins share many features with MV glycoproteins that make them so suitable for pseudotyping of lentiviral vectors.

7.2. Pseudotyping of lentiviral vectors with TPMV glycoproteins

After characterization of TPMV glycoprotein variants they were screened in all combinations for their ability to pseudotype lentiviral vectors. Screening revealed that the F protein variant with a 6-amino acid tail (FΔ32) together with the H variant with a tail length of 14 aa (HΔ80αCD20) resulted in the highest vector titers (section 6.5.2). As already mentioned for the fusion assays, truncations of the long cytoplasmic tail of TPMV H were surprisingly well tolerated. Although the fusogenicity of the protein was reduced when the cytoplasmic tail was truncated, there was no complete loss of function when a certain tail length was reached. In contrast to TPMV H, no functional vectors could be generated with TPMV F proteins with truncations of more than 33 amino acids. Interestingly, transduction was achieved with FΔ33 but not with FΔ34 or FΔ35. Apparently, a difference of only one amino acid prevents functional LV-pseudotyping. This could be due to a loss of function of these proteins or to low or even no incorporation levels into LVs. But as fusion assays have demonstrated that F proteins with short CT do not form syncytia, there is probably a loss of function of these F variants. Replacing the CT of TPMV F with that of truncated MV F protein

also did not lead to a functional protein,. An interesting observation was that even TPMV glycoproteins without any CT truncations were able to pseudotype LVs, although titers were low. This is in contrast to MV-pseudotypes. No vectors could be generated with full-length glycoproteins. Different results were also obtained regarding the length of the CT. For the MV F protein, a truncation of the cytoplasmic tail to three amino acids was found to result in the highest vector titers (Frecha et al., 2008; Funke et al., 2008b). Similar results had been obtained previously with the F protein of Sendai virus that had a cytoplasmic tail truncation to four amino acids (Kobayashi et al., 2003). For the MV H protein, conflicting results have been published: while one group found a truncation to 16 amino acids most effective (Funke et al., 2008b), another group reported a truncation to 10 amino acids to be optimal (Frecha et al., 2008). An explanation for the differences between MV and TPMV could be that the cytoplasmic tails have no sequence similarities. In conclusion, it was not possible to directly transfer the results obtained for MV to TPMV but CT optimal truncations of both glycoproteins are approximately in the same range. For the F proteins, keeping the CT as short as possible was most effective to maintain function. Another difference to the MV system is that the attempts to increase vector titers by using different ratios of the TPMV F and H plasmids during vector production were not successful. For MV it was reported that a H:F plasmid ratio of 1:7 increased titers by up to ten-fold compared to the same amounts of H and F plasmids. In cells infected naturally with MV, there is a gradient of mRNA due to the MV gene order. Accordingly, more F than H mRNA is produced in MV infected cells (Cattaneo et al., 1987b; Plumet, Duprex, and Gerlier, 2005). Thus, the situation in natural MV infected cells is simulated by the use of higher amounts of F plasmid. However, an increase of the amount of TPMV F plasmid had no effect on vector titer although gene order in the TPMV genome is the same as for MV.

Regarding stability assays performed in this thesis it is obvious that the TPMV-pseudotypes are not as stable as MV-pseudotypes. But finally, vector titers similar to those in the MV system were achieved. Compared to lentiviral vectors pseudotyped with VSV, these titers are still low and for using them in gene therapy trials, vector production has to be further improved. To further increase vector titers, a more detailed understanding of the requirements for glycoprotein incorporation into lentiviral particles and of the molecular biology of TPMV will probably be necessary.

To investigate the specificity of the TPMV-pseudotyped vectors, different cell lines were transduced with different vector types (section 6.5.5). There was virtually no background transduction with TPMV-pseudotypes, demonstrating the absence of a receptor for TPMV and the CD20-mediated specific entry of the vectors. This is in contrast to MV-pseudotypes, where some background transduction could be observed. This might be due to incomplete mutation of the CD46/SLAM contact residues or other receptors, e.g. nectin-4 (Noyce et al., 2011). The MV glycoproteins used as controls in this thesis are so far blinded against this receptor. Specificity of TPMVαCD20-LVs was furthermore demonstrated by transduction of a mixed cell population of CD20-positive and –negative cells (section 6.7). TPMV-pseudotyped vectors selectively entered CD20-positive cells while VSV-G pseudotyped vectors do not discriminate between the two cell lines.

An important point of this thesis was the transduction of primary human B lymphocytes. Previous reports described that lymphocytes require stimulation with cytokines or other factors for transduction with lentiviral vectors (Serafini, Naldini, and Introna, 2004; Unutmaz et al., 1999). Remarkably, transduction of stimulated human B cells with LV-TPMVαCD20 resulted in 65.0 % GFP-positive cells (section 6.8.1). Regarding LVs pseudotyped with VSV-G, they are able to transduce most target cells, but transduction levels of human B cells even under optimized conditions are low (Bovia et al., 2003; Janssens et al., 2003). In this study when using an MOI of 100 only 13.2 % of the stimulated B cells were GFP-positive after transduction. Hence, the retargeted TPMVαCD20 glycoproteins are significantly more efficient in mediating gene transfer to primary human B cells than the VSV glycoprotein.

Gene transfer into quiescent lymphocytes might be of even more importance for gene therapy approaches than in activated lymphocytes, since they are involved in many diseases, like inherited immunodeficiencies, cancer and autoimmune diseases (Frecha et al., 2010). Quiescent lymphocytes remain in the G0 phase of the cell cycle until they are stimulated. During this time, the cell size is reduced and transcription and translation rates as well as metabolism are down regulated (Buchholz, Mühlebach, and Cichutek, 2009). Viral infection of cells in the G0 phase does not lead to the respective post-entry steps like reverse transcription of the virus genome and transport to the nucleus. Consequently, the transgene is not integrated in the host genome (Korin and Zack, 1998; Yoder et al., 2008). After stimulation, for

example by cytokines or receptor binding, the cell enters the G1 phase and down regulation is reversed.

Recently, efficient transduction of quiescent primary human lymphocytes with measles virus pseudotyped LVs was reported independently by two different groups (Frecha et al., 2011; Zhou et al., 2011). Unexpectedly, binding to both natural receptors (CD46 and SLAM) of the MV vaccine strain seems to be necessary. However, lentiviruses pseudotyped with a mutated MV H protein were unable to mediate entry via CD46 or SLAM, but when targeted with a αCD20-scAb could also be used to transduce quiescent B cells (Funke et al., 2008b). In this thesis it was shown that LVs pseudotyped with TPMV glycoproteins carrying the same αCD20-scAb also mediate efficient gene transfer into quiescent primary human B cells (45.2 % GFP-positive cells) Efficiency was even higher than MVαCD20-LVs that were used with the same MOI as control (35.5 % GFP-positive cells) (section 6.8.2). The quiescent lymphocytes were not transduced by VSV-G-pseudotyped LVs, even when a high multiplicity of infection (MOI 100) was used.

For the targeted MV-pseudotyped vectors two mechanisms were proposed (Buchholz, Mühlebach, and Cichutek, 2009) that mediate transduction of quiescent lymphocytes. Regarding the first mechanism it has been speculated that residual binding of the H protein to CD46 and SLAM might induce changes in the actin rearrangement and microtubule formation of the target cells that make them susceptible for lentiviral transduction (Buchholz, Mühlebach, and Cichutek, 2009). Another hypothesis is that binding of the retargeted MV H protein to CD20 acts as a proliferative stimulus and as a consequence, resting B cells are activated and become susceptible for transduction. The results obtained in this thesis using retargeted TPMV-pseudotyped vectors exclude the first hypothesis as TPMV does not enter cells via the receptors CD46 and SLAM. But the retargeted TPMV H protein carries the same αCD20-scAb as the retargeted MV H protein, confirming that binding of the scAb to CD20 is sufficient to mediate transduction of quiescent primary human B cells. This observation supports the hypothesis that binding to CD20 induces an activation signal, leading to susceptibility towards vector entry.

The natural role of the CD20 molecule is still not fully understood. Although an anti-CD20, B-cell-specific monoclonal antibody (Rituximab) has been approved for several years for treatment of immune diseases like Non-Hodgkin's lymphoma and rheumatoid arthritis, the underlying molecular mechanism has not been completely

resolved. However, further investigation of the CD20 molecule and post-entry steps of pseudotyped lentiviral vectors are necessary to reveal the molecular mechanism of the CD20 molecule.

At the beginning of this thesis it was postulated that the TPMV glycoproteins are similar enough to MV glycoproteins to provide all properties for efficient pseudotyping but different enough to escape neutralization by MV antibodies. To verify this hypothesis neutralization assays with TPMV-pseudotyped vectors were performed (section 6.8.3). Serum containing MV antibodies, either due to vaccination or infection, was used in these experiments. Since the TPMV glycoproteins only have low similarity to MV glycoproteins in their amino acid sequences (TPMV F: 33.4 %; TPMV H: < 20 %), no cross-reactivity was expected. Indeed, compared to the control vector pseudotyped with non-targeted MV glycoproteins, the TPMV-pseudotyped vectors escape neutralization. There is a slight decrease of the relative transduction efficiency for LV-TPMVαCD20 but since this effect was also observed for MV-negative serum with increasing serum concentrations, this might be due to other factors than cross-reactive antibodies, for example the complement system. Interestingly, the retargeted MVαCD20 vectors were not neutralized as much as the non-targeted vectors, suggesting that the scAb displayed on the MV H protein provides a shielding effect. But with higher antibody concentrations the shielding effect is ablated. This experiment demonstrates that glycoprotein exchange of pseudotyped vectors with glycoproteins of an animal virus provides an escape option from neutralizing antibodies. A similar effect was recently described for a mutant MV where the MV glycoproteins were replaced by the glycoproteins of the animal paramyxovirus *Canine distemper virus* (CDV). In an animal model it was shown that the therapeutic effect of the envelope-exchanged virus was not affected by MV antibodies (Miest et al., 2011). Now it has to be investigated if TPMV-pseudotyped LVs are also able to escape MV neutralizing antibodies *in vivo*.

7.3. Outlook

The results obtained in this thesis demonstrate that it is possible to generate lentiviral vectors with paramyxovirus envelope proteins of a non-human virus that will not be neutralized by preexisting antibodies in patients. This is an important advantage towards MV glycoproteins used for pseudotyping. The TPMV glycoproteins possess all properties of MV glycoproteins making them interesting for pseudotyped LVs for gene therapy. They can be retargeted, they are incorporated into LVs when their CTs are truncated and the titers obtained are similar to those obtained with MV glycoproteins. Additionally, TPMV proteins escape neutralization by MV antibodies. This is also the first time, that a LV pseudotyped with other proteins than MV proteins could achieve transduction of quiescent primary human B cells.

One potential disadvantage of these newly developed vectors is their lower stability compared to MV-based LVs. This might be critical when it is necessary for gene therapy to produce large amounts of vectors that can be stored for a longer period. To overcome this problem, possibly further protein modifications are necessary to increase titers and stability. Since TPMV is an animal paramyxovirus, the TPMV glycoproteins probably have to be adapted to the human transcription and translation machinery. This can be done for example by modification of the codon usage. For the generation of Human Papilloma virus (HPV) virus-like particles it was shown that codon optimization increased protein-levels by about 100-fold (Mossadegh et al., 2004). Converting the codons of TPMV glycoproteins to those more common in human genes might increase protein expression and thereby incorporation levels into the viral envelope. Another attempt to increase stability and titers is to exchange the cytoplasmic tails of the TPMV glycoproteins. It has recently been reported that LVs pseudotyped with rabies virus glycoproteins show increased incorporation levels when the rabies CT is replaced by the CT of VSV (Carpentier et al., 2011). Since VSV-G-pseuodtyped LVs are very stable, it might be possible to increase stability of TPMV-pseudotypes by exchanging the cytoplasmic tail of the F protein as it was done with chimeric rabies/VSV-G-pseudotyped vectors.

For the TPMV H protein it was shown that retargeting with αCEAscAb and αCD20scAb is possible, suggesting that the TPMV system is as versatile as the MV system. Since most cell types targeted for gene therapy express unique surface antigens against which scAbs can be generated, the results obtained in this thesis

open the exciting perspective of targeted lentiviral transduction of any target tissue after systemic administration of gene therapy vectors.

TPMV vectors will certainly induce neutralizing antibodies when administered systemically to patients. Since in many cases repeated administration of gene therapy vectors will probably be necessary for efficient transduction of the target tissue, it might be reasonable to further pursue the identification of other animal paramyxovirus envelope proteins that, like TPMV, can be targeted and used to pseudotype lentiviral vectors.

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