Daniel Alexandre Fernandes Mestre

Degree in Cell and Molecular Biology

A Novel Generation of Lentiviral Vectors for Gene Therapy

Dissertation to obtain Master Degree in Biotechnology

Supervisor: Ana Sofia Coroadinha, Assistant Researcher, ITQB NOVA/IBET

Juri:

President: Prof. Doctor Carlos Alberto Gomes Salgueiro
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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*"**The only place success comes before work is in the dictionary"** Vince Lombardi*

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Preface

This master thesis is within the scope of the project PTDC/EBB-EBI/118621/2010; entitled “A novel generation of packaging cell lines for the manufacture of lentivirus based biopharmaceuticals” funded by the Portuguese Fundação para a Ciência e a Tecnologia (FCT).

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Abstract

The use of lentiviral vectors based on the *Human immunodeficiency virus 1* in gene therapy clinical trials has been growing due to their ability to transduce and permanently modify both dividing and non-dividing cells and their integration pattern considered to be safer relatively to that of gammaretroviral vectors. To take advantage of the full potential of lentiviral vectors for gene therapy applications, stable producer cell lines are desirable. However, its development has been hampered by the cytotoxicity of some viral components, namely the viral protease.

In this work, a genetically modified and less toxic protease was evaluated for lentiviral vector production. Although less cytotoxic, this modified protease exhibits reduced proteolytic activity. While for envelope glycoproteins which do not require proteolytic processing – like VSV-G – reduced protease activity did not impact vector titers, the conjugation of this protease with envelope glycoproteins typically used in stable cell line development – 4070A, GaLV10A1 and RD114A – resulted in reduced titers. Therefore, we have engineered the cleavage site of the HIV-1 protease in these envelope glycoproteins. Engineering the protease cleavage site in GaLV10A1 allowed rescuing viral titers to similar levels of those obtained with the wild-type HIV-1 protease in a transient transfection production, with a 5.5- to 36.7-fold increase. The engineered envelope glycoproteins are now being implemented in stable producer cell lines, expressing the modified protease.

This work contributes for the development of a novel stable packaging cell line for continuous lentiviral vectors production, pioneering the use of a modified protease and providing new chimeric envelope glycoproteins, namely GaLV10A1^{giflet}. Additionally, this thesis opened the door for the use of a novel high-titer envelope glycoprotein – GaLV10A1$^{\Delta R}$ – for development of stable producer cell lines by cell receptor knock out strategy.

Keywords: Lentiviral vectors; Modified HIV-1 protease; Envelope glycoprotein engineering; Packaging cell lines.

Resumo

O uso de vetores lentivirais baseados no *Vírus da imunodeficiência humana* em ensaios clínicos de terapia génica tem vindo a aumentar devido à sua capacidade de transduzir e modificar permanentemente tanto células não quiescentes como quiescentes, e ao seu padrão de integração considerado mais seguro do que o dos vetores retrovirais. Para tirar partido de todo o potencial destes vetores em terapia génica, são desejáveis linhas celulares produtoras estáveis. Porém, o seu desenvolvimento tem sido dificultado pela citotoxicidade de alguns componentes virais, nomeadamente a protease.

Neste trabalho de Mestrado, uma protease geneticamente modificada e menos tóxica foi avaliada para produção de vetores lentivirais. Embora menos citotóxica, esta protease é menos ativa. Enquanto para glicoproteínas do invólucro que não requerem processamento proteolítico – VSV-G – a menor atividade da protease não afetou o título do vetor, a conjugação desta protease com glicoproteínas utilizadas tipicamente no desenvolvimento de células produtoras estáveis – 4070A, GaLV10A1 e RD114A – resultou num título menor. Assim, foi alterado o local de clivagem da protease nestas glicoproteínas por meio de engenharia genética. A modificação dos locais de clivagem da protease no GaLV10A1 permitiu recuperar os títulos virais para níveis semelhantes aos obtidos com a protease *wild-type*, em produção transiente, com um aumento de 5,5 a 36,7 vezes. As glicoproteínas modificadas estão, de momento, a ser implementadas em linhas celulares produtoras estáveis, que expressam a protease modificada.

Este trabalho contribui para o desenvolvimento de uma nova linha celular "empacotadora" estável para a produção contínua de vetores lentivirais, descrevendo primeiramente o uso da protease modificada e fornecendo novas glicoproteínas quiméricas, nomeadamente GaLV10A1^{giflet}. Além disso, esta tese abriu a porta para o uso de uma nova glicoproteína modificada com maior título – GaLV10A1$^{\Delta R}$ – para o desenvolvimento de linhas celulares produtoras estáveis recorrendo à estratégia de *knock out* do recetor celular de GaLV10A1.

Termos chave: Vectores lentivirais; Protease de HIV-1 modificada; Engenharia de glicoproteínas do invólucro; Linhas celulares "empacotadoras".

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
BiTet	Bidirectional Tet-dependent
CA	Capsid protein
CMV	*Cytomegalovirus*
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR-Cas9	CRISPR-associated protein Cas9
CT	Crossing threshold
DMEM	Dulbecco's modified Eagle's medium
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
Env	Envelope glycoprotein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIV	*Feline immunodeficiency virus*
GaLV	*Gibbon ape leukemia virus*
HBB2	Hemoglobin subunit beta-2
HEK	Human embryonic kidney
HIV-1	*Human immunodeficiency virus 1*
I.P.	Infectious particles
IN	Integrase
LDL	Lipoprotein lipase
LTR	Long terminal repeat
LV	Lentiviral vectors
MA	Matrix protein
MLV	*Murine leukemia virus*
M-PER	Mammalian protein extraction reagent
NC	Nucleocapsid protein
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffer saline
PCL	Packaging cell line
PEI	Polyethylenimine
PGK	Human phosphoglycerate kinase 1
PIC	Pre-integration complex

PPT	Polypurine tract
PR	Protease
RBG	Rabbit beta-globin intron
RCL	Replication-competent lentiviruses
RD114	endogenous *Feline leukemia virus*
Rev	Regulator of expression of viral proteins
RMCE	Recombinase-mediated cassette exchange
RRE	Rev Responsive Element
RSV	*Rous sarcoma virus*
RT	Reverse transcriptase
RT-qPCR	Real-time quantitative PCR
rtTA	Reverse tetracycline transactivator
SCID	Severe combined immunodeficiency
SIN	Self-inactivating
SIV	*Simian immunodeficiency virus*
SU	Surface
SV40	*Simian virus 40*
T.P.	Total particles
T.U.	Transduction units
TAR	Trans-activator response element
Tat	Trans-activating regulatory protein
TBS	Tris-buffered saline
TM	Transmembrane
TRE	Tetracycline responsive element
VSV-G	Vesicular stomatitis virus G glycoprotein
WT	Wild-type
zeocin^R	Zeocin resistance marker

1. Introduction

1.1. Therapeutic potential of viral vectors for gene therapy

Gene therapy, the treatment or prevention of a disease by genetic material transfer into an individual's cells or tissues, is considered a revolution in the field of medicine, applicable to a wide range of diseases, including cancer, monogenic, infectious and cardiovascular diseases[1–3] (**Figure 1.1A**). The increasing incidence of several unmet medical needs and chronic diseases across the world has made gene therapy an attractive market with high growth. According to a new report by Visiongain, gene therapy market will achieve revenues of 204 million dollars in 2020, with the potential to expand to 2026 as new genetic treatments reach the market [4]. Currently, most of gene therapy products are in clinical development, although some are already under commercialization [5].

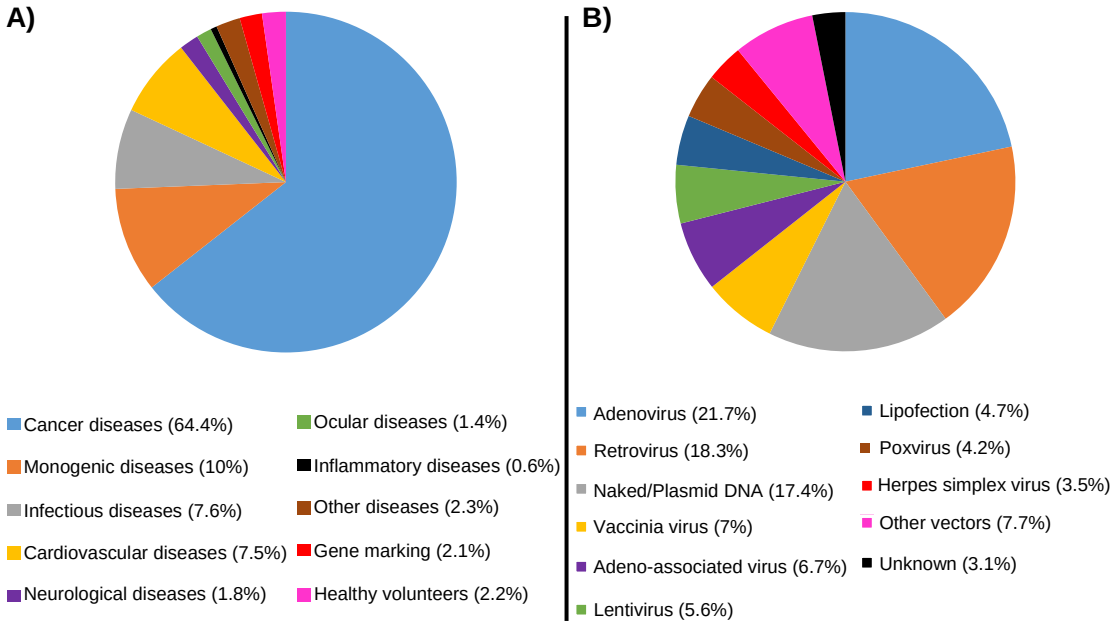

Figure 1.1 – Gene therapy clinical trials. (Source: *The Journal of Gene Medicine*[2]) **A)** Diseases addressed by gene therapy clinical trials. **B)** Vectors used in gene therapy clinical trials.

The first gene therapy clinical trial was initiated in 1990, for the treatment of severe combined immunodeficiency (SCID). Nowadays, more than 2300 clinical trials have been conducted or are still ongoing [2]. From these, viral vectors represent more than 70% of gene delivery vehicles, with vectors based on *Adenovirus*, *Vaccinia virus*, *Herpes simplex virus*, *Adeno-associated virus*, *Retrovirus* and *Lentivirus* being the mostly used [2,6] (**Figure 1.1B**). Each of these viral vectors is characterized by a set of different properties that make then suitable for specific applications [7].

Adenoviruses have shown a real promise for cancer therapy and have been the most widely used viral vector for gene therapy clinical trials [2]. However, their use is decreasing. The major drawback of these viruses is their high immunogenicity, proven to be potentially lethal [8]. In

2003, an *Adenovirus* based vector for the treatment of head and neck squamous cell carcinoma – Gendicine® – was the first gene therapy product released to the market, after approval by the China Food and Drug Administration (FDA) [9]. Two years later, the China FDA granted market approval to another *Adenovirus* based vector – Oncorine™ – for the treatment of late-stage refractory nasopharyngeal cancer in combination with chemotherapy [3].

Vaccinia viruses have been mostly used for the treatment of cancer and vaccination, because of their intense short-lasting transgene expression and cytolytic nature [6]. They were firstly used in 1995 as an immunotherapy for cervical cancer [10], and they have been used to immunize against infectious diseases [2,11]. The main disadvantages of *Vaccinia virus* based vectors are the potential cytotoxicity and high immunogenicity.

Herpes simplex viruses are an interesting gene delivery vehicle due the high infectivity, ability to transduce and persist in a latent state in both dividing and non-dividing cells [6]. These vectors accommodate the largest gene cargos among the viral vectors (up to 50 kb heterologous DNA). Since *Herpes simplex viruses* are neurotropic, they have been successfully used against neurological disorders, although cancer therapy is the predominant application in clinical trials [2]. Yet, 70 to 80% of the population of low socioeconomic status and 40 to 60% from improved socioeconomic status carries latent *Herpes simplex virus* infection [12], exhibiting a specific immune response that can efficiently inactivate vector particles and eliminate transduced cells with exposed viral proteins. Another safety concern is the reversion of replication deficiency by homologous recombination between the *Herpes simplex virus* based vector and the wild-type (WT) genome present in latently infected cells [6]. In 2015, the first oncolytic virus in the Western world, a *Herpes simplex virus* based vector for advanced melanoma treatment – Imlygic® – was approved for use by the USA FDA and recommended for marketing approval in Europe [13].

Adeno-associated virus were firstly used in clinical trials for treatment of cystic fibrosis in 1994 [14]. Their ability to efficiently transduce a wide variety of dividing and non-dividing cells extended their applications to hemophilia, ocular diseases, Alzheimer's and Parkinson's disease, muscular dystrophies, cardiac failure, dyslipidemia and cancer [15]. Their main drawback is the small genetic payload they can accommodate (up to 5 kb). Yet, *Adeno-associated virus* based vectors have been experiencing the highest growth rates in gene therapy clinical trials [2], and will likely take an important segment of the market in the next years. Glybera®, a recombinant *Adeno-associated virus* based vector for the treatment of lipoprotein lipase (LDL) deficiency, was the first gene therapy product being commercialized in the European market, after approval in 2012 by the European Medicines Agency (EMA) [16].

Gammaretroviruses, commonly known as retroviral vectors, were the first class of viral vectors to be developed and are still one of the most used. Together with their complex counterparts, *Lentiviruses*, *Gammaretroviruses* are currently the blockbuster vectors for the treatment of monogenic, infectious diseases and gene marking clinical trials [2,17]. The most attractive features of these vectors are the minimal immune response, capacity for a large genetic payload (up to 9 kb), and their ability to permanently integrate into a target cell genome, sustaining a long-term transgene expression [18]. However, *Gammaretrovirus*es transduction is limited to

dividing cells. Additionally, there is a risk of insertional mutagenesis and oncogenesis associated with the therapeutic use of these vectors [7,19]. This year EMA granted market approval to the second gene therapy product in the European market – Strimvelis™ – a retroviral vector for the treatment of SCID [20].

In contrast to *Gammaretroviruses*, *Lentiviruses* have a distinctive ability to integrate into the genome of non-dividing cells [21] and their integration pattern is considered to be safer, with a less risk of insertional mutagenesis [22]. Therefore, lentiviral vectors (LV) overcome several problems of retroviral vectors, resulting in improved biosafety and performance. Together with *Adeno-associated virus* based vectors, LV are the viral vector experiencing the highest growth rate in clinical trials [2] and are expected to take over retroviral vectors in gene therapy in years to come.

1.2. Biology of *Lentiviruses*

Lentiviruses are human and animal pathogens that are characterized by long incubation periods and persistent infection [23]. These viruses have garnered attention from both scientists and public since 1981, when the first cases of the new epidemic of acquired immunodeficiency syndrome (AIDS) were observed [24,25]. Later, *Human immunodeficiency virus 1* (HIV-1) was isolated and identified as the cause of AIDS [26,27]. Since then, the biology of HIV-1 has been intensively studied. Today it is one of the best understood viruses.

Lentiviruses are classified as one of the seven genus of the family *Retroviridae*, composed by nine virus species, which includes HIV-1, HIV-2, *Simian immunodeficiency virus* (SIV) and *Feline immunodeficiency virus* (FIV) [28]. All *Retroviruses* share similarities in structure, composition and replicative properties [29]. These enveloped viruses harboring a genome of two copies of linear positive-sense single stranded RNA with the size of 7-13 kb are mainly characterized by an unique replicative strategy – reverse transcription – where the viral RNA is reverse transcribed into double stranded DNA, followed by stable integration into the host genome [29,30]. Based on the genome structure, *Retroviruses* are classified as simple or complex. *Lentiviruses* are included in the complex *Retroviruses*, with a more complex genome and additional regulation steps in their life cycle.

Lentiviruses virions measure 100 to 120 nm in diameter and the genomic RNA is associated with the nucleocapsid protein (NC), enclosed within a protein core composed by capsid protein (CA) containing enzymes required for viral infection, such as integrase (IN), protease (PR), reverse transcriptase (RT) and accessory proteins **(Figure 1.2A)**. The matrix protein (MA) forms a spherical shell outside the core, which in turn is surrounded by the lipid bilayer of the virion envelope derived from the host cellular membrane. This lipid bilayer contains the viral envelope glycoproteins (Env) responsible for recognizing specific cell surface receptors on the host cell, allowing virus entry. Env is formed by the surface (SU) subunit which binds to the cell surface receptors and the transmembrane (TM) subunit present as a protein anchor into the lipid membrane [30].

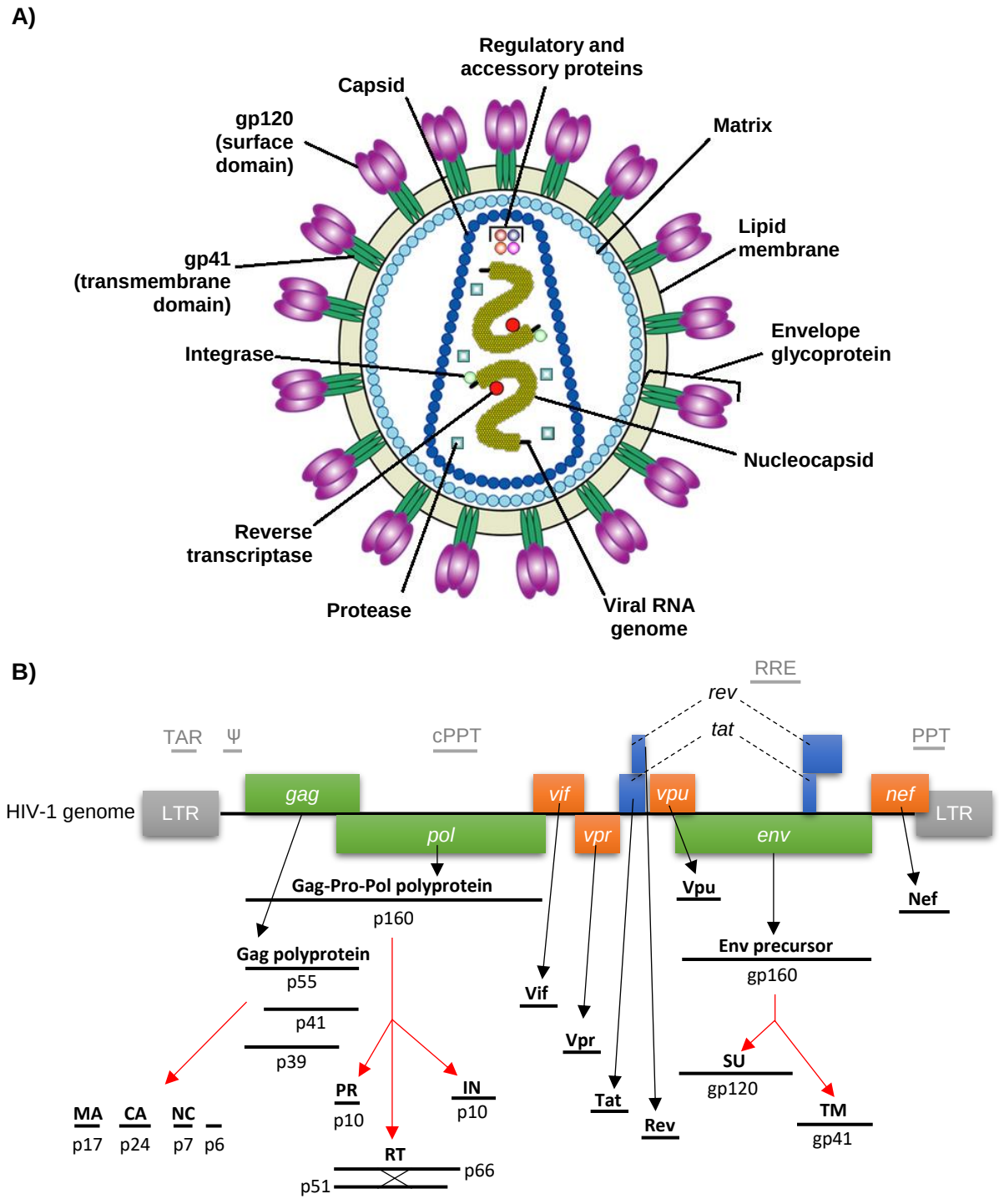

Figure 1.2 – HIV-1 structure and genome. A) Schematic representation of HIV-1 particle structure. Adapted from US National Institute of Allergy and Infectious Diseases. http://web.archive.org/web/20050531012945/http://www.niaid.nih.gov/factsheets/howhiv.htm (Accessed: 5th August 2016). **B)** HIV-1 genome and encoded proteins. Green, orange and blue boxes refer to structural, accessory or regulatory genes, respectively. *Cis*-acting components are indicated in grey. Translation products are indicated with black arrows. Red arrow represents polyprotein processing by the viral protease. Adapted from [31]. HIV-1: *Human immunodeficiency virus 1*; LTR: long terminal repeat; TAR: trans-activator response element; ψ: packaging signal; PPT: polypurine tract; cPPT: central polypurine tract; RRE: Rev Responsive Element; MA: matrix protein; CA: capsid protein; NC: nucleoprotein; PR: protease; RT: reverse transcriptase; Env: envelope glycoprotein; SU: surface subunit; TM: transmembrane subunit.

HIV-1 genome has about 9-10 kb and is constituted by several non-coding sequences that control gene expression and protein synthesis, and nine genes that code for regulatory and accessory proteins in addition to the structural and enzymatic proteins shared among all *Retroviruses* [32] (**Figure 1.2B**).

The *gag* gene encodes a 55 kD polyprotein (Gag) that is cleaved by the viral protease, during and after the release of the progeny virions, into the main structural proteins MA, CA and NC. The *gag-pol* sequence encodes a 160 kD polyprotein (Gag-Pro-Pol), resulting from a ribosomal frameshift event, that after cleavage by the viral protease during the virus maturation gives rise to PR, RT and IN enzymes. These enzymes are responsible for proteolytic cleavage of most viral polyproteins, reverse transcription of the viral RNA into DNA and integration of viral DNA into the host genome, respectively. The *env* gene encodes an Env precursor (gp160), that is cleaved by cellular proteases into the SU and TM subunits (gp120 and gp41, respectively) [31,33].

The remaining six HIV-1 genes encode proteins not found in simple *Retroviruses*. Two of these genes encode regulatory proteins Tat (trans-activating regulatory protein) and Rev (regulator of expression of viral proteins). Tat increases the transcription of the HIV-1 genome several hundredfold by forming a complex with transcriptional factors that interacts with RNA polymerase II, increasing its processivity [34]. Rev mediates the nuclear export of unspliced or singly unspliced mRNA, by multimerizing along the Rev Responsive Element (RRE), and binding to cellular factors. The function of Rev is crucial for viral replication, to overcome the cell's default splicing mechanism, necessary for full-length mRNA transcripts of the genome to be packaged into progeny virions and for the proper translation of *gag-pol* and some HIV-1 accessory proteins [35].

The final four genes encode accessory proteins: Vif, Nef, Vpr and Vpu. These proteins are not crucial for viral replication *in vitro*, but all play one or more roles in disease progression or pathogenesis in man [31].

The HIV-1 genome contains non-coding *cis*-acting sequences that play important roles in viral replication [29]. The long terminal repeat (LTR) is a regulatory sequence of DNA found at both the 5' and 3' ends of the provirus which contain elements required to drive gene expression, reverse transcription and integration into the host cell genome. In the 5' LTR there is the trans-activator response element (TAR) that interacts with the complex formed by Tat and transcriptional factors. In addition to the polypurine tract (PPT) at the beginning of the 3'LTR, lentiviruses have an additional central PPT (cPPT), both of which are used for positive strand DNA synthesis during reverse transcription [31]. The packaging signal (ψ) allows specific packaging of the viral genomic RNA into progeny virions [36]. RRE is a structured RNA element within the *env* gene, recognized by Rev.

The life cycle of HIV-1 is illustrated in **Figure 1.3**. The main difference between the simple *Retroviruses* (like *Gammaretroviruses*) and *Lentiviruses* is the capacity of the latter to integrate their genomes into the nuclear DNA without requiring cell division [29]. Therefore, lentiviral vectors transduce dividing and non-dividing cells, oppositely to retroviral vectors that only transduce the first ones, making lentiviral vectors a better choice for gene therapy. For gene therapy

applications, the ideal viral vectors harness the infection pathway without leading to their replication and toxicity [7]. This assumes the use of packaging systems with a split genome approach for replicative deficient lentiviral vector production.

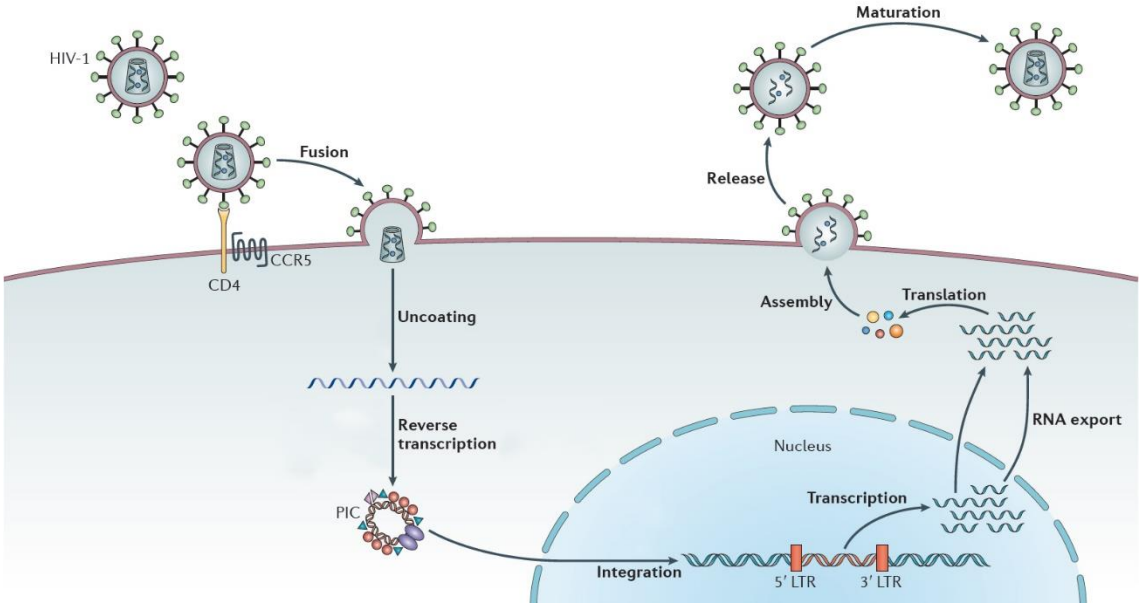

Figure 1.3 – Schematic overview of the HIV-1 replication cycle. The figure illustrates the main steps in the HIV-1 replication cycle. HIV-1 infection begins with virion binding to the CD4 receptor and co-receptors. Viral entry occurs by fusion of the viral membrane with the host cell membrane. Uncoating of the viral capsid takes place and HIV-1 RNA is reverse transcripted into DNA. The pre-integration complex (PIC) is formed and double-stranded DNA product is translocated into the nucleus and integrated into the chromosomal DNA by the integrase, creating the provirus. The integrated viral DNA is subsequently transcribed and translated to form new viral RNA and viral proteins. Viral components are transported to the cell surface to assemble into virus particles. The progeny virus particles bud off and are released. During maturation, proteolysis by the viral protease generates infectious virions. HIV-1: *Human immunodeficiency virus 1*; PIC: pre-integration complex; LTR: long terminal repeat. Adapted from [37].

1.3. Design of lentiviral vector packaging systems

The use of a highly pathogenic human virus in gene therapy applications raises series biosafety concerns. Therefore, the design of packaging systems has evolved in order to increment the efficiency and the safety of lentiviral vector while minimizing the possibility of replication-competent lentiviruses (RCL) during vector production [38,39]. Currently, four generations of lentiviral vectors are considered.

The first generation, developed by Naldini and co-workers, consisted in a three expression cassettes system [21]. The packaging cassette had all structural, accessory and regulatory proteins, with the exception of the envelope glycoprotein. The transgene cassette was composed by the 5'LTR, the packaging signal, the RRE *cis*-acting region and the transgene under the control of a heterologous promoter. In the envelope glycoprotein expression cassette, the native HIV-1 envelope glycoprotein was replaced by the vesicular stomatitis virus G glycoprotein (VSV-G). With this system, good titers were easily achieved, but the poor safety level could not be accepted for a human and potentially lethal pathogen. RCL could be generated with three homologous recombination events between the viral sequences of the packaging and transgene

cassette or endogenous retroviral sequences within the producer cells. Additionally, the presence of the LTR promoter could activate neighboring cellular genes, and the presence of all accessory HIV-1 genes, which are incorporated into the viral particle, enhanced the immunogenicity of the vector [38].

In the second generation [40], all accessory genes were deleted from the three plasmid system, without negative effects on vector titer. By removing the accessory genes (*vpr*, *vif*, *vpu* and *nef*), the generation of RCL became less probable and, if generated, would be unlikely to be pathogenic [32].

The third and still most widely used generation was developed by Dull and co-workers [41] (**Figure 1.4**)**.** It is characterized by the deletion of the *tat* gene from the packaging plasmid and *rev* gene placed in an independent plasmid. The *tat* sequence was replaced by a chimeric 5'LTR with a heterologous viral promoter/enhancer, such as those of *Cytomegalovirus* (CMV) or *Rous sarcoma virus* (RSV). Therefore, lentiviral vector expression is independent of Tat. Rev is maintained but is provided by an independent non-overlapping plasmid. Thus, this system has a total of four constructs, increasing the number of homologous recombination events required for RCL formation. Another feature of this generation is the partial deletion of the 3'LTR in the transgene cassette, leading to transcriptional inactivation of the LTR promoter, after reverse transcription. These vectors are called self-inactivating (SIN) vectors. This inactivation increases safety and reduces concerns related to insertional mutagenesis in the neighboring sequences that can lead to the transactivation or up-regulation of neighboring genome sequences, such as oncogenes [39].

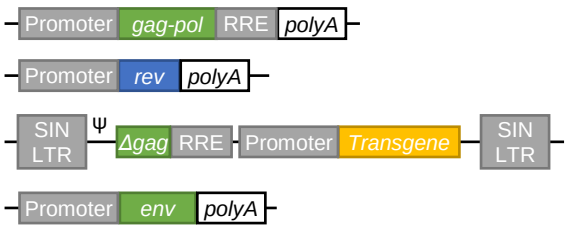

Figure 1.4 – Third generation lentiviral vector packaging system [41]**.** From top to bottom: packaging cassette, *rev* independent cassette, transgene cassette, envelope glycoprotein (usually VSV-G) cassette.

A fourth generation of lentiviral vectors, Rev independent, has also been developed by means of replacing RRE with heterologous viral sequences or by codon-optimization [17]. These packaging systems are not, however, easily available for the research community. Also, the reported titers are one to two logs lower than that of the second or third generation systems.

1.4. Pseudotyping lentiviral vectors

The envelope glycoprotein defines the tropism of the virus by interacting with specific cell surface proteins and promoting the entrance of viruses into the host cell. The natural tropism of

HIV-1 envelope glycoprotein is restricted to $CD4^+$ cells, thus limiting its gene therapy applications to $CD4^+$ cells like macrophages or T cells [42]. However, lentiviral vectors have the ability to incorporate in their viral particles envelope glycoproteins from other viruses. This feature, denominated pseudotyping, allows the manipulation of vector tropism [32,38].

The most used pseudotyping envelope glycoprotein in lentiviral vectors is VSV-G, due to its wide tropism (possibly pantropism), with high titers achieved and improved vector stability, allowing the concentration of the particles by ultracentrifugation and resistance to freeze-thaw cycles [43]. Despite all the advantages, VSV-G is toxic to producer cells, posing a challenge to stable production of lentiviral vectors pseudotyped with this envelope glycoprotein (discussed later). Moreover, the broad tropism of VSV-G can be an impediment for targeted transduction of specific tissues, for example, for *in vivo* applications [43]. Another limitation to its use for *in vivo* application is the inactivation of VSV-G by human complement present in the blood [44].

Several alternative envelope glycoproteins have been studied and are also suitable for pseudotyping lentiviral vectors, for example, the amphotropic *Murine leukemia virus* (MLV) 4070A envelope glycoprotein which is able to transduce most cells [32]. Other envelope glycoproteins have been engineered to pseudotype lentiviral vectors with increased efficiency, for example the chimeric envelope glycoproteins RD114A [45,46] and RDpro [47,48] derived from the endogenous *Feline leukemia virus* (RD114) and GaLV10A1[49] derived from the *Gibbon ape leukemia virus* (GaLV).

Despite specific advantages and disadvantages, each envelope glycoprotein confers a different set of properties to the lentiviral vector, and so each pseudotype may have its own potential niche [43,50].

1.5. Production of lentiviral vectors

For research and clinical purposes, Human Embryonic Kidney (HEK) 293 and its derivate HEK 293T cell lines have been the most used cell substrates for large scale production of lentiviral vectors [51]. HEK 293T cells, due the presence of the *Simian virus 40* (SV40) large T antigen, are preferable because they show superior growth, transfection efficiency and vector productivity in comparison to HEK 293 cells [52]. For research purposes, other human or monkey derived cells have been used (other 293 derived clones, HeLa, HT1080, TE671, COS-1, COS-7, CV-1), although most of them showed reduced vector production titers [52].

Lentiviral vectors can be produced by transient transfection or stable producer cell lines (**Figure 1.5**).

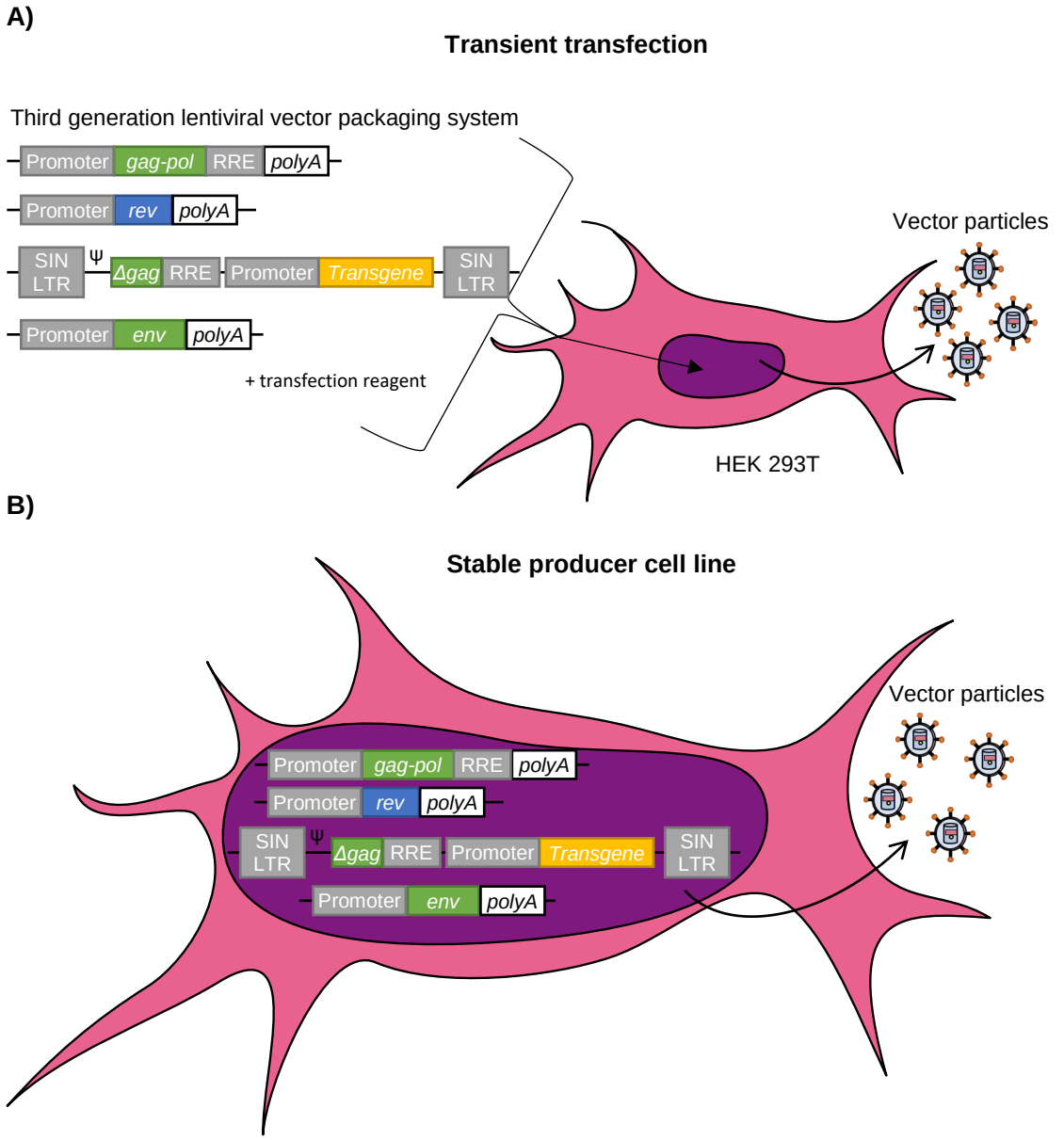

Figure 1.5 – Transient transfection and stable production of lentiviral vectors. A) Transient production after transfection of plasmids from third generation lentiviral vector packaging system into HEK 293T cells. **B)** Stable production from packaging cell lines that constitutively express the viral components.

In transient transfection production, cells are co-transfected with the viral constructs (**Figure 1.5A**). Between 24 to 72 hours post-transfection, the lentiviral vectors present in the supernatant are harvested.

Stable production relies on packaging cell lines (PCLs) in which all of the components necessary to produce vectors are integrated into the cells' genome (**Figure 1.5B**). To establish a PCL for stable production, the packaging, envelope and transgene constructs are inserted one-by-one, followed by clonal selection in between insertions, making the development of stable cell lines a tedious and long lasting process that can take up to one year for a fully developed and characterized cell platform [17]. Most LV batches, including those used in clinical trials to date, have

been produced by transient transfection of HEK 293T cells with multiple plasmids. Transient transfection production is faster compared to the time frame necessary to develop a stable packaging cell line, presenting very competitive titers (up to 10^7 infectious vectors *per* milliliter). Yet, for large-scale production means, transient systems are cumbersome since are difficult to standardize, scale-up, time and cost-ineffective and exhibit high batch-to-batch variability [17,52]. Stable producer cell lines will facilitate the transition from clinical to market, because of the easy scalability, increased safety and well characterized production process [51].

The establishment of a stable packaging cell line for lentiviral vector production has been hampered by the cytotoxicity of viral proteins like Tat, Nef, Vpr and protease [53]. From these, only the protease is still required in the current packaging systems. HIV-1 protease mediates its cytotoxicity *in vitro* and *in vivo* by cleaving and activating procaspase 8, leading to mitochondrial release of cytochrome c, activation of the downstream caspases 9 and 3 and nuclear fragmentation [54,55]. Due to the cytotoxic and cytostatic effects of HIV-1 *gag-pol* genes, high expression of these genes in packaging cell lines has also been challenging. Additionally, the most widely utilized envelope glycoprotein for lentiviral vectors, VSV-G, is highly cytotoxic [56].

At small-scale and for research purposes, transient production by plasmid transfection has been the first choice to cope with the cytotoxic proteins [17]. For large-scale production purposes, conditional packaging systems have been developed in which the expression of toxic proteins are controlled by inducible systems [52]. The first and more frequently used inducible systems are based on the well-defined and characterized tetracycline-inducible expression system [57]. In the Tet-on and Tet-off systems, addition or removal, respectively, of the tetracycline/doxycycline antibiotic in the culture medium triggers gene transcription. At the beginning, the titers obtained using conditional packaging cell lines were low, but further improvements led to similar levels compared to transient production titers [52]. As it happens in transient transfection production, these systems can only produce lentiviral vectors for a few days after induction due to the cytotoxicity of induced viral components. Also, additional purification steps of the viral preparations are required to eliminate inducing agents, hindering the scaling-up of conditional systems for clinical-grade lentiviral vectors production [17]. In addition, packaging cells have often been shown to be instable due to leaky expression of the toxic proteins [51].

Constitutive packaging cell lines are a preferable production system. These cells however are more difficult to obtained than inducible cells, due to the previously discussed cytotoxicity of some viral components. The unfeasible use of VSV-G and the selection of highly *gag-pol* expressing cells seem to be the main challenges [52]. Until the date, four constitutive packaging cell lines have been reported (**Table 1.1**).

Table 1.1 – Constitutive packaging cell lines for lentiviral vector production.

Packaging cell line	LV generation	Envelope glycoprotein	Titer (T.U./ml)	Delivery of viral components
STAR [47]	Second	RDpro GaLV10A1 MLV 4070A	8.5×10^6 1.6×10^6 1.2×10^7	Codon-optimized *gag-pol* inserted by MLV vector transduction
WinPac [58]	Third	RDpro	1×10^6	MLV vector transduction combined with RMCE technology
RD2-MolPack-Chim3 [59]	Second	RD114A	1×10^7	Integrating vectors (chimeric baculo-AAV and LV)
RD3-MolPack-GFP [60]	Third			

T.U.: Transduction units; LV: Lentiviral vectors; MLV: Murine Leukemia Virus; AAV: *Adeno-associated virus* vector; RMCE: recombinase-mediated cassette exchange.

Ikeda and co-workers developed, in 2003, the first continuous packaging cell line, STAR [47], producing second generation lentiviral vectors for up to three months in culture with a titer of 10^7 TU/ml. They used a strategy of codon optimization of HIV-1 *gag-pol* and the envelope glycoprotein derived from the RD114 with the R-peptide cleavage site replaced with that of the HIV-1 matrix/capsid (MA/CA) in Gag (RDpro) [47]. Later, they tested the chimeric envelope glycoproteins derived from GaLV with an MLV 10A1 cytoplasmic tail (GaLV10A1) and the 4070A, demonstrating that VSV-G can be replaced by others envelope glycoproteins to avoid its cytotoxicity [61]. However, significant titers could only be obtained after MLV vector transduction of the optimized *gag-pol*. This procedure raises biosafety issues, due to the increasing chance of generating RCL by homologous recombination, posing further concerns of co-packaging [39]. This risk was later reduced in the development of the WinPac cells [58], by using the MLV vector transduction combined with the recombinase-mediated cassette exchange (RMCE) technology [62]. WinPac cells with the RDpro can continuously produce third generation lentiviral vectors at titers in the order of 10^6 TU/ml [58].

Another constitutive packaging technology, RD-MolPack cells [59,60], were developed by MolMed S.p.A, Milan. Unlike STAR and WinPac cells, the envelope glycoprotein used in this packaging system is a chimeric RD114 envelope glycoprotein with the 4070A cytoplasmic tail (RD114A). RD-MolPack technology is characterized by the sequential insertion of the viral genes by integrating vectors. Packaging genes were introduced by infecting 293T cells with a chimeric baculo-AAV vector. The remaining components were delivered by LV transduction. Similarly to STAR cells, the use of MLV or lentiviral vectors in the construction of packaging cell lines raises biosafety issues [39]. RD2-MolPack and RD3-MolPack cells produce second and third generation lentiviral vectors, respectively, at titers in the order of 10^7 TU/ml [59,60].

As observed in the reported constitute packaging cell lines, non-toxic *Gammaretroviruses* envelope glycoproteins can be used to pseudotype lentiviral vectors to overcome the significant issue of VSV-G. However, the cytotoxicity induced by the HIV-1 protease is still to be addressed. Additionally, the described constitutive packaging cell lines are shown to be unstable and exhibited an abnormal morphology. In our lab we have observed this with STAR cells.

1.6. Aim and strategy

The aim of this work was to provide new tools for the development of a novel lentiviral vector packaging cell line for the constitutive production of these vectors. In this context, we generated a point mutation in the active site of the HIV-1 protease. This mutation consisted in changing amino acid 26 from a threonine to a serine (T26S). According to Konvalinka and co-workers, this mutation led to a 5- to 10-fold decrease in the protease activity compared to the WT HIV-1 protease and, consequently, to a reduced cytotoxicity without effect on virus maturation and infectivity [63]. Yet, the authors did not report the use of T26S HIV-1 protease for lentiviral vector production. Thus, in this work we aimed to minimize cytotoxicity induced by the viral protease in lentiviral vector production by using the less toxic T26S HIV-1 protease.

The first objective consisted on the validation of T26S HIV-1 protease for lentiviral vector production. To accomplish this, proteolytic processing of Gag-Pro-Pol and Gag polyproteins by the protease was assessed and transient transfection production with third generation lentiviral vector packaging was performed. Additionally, we have tried to elucidate the cytotoxicity mediated by the HIV-1 protease using an inducible system.

Since the commonly used envelope glycoprotein VSV-G cannot be used to establish a packaging cell line for stable and constitutive production of lentiviral vectors, we evaluated lentiviral vector production with non-cytotoxic *Gammaretroviruses* envelope glycoproteins, using 4070A, RD114A [45,46] and GaLV10A1 [49]. In order to enhance viral titers conjugated with T26S HIV-1 protease, the cleavage site of the HIV-1 protease in the TM subunit was engineered.

After evaluation of lentiviral vector production using T26S HIV-1 protease and engineered envelope glycoproteins, these components will be used to establish a stable producer cell line and viral titers will be assessed (the establishment of the stable producer cell line was already started in this thesis).

2. Materials and Methods

2.1. Plasmids

Primers and templates for all the plasmids constructed in this work are listed in **Table A.1** in annexes. A schematic representation of the constructed plasmids main transcriptional units is provided in **Figure A.1** in annexes.

pMDLg/pRRE is a third generation lentiviral vector packaging plasmid expressing HIV-1 *gag-pol* under the control of a CMV promoter, and RRE, a binding site for the Rev protein which facilitates export of the RNA from the nucleus. *pRSV-Rev* is a third generation lentiviral vector packaging plasmid containing the second and third exons of HIV-1 *rev* under the control of RSV U3 promoter. These plasmids were kindly provided by Dr. Didier Trono through Addgene plasmid repository (Cambridge, MA, USA) (plasmids # 12251 and # 12253, respectively) and are described in [41].

pRRLSIN-CMV-GFP is a third generation lentiviral transgene plasmid, driving the expression of enhanced green fluorescent protein (eGFP) from the CMV promoter. This plasmid is an in-house constructed plasmid, derived from *pRRLSIN.cPPT.PGK-GFP.WPRE*, kindly provided by Dr. Didier Trono through Addgene plasmid repository (plasmid # 12252) where the human phosphoglycerate kinase 1 (PGK) promoter was replaced by the CMV promoter.

pMDLg/pRRE^{T26S} and *pMDLg/pRRE^{D25N}* are in-house constructed plasmids derived from *pMDLg/pRRE*, with the mutations T26S and D25N in the HIV-1 protease active site, respectively. The T26S mutation is described to cause reduced proteolytic activity and loss of protease-mediated cytotoxicity [63]; the D25N mutation inactivates the active site of the protease [64].

All previously described plasmids were used to produce lentiviral vector by transient transfection as reported in [41], pseudotyped with different envelope glycoproteins.

pMD2.G encodes VSV-G under the control of the CMV promoter. This plasmid was kindly provided by Dr. Didier Trono through Addgene plasmid repository (plasmid # 12259).

phGaLV10A1 encodes GaLV10A1 and zeocin resistance marker under the transcriptional control of the CMV promoter and contains rabbit beta-globin (RBG) and hemoglobin subunit beta-2 (HBB2) introns upstream the start codon. GaLV10A1 is a modified GaLV envelope glycoprotein with the substitution of the cytoplasmic tail by that of the MLV clone 10A1 [49]. This plasmid was kindly provided by Dr. Otto Merten (Généthon, Évry, France).

pCMV-GaLV10A1 was constructed by removing a 19 nucleotides sequence prior to the start codon of *galv10a1* from *phGaLV10A1* by inverse PCR.

pCMV-4070A and *pCMV-RD114A* encode envelope glycoprotein 4070A of the amphotropic MLV and a modified RD114 envelope glycoprotein, RD114A[45,46], with the substitution of the cytoplasmic tail by that of the 4070A, respectively. *pCMV-4070A* and *pCMV-RD114A* were derived from *phGaLV10A1* in which *galv10a1* was removed by *EcoRI* and *KasI* restriction and replaced by *4070a* and *rd114a*, respectively. *4070a* and *rd114a* were amplified by PCR from *pMonoZeo-4070A* (in-house constructed plasmid, described in [65]) and *pLTR-RD114A*,

kindly provided by Jakob Reiser through Addgene plasmid repository (plasmid # 17576, described in [46]).

pCMV-4070A$^{\Delta R}$, *pCMV-GaLV10A1$^{\Delta R}$* and *pCMV-RD114A$^{\Delta R}$* encode 4070A, GaLV10A1 and RD114A, respectively, with the deletion of the R-peptide from the cytoplasmic tail. Each plasmid was amplified by inverse PCR from the parental plasmids previously described, to remove the nucleotides coding for the R-peptide of the cytoplasmic tail of the envelope glycoprotein genes.

pCMV-4070A^{pro}, *pCMV-GaLV10A1^{pro}* and *pCMV-RD114A^{pro}* encode 4070A, GaLV10A1 and RD114A, respectively, in which the R-peptide cleavage site sequence was replaced by that of the HIV-1 matrix/capsid (MA/CA) in HIV-1 Gag. Each plasmid was amplified by inverse PCR from the parental plasmids to substitute the natural R-peptide cleavage site sequence – VQAL↓VLTQ (amino acid sequence) – with the cleavage site of MA/CA – SQNY↓PIVQ.

pCMV-4070A^{giflet}, *pCMV-GaLV10A1^{giflet}* and *pCMV-RD114A^{giflet}* encode 4070A, GaLV10A1 and RD114A, respectively, with a synthetic R-peptide cleavage site sequence reported as the most efficiently cleaved peptide site – GSGIF↓LETSL [66] – by the HIV-1 protease. The construction of these plasmids was conducted in two steps to replace the natural R-peptide cleavage site sequence with the synthetic sequence. In the first step, each plasmid was amplified by inverse PCR from the parental plasmids to substitute the sequence VLTQ of the natural R-peptide cleavage site with the LETSL. In the second step, the same approach was used to replace the VQAL sequence with the GSGIF sequence.

pTet-GFP/PGK-Zeo encodes eGFP under the transcriptional regulation of a bidirectional Tet-dependent (BiTet) promoter and a zeocin resistance marker from the PGK promoter. The backbone was derived from phGaLV10A1, where *galv10a1* was removed by inverse PCR and replaced by *egfp* amplified from *pRRLSIN.cPPT.PGK-GFP.WPRE* by PCR. The PGK promoter was amplified by PCR from *pRRLSIN.cPPT.PGK-GFP.WPRE* and inserted 20 nucleotides upstream of the zeocin resistance marker. The BiTet promoter was isolated from *pTargLox-polyATetVSV-G-FerHprom* (in-house constructed plasmid) by *BamHI* restriction to replace the *CMV-RBG-HBB2* sequence removed by inverse PCR.

pTet-HIV1 PR^{WT}/PGK-Zeo, *pTet-HIV1 PR^{T26S}/PGK-Zeo* and *pTet-HIV1 PR^{D25N}/PGK-Zeo* expresses the WT, T26S and D25N HIV-1 proteases under the control of the BiTet promoter, respectively. The coding sequences for the proteases were amplified from the respective *pMDLg/pRRE* plasmids by PCR and replaced *egfp* in *pTet-GFP/PGK-Zeo* (removed by *EcoRI* restriction). These plasmids were used to develop stable cell lines with inducible expression of HIV-1 proteases.

pRRLSIN-mCherry-hPGK-GFP is a lentiviral transgene plasmid, driving the expression of mCherry protein from the LTR promoter and eGFP from the LTR and PGK promoters. This plasmid was constructed by Hélio Tomás (ACT Unit IBET/ITQB NOVA, Oeiras, Portugal), and was used to establish stable cell lines for lentiviral vector production.

2.2. Cloning procedure

All PCR reactions were performed with Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy, Vantaa, Finland), using the conditions suggested by the manufacturer. The restriction enzymes (New England Biolabs, Ipswich, MA, USA) were used with the appropriate buffer according to the manufacturer's instructions. Generated fragments by restriction or PCR reactions were isolated by 0.7% (w/v) agarose gels (NZYTech, Lisbon, Portugal), and purified with NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Cloning reactions were performed using In-Fusion® HD Cloning Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) following manufacturer's instructions.

2.3. Bacterial strains and culture media

Escherichia coli (*E. coli*) Stellar™ (Clontech Laboratories, Inc.), NZY5α (NZYTech) and One Shot® Stbl3™ (Life Technologies, Carlsbad, CA, USA) competent cells were used for the production of the plasmids. Transformation procedures were performed under the manufacturer's instructions.

The liquid and agar cultures were performed with Terrific Broth (Fast-Media® TB; InvivoGen, San Diego, CA, USA) and Luria Broth (Fast Media® LB; InvivoGen), respectively, supplemented with the appropriate antibiotic (ampicilin, kanamycin, blasticidin or puromycin). The media were prepared using ultrapure water (Milli-Q®, Merck Millipore, Billerica, MA, USA).

2.4. Plasmid purification and quality control

Plasmid purification was performed at small-scale purification (yields up to 20 µg of DNA) with GeneJET Plasmid Miniprep Kit (Thermo Scientific™, Waltham, MA, USA) and larger scale purification (yields up to 500 µg of DNA) with Genopure Plasmid Maxi Kit (Roche Applied Science, Penzberg, Germany) following manufacturers' instruction. Working bacteria banks for each plasmid were generated and stored at -20ºC with 15-20% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA).

The DNA concentration was determined using Nanodrop™ 2000C Spectrophotometer (Thermo Scientific™). Plasmid purity was assessed by the Abs_{260nm}/Abs_{280nm} and Abs_{260nm}/Abs_{230nm} ratios. Plasmid integrity was assessed with 0.7% (w/v) agarose gels (NZYtech). All generated plasmids were sequenced using GATC Biotech services (Constance, Germany).

2.5. Cell lines and culture conditions

HEK 293T (ATCC CRL-11268), is a Human Embryonic Kidney 293 derived cell line that constitutively expresses the SV40 large T antigen. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco™, Carlsbad, CA, USA), supplemented with 10% (v/v) Fetal

Bovine Serum (FBS) (Gibco™) at 37 °C in a humidified atmosphere containing 8% CO_2. All cells were cultured under adherent conditions (t-flask) (Starstedt, Nümbrecht, Germany).

2.6. Determination of cell concentration and viability

Cell concentration and viability were determined by the trypan blue exclusion assay, using a 0.1% (v/v) Trypan Blue (Sigma-Aldrich) solution in Phosphate Buffer Saline (PBS) (Gibco™). Cell count was performed in a Fuchs-Rosenthal hemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany) on an inverted microscope (Olympus, Tokyo, Japan).

2.7. Cell transfection procedure

For transfection procedure, cells were seeded at 5×10^4 cells/cm^2. After 24 hours, transfection was carried using polyethylenimine (PEI, Linear 25kDa) (Polysciences, Inc., Warrington, PA, USA) at 1:1.5 (w/w) ratio of DNA:PEI. A total of 5 µg of plasmid *per* million of cells was used. PEI transfection solution and plasmid mix solution were prepared in serum-free DMEM. Plasmid mix solution was filtered through 0.22 µm-pore-size cellulose acetate filter to the PEI transfection solution. After 10 to 15 minutes incubation at room temperature, transfection solution was added to the cells.

2.8. Establishment of stable cell lines with inducible HIV-1 protease gene expression

To establish stable cell lines with inducible HIV-1 protease gene expression, *pTet-HIV1 PR^{WT}/PGK-Zeo*, *pTet-HIV1 PR^{T26S}/PGK-Zeo* and *pTet-HIV1 PR^{D25N}/PGK-Zeo* were separately transfected into HEK 293T cells as previously described. *pTet-GFP/PGK-Zeo* was used as control. The transfection procedure was performed in a 6-well plate. 48 hours post-transfection, cells were transferred into a 25 cm^2 t-flask and zeocin selection was started (200 µg/ml; InvivoGen). Medium was regularly exchanged during 4 weeks. Complete selection was considered when the cells in the t-flask reached confluence after standard splitting dilutions (1:15 and 1:30) in the following 3 or 4 days (respectively).

2.9. Growth study and induction conditions

Cells with inducible HIV-1 WT, T26S and D25N proteases gene expression were seeded at 2×10^4 cells/cm^2 in 6-well plates. 12 hours after seeding, gene expression was induced by 1 µg/ml of doxycycline (Sigma-Aldrich) added every 24 hours. Cells were counted every 10 to 14 hours during the following 6 days. Protein and RNA extraction was conducted two days after induction.

2.10. Protein extraction and quantification

Cells were harvested and pelleted at 300 × *g* for 10 minutes and washed with cold PBS. A total of 2 × 10^6 cells was used for protein extraction. Two protocols were performed, depending on the use of the extracts. For HIV-1 protease activity assay, cells were ressuspended with 100 µl of hypotonic buffer (10mM Tris-HCl, pH 7.8, 10 mM NaCl) *per* million of cells and incubated on ice for 20 minutes. Cell pellets were disrupted with freeze and thaw cycle (3 times) in liquid nitrogen and water bath at 37ºC. For HIV-1 polyprotein processing analysis by western blotting, cells were lysed in 100 µl Mammalian Protein Extraction Reagent (M-PER) (Thermo Scientific™) *per* million of cells. cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) was added to M-PER. The mixture was shaken for 10 seconds in the vortex and placed in ice for 5 minutes. This last step was repeated 2 times. Extracts were clarified by centrifugation at 13500 × *g*. Samples were frozen at -20ºC for short-term and -80ºC for long-term storage. Total protein quantification was performed with Pierce™ BCA Protein Assay Kit (Thermo Scientific™), according to manufacturer's instructions.

2.11. Western blot analysis of HIV-1 polyprotein processing

Cells were transfected with *pRSV-Rev* and *pMDLg/pRRE* and its variants (WT, T26S and D25N), as previously described. A plasmid DNA ratio of 1:3 was used, respectively. 48 hours later, protein extracts were performed for HIV-1 polyprotein processing analysis by western blotting, as previously described.

For protein electrophoresis separation, NuPAGE® electrophoresis system (Life Technologies) was used. Samples were prepared in denaturing conditions according to manufacturer's instructions. Samples were resolved on a NuPage® 4-12% Bis-Tris gel with NuPAGE® MES SDS Running Buffer, at 180 V for 35 minutes. Protein transfer into nitrocellulose membrane was performed in Trans-Bot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA) for 30 minutes, according to manufacturer's instructions. Membranes were blocked with blocking solution 0.1% (w/v) tween 20 (Sigma-Aldrich) and 5% (w/v) skim milk powder (Sigma-Aldrich) in Tris-Buffered Saline (TBS) (Sigma-Aldrich), for 30 minutes at room temperature and incubated overnight with primary antibody, anti-HIV1 p24 antibody [39/5.4A] (ab9071) (Abcam, Cambridge, UK), diluted 1:2000 in blocking solution with gentle agitation. Membranes were washed 3 times with washing solution 0.1% (w/v) tween 20 in TBS and incubated with secondary antibody, horseradish peroxidase-linked ECL Anti-Mouse IgG (NA931) (GE Healthcare, Little Chalfont, UK) diluted 1:5000 in blocking solution for 2 hours at room temperature. Chemiluminescence detection was performed by incubating the membranes with Amersham™ ECL™ prime western blotting detection reagent (GE Healthcare), according to manufacturer's instructions, and analyzed under ChemiDoc XRS System (Bio-Rad).

2.12. Measurement of HIV-1 protease activity

To measure HIV-1 protease activity in the protein extracts of cells with inducible HIV-1 WT, T26S and D25N proteases gene expression, the HIV-1 protease activity fluorometric assay kit (BioVision, Inc., Milpitas, CA, USA) was used, following the manufacturer's instructions.

2.13. RNA extraction and real-time quantitative PCR for gene expression

To assess gene expression of cell surface receptors SLC20A2, SLC1A5 and SLC20A1 (receptors for 4070A, RD114A and GaLV10A1, respectively) HEK 293T cells were inoculated as previously described. 48 hours later, total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA yields were quantified using Nanodrop 2000C Spectrophotometer. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) using 2 µg of total RNA and anchored-oligo$(dT)_{18}$ primers for total mRNA reverse transcription, according to manufacturer's instructions. The cDNA was aliquoted and stored at -20ºC until further use. For real-time quantitative PCR (RT-qPCR), *RPL22* was chosen as a control gene. Forward and reverse primer sequences are listed in **Table A.1** in annexes. RT-qPCR was conducted on a thermocycler LightCycler® 480 Real-Time PCR System (Roche Applied Science) using LightCycler® 480 SYBR Green I Master (Roche Applied Science). Gene expression was quantified after normalization to *RPL22* using $2^{-\Delta CT}$ method.

2.14. Lentiviral vectors production and titration

2.14.1. Transient production

For transient production of lentiviral vectors, the third generation lentiviral packaging system and the transfection procedure described in [41] were used. The transfection procedure was conducted using PEI as previously described. HEK 293T cells were seeded at 5×10^4 cells/cm^2 in 25 cm^2 t-flask 24h prior to transfection. A total of 4.65 µg of plasmid DNA *per* million cells was used for the transfection of one t-flask: 1 µg of *pMDLg/pRRE* or its variants (T26S and D25N) and 0.25 µg of *pRSV-Rev* (providing the packaging functions), 2.5 µg of *pRRLSIN-CMV-GFP* (providing the transgene vector) and 0.9 µg of envelope glycoprotein encoding plasmid. After 20 to 24 hours post transfection, the medium was replaced with 4 ml of DMEM supplemented with 10% (v/v) FBS. For titration of total particles by nanoparticle tracking analysis, serum-free DMEM was used in this step. After an additional production period of 24 hours, the medium containing the viral vectors was harvested, filtered through 0.45 µm-pore-size cellulose acetate filter for clarification, aliquoted and stored at -80ºC until further use. To assess transfection efficiency, transfected cells were harvested and analyzed for GFP fluorescence by flow cytometry (CyFlow® Space, Sysmex Corporation, Kōbe, Japan).

2.14.2. Titration of infectious particles

For titration of infectious particles (I.P.), HEK 293T cells were seeded at 5×10^4 cells/cm^2 in 24-well plates 24 hours before infection. Transduction was performed by removing the cell supernatant and infecting cells with 0.2 ml of viral supernatants at several dilutions performed in DMEM supplemented with 10% (v/v) FBS containing 8 µg/ml of polybrene (Sigma-Aldrich). For normal transduction protocol, cells were incubated at 37 ºC overnight after which 0.5 ml of DMEM supplemented with 10% (v/v) was added. For spin inoculation protocol, the plates were centrifuged at 1200 × *g*, 25 ºC for 2 hours after which 0.5 ml of fresh supplemented DMEM was added and cells were incubated at 37 ºC. Two days after infection, cells were harvested and analyzed for GFP fluorescence by flow cytometry (CyFlow® Space).

The I.P. titer was determined taking into account the percentage of GFP positive cells, the number of cells determined at infection time and the dilution factor of the viral supernatant. Infections that rendered 2-20% of infected cells were considered for titer calculations. Viral titers, defined as infectious particles per milliliter (I.P./ml) were calculated using the following equation:

$$\frac{\text{I. P.}}{\text{ml}} = \frac{\%\text{GFP positive cells} \times \text{number of cells at infection time} \times \text{dilution factor}}{100 \times \text{infection volume (ml)}}$$

2.14.3. Titration of total particles

Physical (total) particles (T.P.) in the serum-free viral supernatant were assessed by Nanoparticle Tracking Analysis (NTA) using NanoSight® NS500 (Malvern Instruments Ltd, Malvern, UK), following the manufacturer's instructions.

For viral supernatants containing serum, a p24 enzyme-linked immunosorvent assay (ELISA) – INNOTEST HIV Antigen mAb (Fujirebio Diagnostics, Inc., Malvern, PA, USA) – was used to quantify total particles, according to the manufacturer's instructions.

2.15. Establishment of stable cell lines for lentiviral vector production

HEK 293T cell line constitutively expressing HIV-1 *gag-pol* with T26S mutation and *rev*, developed by Hélio Tomás (ACT Unit IBET/ITQB NOVA), was used to establish stable cell lines for lentiviral vector production with T26S HIV-1 protease and engineered envelope glycoproteins. Blasticidin (15 µg/ml, InvivoGen) and hygromicin B (150 µg/ml, InvivoGen) were the antibiotics used for the selection of clones expressing *gag-pol* and *rev*, respectively. Cells were transfected with *pRRLSIN-mCherry-hPGK-GFP* (providing the transgene vector), as previously described. 48 hours post-transfection, cells were transferred into a 25 cm^2 t-flask and puromycin selection was started (0.5 µg/ml; InvivoGen). Medium was regularly exchanged during 3 weeks, until the cells in the t-flask reached confluence after standard splitting dilutions (1:15 and 1:30) in the following 3 or 4 days (respectively). A new antibiotic selection step was performed with blasticidin, hygromicin B and puromycin for one week.

3. Results

3.1. Evaluation of T26S HIV-1 Protease for lentiviral vector production

To minimize the cytotoxicity of the viral components for the development of a packaging cell line for stable lentiviral vector production, a point mutation was generated in the active site of the HIV-1 protease. This mutation consisted in changing amino acid 26 from a threonine to a serine (T26S)[63]. Konvalinka and co-workers reported that this mutation resulted in 5- to 10-fold reduction in the proteolytic activity compared to the WT HIV-1 protease and, consequently, lower protease-mediated cytotoxicity without effect on virus maturation and infectivity [63]. In this work, we evaluated the use of the T26S HIV-1 protease to establish a stable cell line for lentiviral vector production. As control, an inactivating mutation was performed by changing amino acid 25 from an aspartic acid to an asparagine (D25N) [64].

3.1.1. Proteolytic processing of Gag-Pro-Pol and Gag polyprotein by T26S HIV-1 protease

To assess the ability of T26S HIV-1 protease to process Gag-Pro-Pol and Gag polypeptide, protein extracts from cells transfected with *pMDLg/pRRE* or its variants (WT, T26S and D25N) were analyzed by western blotting (**Figure 3.1**).

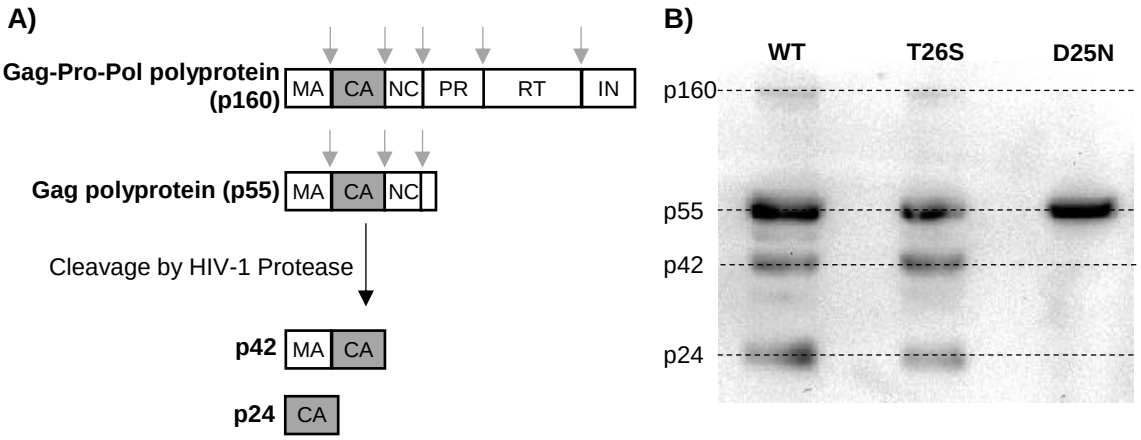

Figure 3.1 – Analysis of Gag-Pro-Pol and Gag proteolytic processing by T26S HIV-1 protease. A) Precursor proteins and cleavage products containing p24 peptide sequence (identified in grey). Grey arrows represent the cleavage sites for HIV-1 protease. MA: matrix; CA: capsid; NC: nucleocapsid; PR: protease; RT: reverse transcriptase; IN: integrase. **B)** Western blotting analysis of Gag-Pro-Pol and Gag proteolytic processing by WT, T26S and D25N HIV-1 proteases. Extracts of HEK 293T cells transfected with *pRSV-Rev* and *pMDLg/pRRE* variants (WT, T26S and D25N) were analyzed by immunoblotting with anti-HIV-1 p24 antibody. Precursor proteins and cleavage products are identified on the left. WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease.

T26S HIV-1 protease showed Gag-Pro-Pol and Gag polyprotein processing similar to the WT HIV-1 protease. As expected, D25N inactivating mutation yielded no Gag-Pro-Pol and Gag polyprotein processing.

3.1.2. Lentiviral vector particles production with T26S HIV-1 protease

The main challenge for stable production of lentiviral vector is the cytotoxicity of the viral protease. According to Konvalinka and co-workers, the T26S mutation in the active site of the HIV-1 protease reduces its cytotoxic activity [63]. However, the authors did not report the use of T26S HIV-1 protease for lentiviral vector production.

To evaluate the ability of T26S HIV-1 protease to support lentiviral vector production, transient production with third generation lentiviral vector packaging [41] pseudotyped with VSV-G was performed and infectious and total particles were assessed (**Figure 3.2**). For total particles quantification, p24 ELISA was performed in viral supernatants.

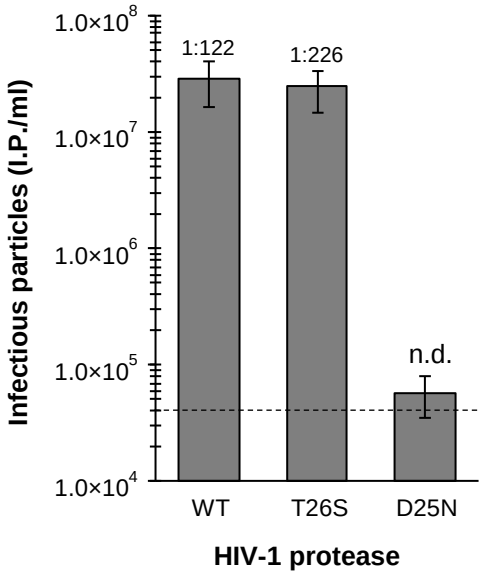

Figure 3.2 – Transient production of lentiviral vector with T26S HIV-1 protease. Infectious particles titers are shown as average ± standard deviation of twelve biological replicates (n=12) for the WT and T26S HIV-1 proteases, and three biological replicates (n=3) for D25N HIV-1 protease. The numbers on top of the bars indicate the ratio of infectious particles per total particles of twelve biological replicates (n=12). Detection limit of 4.0×10^4 I.P./ml is indicated by a dashed arrow. I.P.: infectious particles; WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease, n.d.: not detected.

Infectious vector titer was found to be similar between the WT and the T26S HIV-1 protease (2×10^7 I.P./ml). Regarding total particles, T26S HIV-1 protease additionally showed a decreased ratio of infectious particles *per* total particles by approximately 2-fold. As expected, D25N HIV-1 protease yielded low infectious titer. Total particles were not detected in productions with D25N HIV-1 protease.

Since it was not possible to access total particles quantification of lentiviral vectors produced with D25N HIV-1 protease with p24 ELISA, a parallel production was performed in serum-free conditions to enable total particles quantification by NTA (Nanosight® NS500) (**Figure 3.3**).

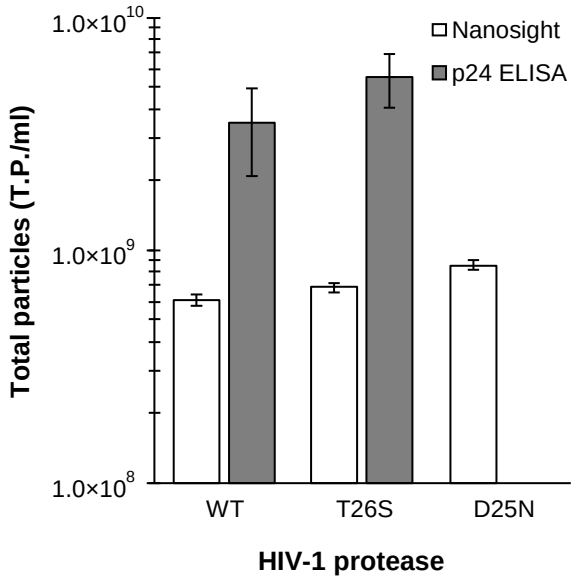

Figure 3.3 – Total particles quantification of lentiviral vector with T26S HIV-1 protease by nanoparticle tracking analysis. Total particles values assessed using Nanosight® NS500 or p24 ELISA are shown as average ± standard deviation of three and two technical replicates, respectively. T.P.: total particles; WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease; ELISA: enzyme-linked immunosorbent assay.

Total particles quantified by NTA were similar between WT and T26S HIV-1 protease and higher values for D25N HIV-1 protease. These results suggest that p24 ELISA detects exclusively p24 in its processed form.

3.1.3. Cytotoxicity assessment of T26S HIV-1 protease

Our first trial to evaluate T26S HIV-1 protease-mediated cytotoxicity was by performing transient transfection of HEK 293T cells with *pRSV-Rev* and *pMDLg/pRRE* and its variants (WT, D25N and T26S). After 48 hours apoptosis was assessed using NucView™ 488 Caspase-3 Substrate and MitoView™ 633 mitochondrial dye (Biotium Inc., Fremont, CA, USA). No differences between HIV-1 proteases or controls were observed (data not shown). We hypothesized that cytotoxicity would be more evident in long term culture not easily seen using a transient transfection. In this context, an expression cassette based on the tetracycline-inducible expression system [57] was constructed to assess the cytotoxicity of the T26S HIV-1 protease in HEK 293T during long term culture (**Figure 3.4** and **Figure A.1C** from annexes).

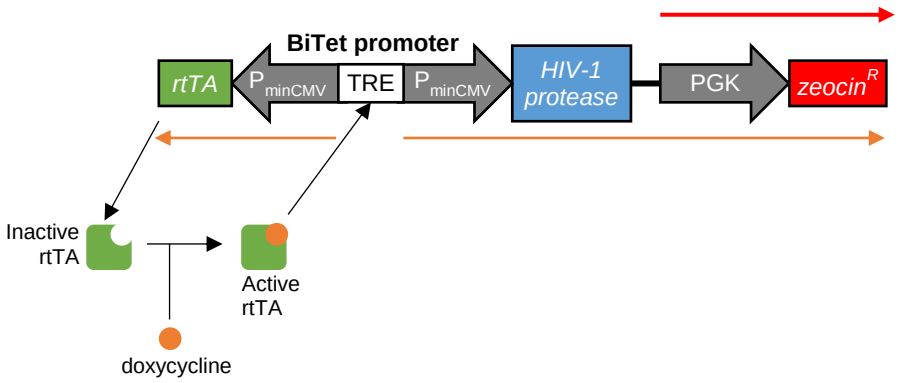

Figure 3.4 – Inducible expression cassette constructed for HIV-1 protease induced gene expression. Orange and red arrow represents inducible and constitutive gene expression, respectively. BiTet: Bidirectional Tet-dependent; P_{minCMV}: minimal *Cytomegalovirus* promoter; PGK: human phosphoglycerate kinase 1 promoter; TRE: Tetracycline responsive element; rtTA: reverse tetracycline transactivator (rtTA), *zeocin*^{R}: zeocin resistance marker.

We choose the available inducible system in our lab, bidirectional Tet-dependent promoter, to control the expression of T26S HIV-1 protease. In this system, doxycycline (a tetracycline analogue) binds to the reverse tetracycline transactivator (rtTA) protein, which is under the control of the BiTet promoter. The resulting rtTA protein is able to bind to DNA in the tetracycline responsive element (TRE), inducing the transcription of T26S HIV-1 protease gene and itself (Tet-on). In this way, T26S HIV-1 protease is expressed after addition of doxycycline to culture medium. In order to select cells transfected with the inducible T26S HIV-1 protease expression cassette, PGK promoter was introduced into the construction to allow constitutive expression of the zeocin resistance marker (*zeocin*^{R}), enabling the selection of cells without induction of the protease expression. As control, WT and D25N HIV-1 protease inducible expression system were also developed. Subsequently, HEK 293T cells were transfected with the plasmids containing the inducible expression cassettes and selected as described in the Materials and Methods section.

To further investigate the protease-mediated cytotoxicity, HIV-1 protease gene expression was induced with 1 µg/ml doxycycline every 24 hours and cell growth was monitored (**Figure 3.5**). Additionally, RNA and protein extracts were performed 3 days after the first induction.

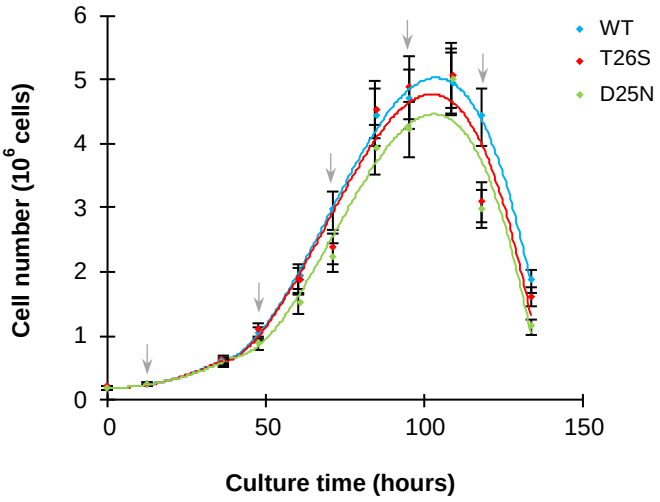

Figure 3.5 – Cell growth of HEK 293T expressing HIV-1 protease. The blue, red and green colors represent cells with inducible expression of WT, T26S and D25N HIV-1 proteases. Grey arrows indicate induction of HIV-1 proteases gene expression conducted with 1 µg/ml doxycycline. Error bars correspond to 10% error for cell counting. WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease.

Cell growth was found to be similar between cells expressing the WT, T26S and D25N HIV-1 proteases. However, HIV-1 protease activity could not be detected in protein extracts (data not shown). Therefore, RT-qPCR was performed to evaluate gene expression of HIV-1 proteases with the bidirectional Tet-dependent promoter (**Figure 3.6**). Cells without induced expression were used as controls.

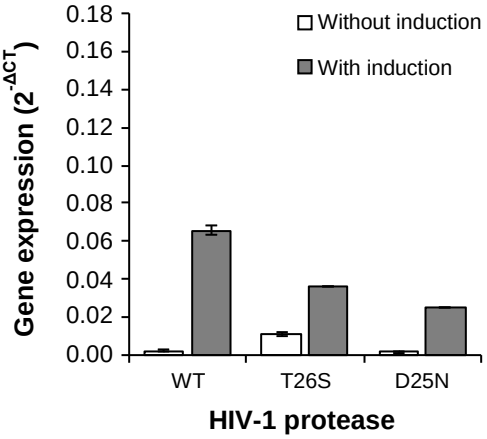

Figure 3.6 – Gene expression of HIV-1 proteases under the control of a bidirectional Tet-dependent promoter. White and grey bars correspond to gene expression without or with doxycycline induction, respectively. Gene expression was quantified after normalization to a control gene (*RPL22*) using the $2^{-\Delta CT}$ method as defined in Materials and Methods. Gene expression levels are show as average expression (relative to the control gene, *RPL22*) ± standard deviation of two technical replicates. WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease; CT: crossing threshold.

For all the HIV-1 proteases, low gene expression with the BiTet promoter was observed. The judgment on the low expression levels related to the high CT (crossing threshold) values obtained (**Table A.3** in annexes).

To evaluate the functionality of the inducible expression system constructed, HEK 293T cells expressing *egfp* under the control of the BiTet promoter were established, similarly as described to previous cells with HIV-1 proteases. eGFP fluorescence intensity was assessed by flow cytometry (CyFlow® Space) after 3 days of continuous induction (**Figure 3.7**). Cells without induced *egfp* expression and HEK 293T were used as controls.

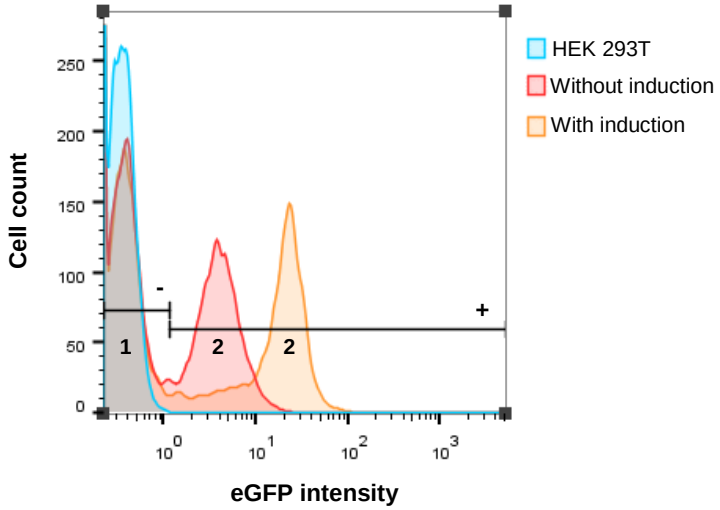

Figure 3.7 – eGFP fluorescence intensity after induction with doxycycline. Blue, red and orange represents HEK 293T, selected cells without and with induction of eGPF gene expression, respectively. Positive cells for eGFP are indicated in "+" gate and negative cells in "-" gate. 1 and 2 indicate two different cell populations. Data was treated using FlowJo™ software.

The results from **Figure 3.7** showed that even without addition of doxycycline, e*gfp* is expressed, revealing a leakiness feature of the bidirectional Tet-dependent promoter. Moreover, eGFP intensity increased only by 4-fold after induction with doxycycline. Additionally, two cells populations (referred as 1 and 2) are observed with the inducible eGFP expression cassette. Population 1 did not show eGFP intensity, suggesting that *egfp* is not being expressed even after induction.

3.2. Lentiviral vector production with T26S HIV-1 protease and non-cytotoxic envelope glycoproteins

VSV-G has been the envelope glycoprotein of choice for lentiviral vector production because of its wide tropism and stability conferred to viral particles [43]. However, due to its cytotoxicity, it cannot be used to establish a packaging cell line for stable and constitutive production of lentiviral vectors. Moreover, non-toxic *Gammaretrovirus* envelope glycoproteins such as the amphotropic MLV 4070A, RD114 and GaLV envelope glycoproteins have been successfully used to pseudotype lentiviral vectors [45–50,61,67].

3.2.1. Evaluation of lentiviral vector production with T26S HIV-1 protease and retroviral envelope glycoproteins

In this work, three plasmids were constructed for the 4070A, chimeric RD114 envelope glycoprotein with the cytoplasmic tail of 4070A (RD114A)[45,46] and chimeric GaLV envelope glycoprotein with the cytoplasmic tail of 10A1 (GaLV10A1)[49] (indicated with * in **Figure 3.9**). To eliminate possible expression bias, these envelopes were cloned in the same expression cassette (**Figure A.1A** in annexes).

Transient production of lentiviral vectors with the T26S HIV-1 protease was performed to evaluate the titers of viral particles pseudotyped with the 4070A, RD114A and GaLV10A1 (**Figure 3.8**). As control, WT HIV-1 protease was also used. For titration of infectious particles, two protocols were performed: standard procedure and spin inoculation. Both use polybrene (8 µg/ml) for vector titer enhancement, but spin inoculation has an additional step – centrifugal inoculation – to potentiate physical contact of viral particles with the target cells [61].

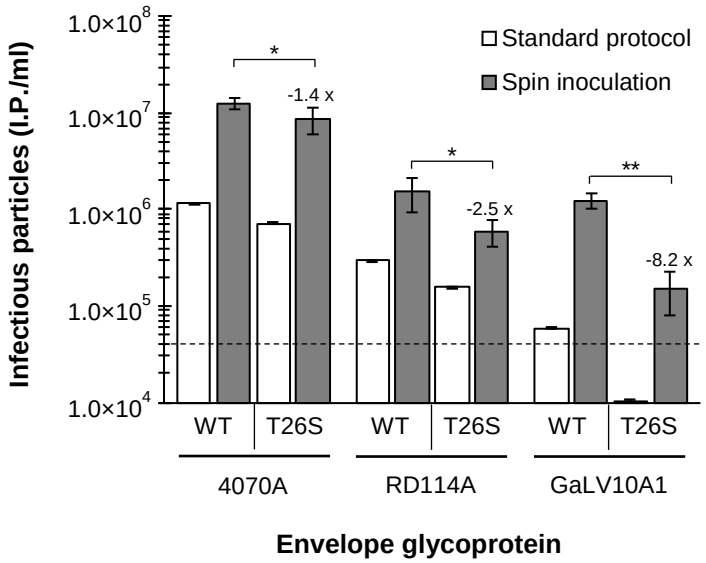

Figure 3.8 – Lentiviral vector production with T26S HIV-1 protease and non-cytotoxic retroviral envelope glycoproteins. White and grey bars represent infectious titers obtained with standard protocol or spin inoculation, respectively. Values from standard protocol are shown as average ± standard deviation of two technical replicates. Values from spin inoculation are shown as average ± standard deviation of three biological replicates (n=3). The numbers on the top of the bars indicate fold change of infectious titer with the T26S HIV-1 protease, obtained by spin inoculation, relatively to the corresponding titer with the WT HIV-1 protease. Fold changes are shown for significant changes based on an one-tailed non-paired t-test, * $p<0.1$ and ** $p<0.01$. Detection limit of 4.0×10^4 I.P./ml is indicated by a dashed arrow. WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease; D25N: D25N HIV-1 protease; I.P.: infectious particles.

For the three envelope glycoproteins evaluated, T26S HIV-1 protease yielded reduced titer relatively to the WT HIV-1 protease. This result was independent of the infection protocol used.

3.2.2. Engineering envelope glycoproteins for enhanced proteolytic processing

Gammaretrovirus envelope glycoproteins, unlike VSV-G, undergo proteolytic processing during virion assembly mediated by the retroviral protease [68]. In this step, a short sequence – R-peptide – is cleaved from the cytoplasmic tail [68]. This cleavage is required for virus entry, since it activates the fusogenic activity of the envelope glycoprotein [67–70].

Since the R-peptide cleavage site in the original envelope glycoproteins was a retroviral sequence, better results were expected with a lentiviral cleavage site for lentiviral vector production, especially considering the putative reduced activity of the T26S HIV-1 protease. Therefore, the influence of the protease cleavage sequence on the R-peptide cleavage site and its impact on viral particles production was studied. To this end, cleavage sites recognizable by the HIV-1 protease were introduced in 4070A, RD114A and GaLV10A1. These envelope glycoproteins shared the retroviral cleavage site – VQAL↓VLTQ – of the cytoplasmic tail of 4070A. Herein, several envelope glycoproteins chimeras were constructed, engineered at the protease cleavage site of the R-peptide, to contain cleavage sequences amenable to HIV-1 proteolytic processing (**Figure 3.9**).

For each envelope glycoprotein, three mutations were performed:

- ΔR: the removal of the R-peptide, generating a truncated cytoplasmic tail [67,70];
- pro: cleavage site sequence of HIV-1 matrix/capsid (MA/CA) – SQNY↓PIVQ;
- giflet: synthetic cleavage site sequence reported as the most efficiently cleaved peptide site by the HIV-1 protease – GSGIF↓LETSL [66].

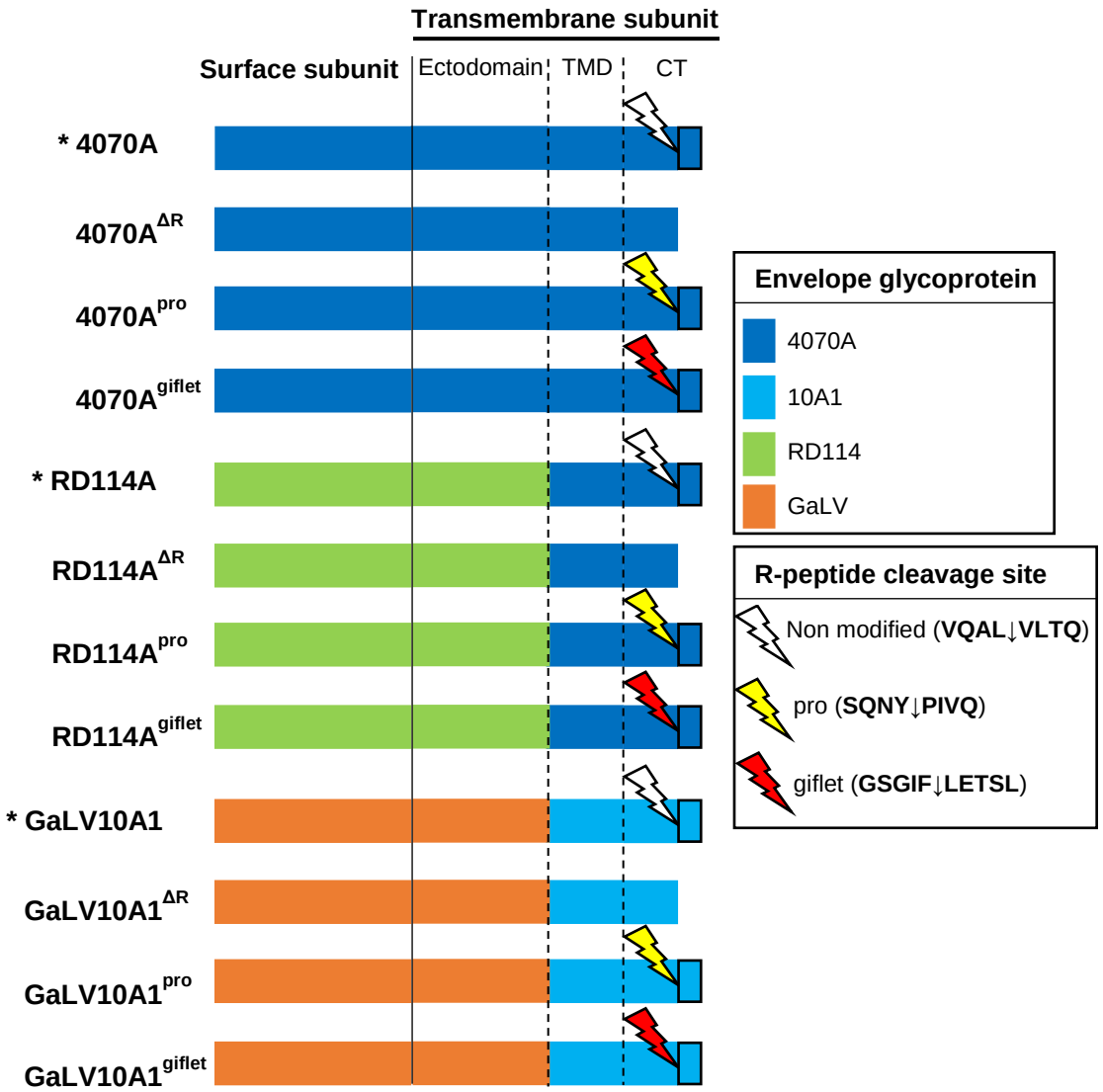

Figure 3.9 – Schematic representation of engineered envelope glycoproteins. On the top are represented the surface and transmembrane subunits and domains of the envelope glycoproteins: ectodomain, transmembrane domain (TMD) and cytoplasmic tail (CT). The blue, sky blue, green and orange bars represent sequences from 4070A, 10A1, RD114 and GaLV envelope glycoproteins, respectively. The square outlined in black is the R-peptide sequence. The white, yellow and red thunder-like shape represent the non modified, pro and giflet mutations on the protease cleavage site of the R-peptide, respectively. *: original envelope glycoproteins used to evaluate lentiviral vector production with T26S HIV-1 protease (**Figure 3.8**).

The engineered envelope glycoproteins were evaluated in transient production of lentiviral vector using T26S HIV-1 protease (**Figure 3.10**). WT HIV-1 protease was used as control. Due to the number of viral samples to be assessed, total particles were quantified by p24 ELISA. For titration of infectious particles, spin inoculation was performed.

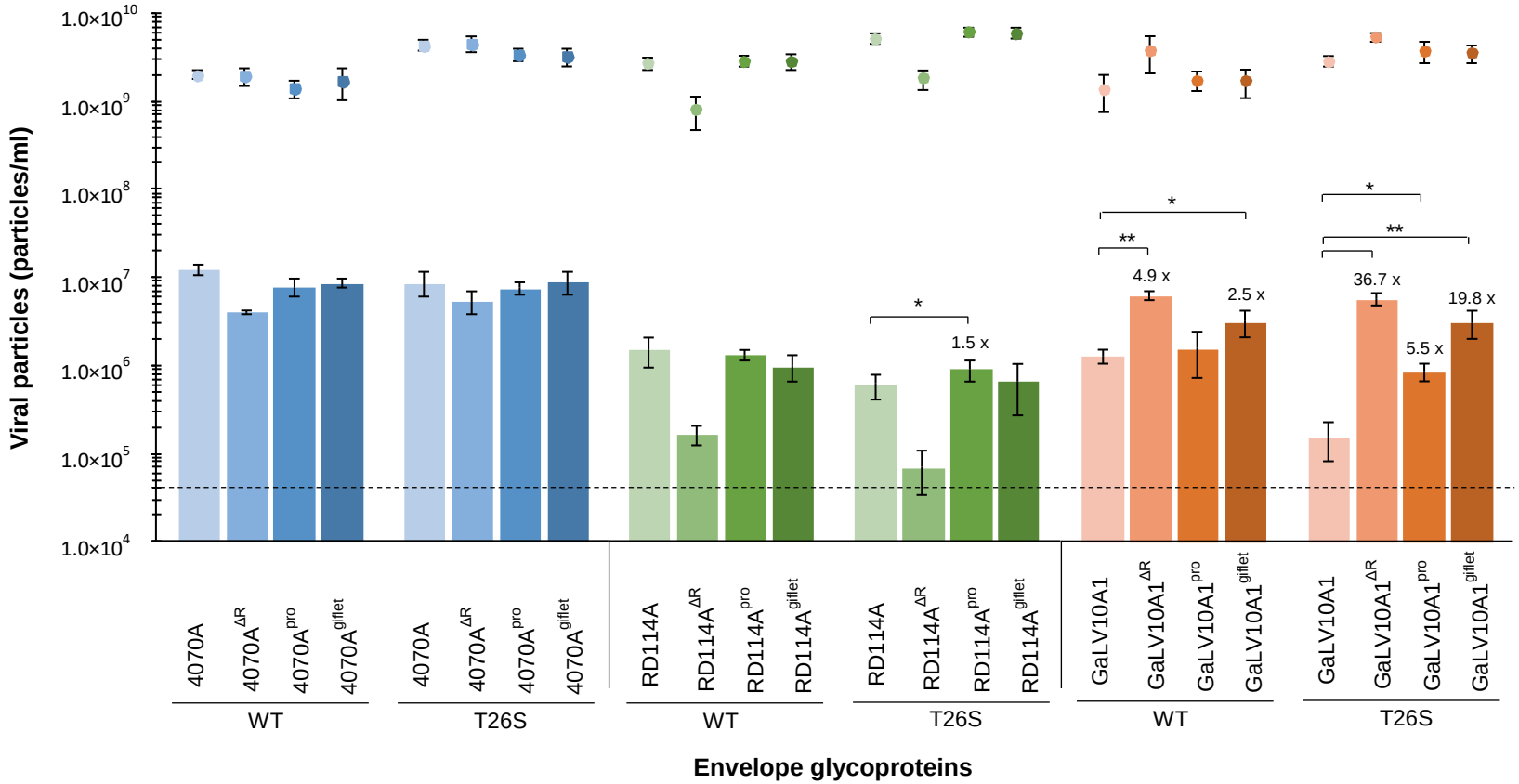

Figure 3.10 – Lentiviral vector production with T26S HIV-1 protease and engineered envelope glycoproteins. The bars correspond to infectious particles and the dots to total particles. The numbers on the top of the bars indicate fold increase of infectious titer relatively to the corresponding non modified envelope glycoprotein. All values are shown as average ± standard deviation of three biological replicates (n=3). Fold increase is shown for significant changes based on a one-tailed non-paired t-test, * $p<0.1$ and ** $p<0.01$. Detection limit of 4.0×10^4 I.P./ml is indicated by a dashed arrow. WT: wild-type HIV-1 protease; T26S: T26S HIV-1 protease.

Mutations in the R-peptide cleavage site were found not to increase infectious titers of lentiviral vectors pseudotyped with 4070A and RD114A, with the exception of the RD114A^{pro}. However, all mutations increased viral titers of GaLV10A1. Remarkably, ΔR mutation in GaLV10A1 envelope glycoprotein exhibited the highest viral titer (around 6 × 10^6 IP/ml), increasing the infectious particles yields by 36.7-fold. This effect was also visible with the WT HIV-1 protease, with a 4.9-fold improvement.

3.2.3. Effect of engineered envelope glycoproteins on producer cell

The results obtained with the engineered envelopes, particularly with GaLV10A1, encourage their use for the establishment of stable cell lines for lentiviral vector production. However, some of the engineered envelopes were expectably cytotoxic, namely those with the ΔR mutation, leading to the syncytium formation. Syncytium are multi-nucleate enlarged cells formed by fusion of an infected cells with neighboring cells. This event is induced by viral proteins expressed at cell surface that are directly fusogenic at the host cell membrane (**Figure 3.11**)

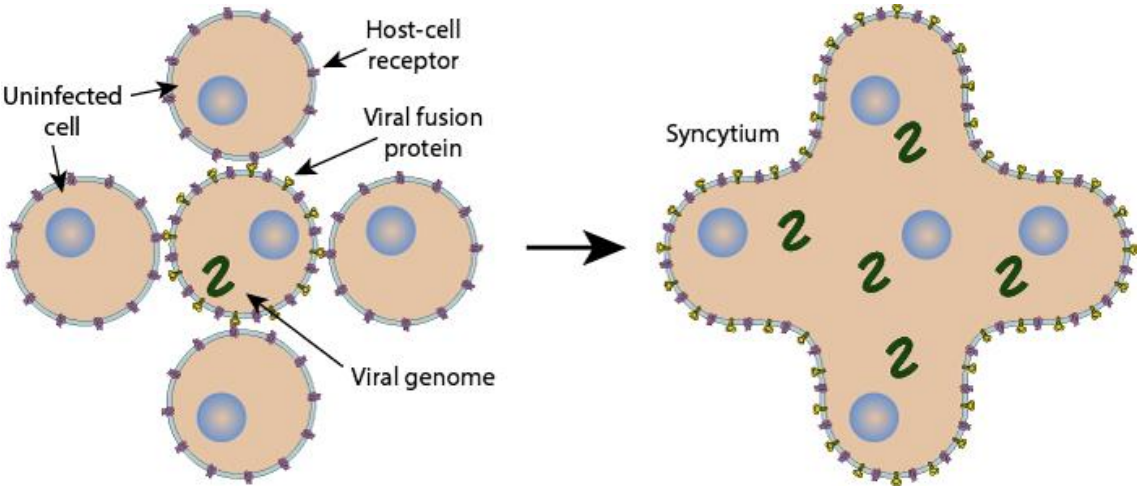

Figure 3.11 – Syncytium formation induced by surface expression of a viral fusogenic protein. Retrieved from ViralZone. http://viralzone.expasy.org/all_by_species/5957.html (Accessed: 30th June 2016).

To evaluate syncytium formation, cells were transfected with the plasmids coding for the engineered envelope glycoproteins. VSV-G and cells not expressing envelope glycoproteins were used as controls. 24 hours post transfection, cells were observed by phase-contrast microscopy (**Figure 3.12**).

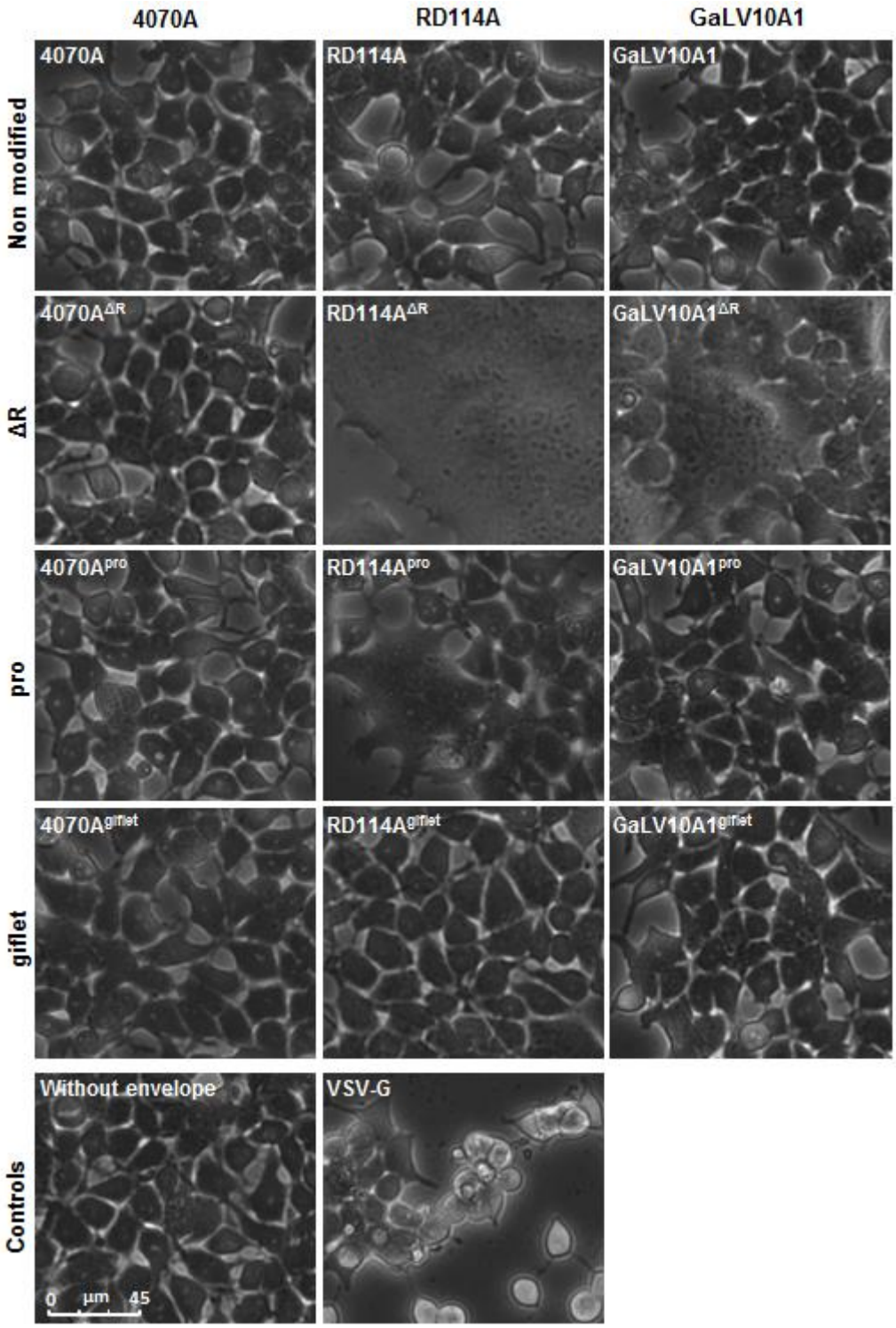

Figure 3.12 – Syncytium formation in HEK 293T cells expressing engineered envelope glycoproteins. Scale bar: 45 µm.

As expected, GaLV10A1$^{\Delta R}$ and RD114A$^{\Delta R}$ induced syncytium formation. Additionally, RD114A^{pro} expression also led to syncytium formation but less noteworthy. However, and contrarily to what could be expected, syncytium formation was not observed in cells expressing

4070A$^{\Delta R}$. Syncytium formation is mediated by the interaction of the envelope glycoproteins present at the cell membrane with neighboring cell's specific surface receptors, leading to cells fusion. Since syncytium formation was not observed in cells expressing 4070A$^{\Delta R}$, we hypothesized that the cell surface receptor for 4070A was not expressed or expressed at low levels. Thus, gene expression of cell surface receptors for 4070A, RD114A and GaLV10A1 – *SLC20A2, SLC1A5* and *SLC20A1*, respectively [71] – in HEK 293T cells was assessed by RT-qPCR (**Figure 3.13**).

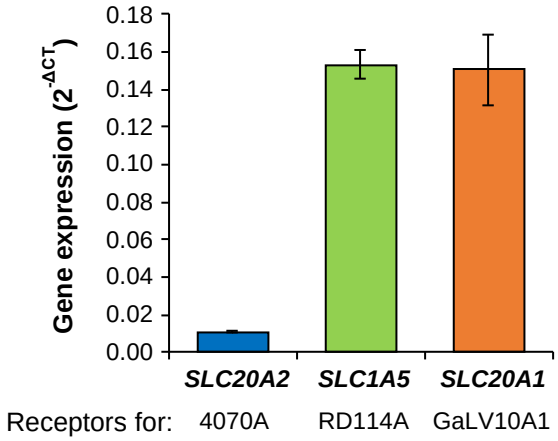

Figure 3.13 – Gene expression of cell surface receptors for envelope glycoproteins in HEK 293T cell line. SLC20A2, SLC1A5 and SLC20A1 are the cell surface receptors for 4070A, RD114A and GaLV10A1, respectively. Gene expression was quantified after normalization to a control gene (*RPL22*) using the $2^{-\Delta CT}$ method as defined in Materials and Methods. Gene expression levels are shown as average expression (relative to the control gene, *RPL22*) ± standard deviation of two technical replicates.

Indeed, cell surface receptors expression showed significantly lower values of *SLC20A2* compared to *SLC1A5* and *SLC20A1*. This results demonstrated that syncytium formation induced by an envelope glycoprotein is correlated with the expression of its specific cell surface receptor. More importantly, they open the door for the use of some of the newly engineered and high-titer envelope glycoproteins, namely GaLV10A1$^{\Delta R}$ in stable producer cell by receptor knock out strategy.

3.3. Establishment of stable producer cell lines for lentiviral vector production with T26S HIV-1 protease

HEK 293T cell line constitutively expressing HIV-1 *gag-pol* with T26S mutation and *rev*, developed by Hélio Tomás (ACT Unit IBET/ITQB NOVA), was used as cell substrate to establish stable cell lines for lentiviral vector production with T26S HIV-1 protease and engineered envelope glycoproteins. Cells were transfected with *pRRLSIN-mCherry-hPGK-GFP* and selected as described in the Materials and Methods section. At this moment, we have successfully established a stable cell line expressing all the necessary components for lentiviral vector production, with the exception of the envelope glycoprotein.

4. Discussion and Conclusions

The use of lentiviral vectors in gene therapy clinical trials, particularly those derived from HIV-1, has been growing due their unique ability to transduce and permanently modify non-dividing cells [21], a feature that distinguishes them from their simple counterparts, gammaretroviral vectors. Additionally, lentiviral vectors show a safer integration pattern, with reduced oncogenic risk compared to retroviral vectors [22], and a more efficiently transduction of hematopoietic stem cells [21,72]. These vectors offer a great promise for gene therapy, and are likely to overtake gammaretroviral vectors in clinical trials. To take advantage of the full potential of lentiviral vectors for gene therapy applications and aid a faster clinical to market transition, robust and standardized means for the production of these vectors are needed. In this context, the use of stable producer cell lines is of great value. However, the establishment of stable producer cell lines for lentiviral vector production has been hampered by the cytotoxicity of some viral components, namely the viral protease [54,55]. In this thesis, we aimed at minimizing the cytotoxicity induced by the viral components in lentiviral vector production. Thus, a point mutation in the active site of the HIV-1 protease was generated, changing amino acid 26 from a threonine to a serine (T26S) [63]. This mutation is reported to reduce proteolytic activity compared to the WT protease by 5- to 10-fold, and yield lower protease-mediated cytotoxicity without affecting virus maturation and infectivity. Therefore, we evaluated lentiviral vector production with T26S HIV-1 protease.

T26S HIV-1 protease showed similar Gag-Pro-Pol and Gag polypeptide proteolytic processing (**Figure 3.1**) and infectious titer yield (2×10^7 I.P./ml) similar to the WT HIV-1 protease in a transient production system with third generation lentiviral vector packaging (**Figure 3.2**). These results indicate that T26S HIV-1 protease proteolytic activity is sufficient to generate mature viral proteins, an essential step for viral infectivity[64]. However, a 2-fold decrease ratio of infectious particles *per* total particles was obtained with T26S HIV-1 protease comparing to the WT HIV-1 protease (**Figure 3.2**), decreasing the quality of LV preparation. This effect could be related to the putative reduced cytotoxicity of T26S HIV-1 protease that leads to an increment in the production of total particles (**Figure 3.3**), however, with less mature (infectious) particles due to the reduced proteolytic activity. This reduction in viral preparation quality was small and should be possible to improve by means of optimizing the stoichiometry of viral components for transient transfection production [73]. More importantly, we have demonstrated for the first time that T26S HIV-1 protease can be used to produce lentiviral vectors with similar titers to the WT HIV-1 protease in a transient transfection system, making it a great candidate for the establishment of a novel stable packaging cell line for continuous LV production.

To study the protease-mediated cytotoxicity, we hypothesized that cytotoxicity would be more evident in long term culture, being a stable cell line the best approach to express the HIV-1 protease and evaluate its cytotoxicity. Instead of a constitutive expression cassette to express the HIV-1 protease, an inducible expression cassette was chosen since stable expression could provide a proliferative advantage to cells with lower HIV-1 protease gene expression. This could bias a fair comparison between the WT HIV-1 protease and its reduced activity mutant T26S.

Therefore, a tetracycline inducible expression promoter (BiTet promoter) was used to control HIV-1 protease gene expression and zeocin selective marker was put under the control of a constitutive promoter (PGK) (**Figure 3.4**). With this system, HIV-1 protease is expressed only after addition of doxycycline in the culture medium (Tet-on), enabling the selection of cells transfected without its expression.

After induction with doxycycline, there was no differences in the cell growth profile between cells expressing WT, T26S or D25N HIV-1 proteases (**Figure 3.5**), providing low evidence of any cytotoxicity. However, HIV-1 protease activity could not be detected in protein extracts (data not shown) and gene expression values were found to be low (**Figure 3.6**). Additionally, a small 4-fold increase was shown for eGFP intensity after induction of gene expression with the constructed inducible expression cassette (**Figure 3.7**). These results indicate that the inducible expression cassette did not induce gene expression at sufficiently high levels to assess HIV-1 protease-mediated cytotoxicity. Moreover, the leaky expression observed for the inducible expression cassette (**Figure 3.7**) is an undesirable feature of the system for the study of cytotoxic proteins, since it could have given proliferative advantage to cells with low copy number of integrated cassettes or integrated into inactive heterochromatin. This effect would result in little or no gene expression controlled by the weak BiTet promoter, but it would be enough to express the *zeocin*^{R} under the control of the PGK promoter at necessary levels to confer antibiotic resistance during cell selection. In order to prevent this possible effect, a tighter control of gene expression is required. Therefore, optimization of the inducible expression cassette for HIV-1 proteases expression is ongoing by replacing the BiTet promoter with a stronger and tighter unidirectional doxycycline inducible Tet-On promoter, with the rtTA protein being provided *in trans*.

Despite the fact that T26S HIV-1 protease provided infectious titer yields similar to WT HIV-1 protease (**Figure 3.2**), the envelope glycoprotein used – VSV-G – was cytotoxic [56], making it impossible to use it to establish stable cell lines for continuous lentiviral vector production. To overcome this limitation, non-toxic *Gammaretrovirus* envelope glycoproteins, commonly used in retroviral vector stable cell line development [17], have been used to pseudotype lentiviral vectors [45–49,61,67]. In line, we evaluated transient LV production with the G*ammaretrovirus* envelope glycoproteins 4070A, RD114A and GaLV10A1 (indicated with * in **Figure 3.9**). However, these envelope glycoproteins require cleavage of the R-peptide from the cytoplasmic tail of TM subunit, catalyzed by the viral protease, to activate the fusogenic activity necessary for virus entry [67–70]. Due to the described reduced proteolytic activity [63], the conjugation of T26S HIV-1 protease with 4070A, RD114A and GaLV10A1 could result in reduced titers compared to the WT HIV-1 protease. This was in fact, observed (**Figure 3.8**), while for VSV-G, which does not require proteolytic processing for viral infectivity, reduced protease activity did not impact vector titers (**Figure 3.2**). Furthermore, the envelope glycoproteins used herein (4070A, RD114A and GaLV10A1) shared the same retroviral R-peptide cleavage site, which was hypothesized to be cleaved less efficiently by the HIV-1 protease, especially considering the putative reduced proteolytic activity of the T26S HIV-1 protease. Therefore, the retroviral cleavage site was

replaced by that of HIV MA/CA (pro mutation) or by a synthetic cleavage sequence (giflet mutation) efficiently cleaved by HIV-1 protease (**Figure 3.9**). As a control, the proteolytic processing required for fusogenic activity of *Gammaretrovirus* envelope glycoproteins was obliterated by removing the R-peptide from the cytoplasmic tail (ΔR mutation). This control allowed to establish the maximum titer that could be obtained with each of the envelope glycoproteins if no proteolytic processing was needed.

Engineering R-peptide cleavage site did not result in increased infectious titers with 4070A and RD114A, with the exception of a small 1.5-fold increase with RD114A^{pro} (**Figure 3.10**). Previous work has shown that the pro mutation enhances cleavage interactions between HIV-1 protease and the RD114 cytoplasmic tail during virion formation [47]. Thus, a higher fold increase was expected with the most efficiently cleaved sequence [66] – giflet mutation – than the pro mutation, which was not observed. RD114A^{pro} also induced unexpected syncytium formation in cells transfected with its encoding plasmid (**Figure 3.12**). A possible explanation for these results is that the pro mutation could have led to conformational changes in the TM subunit that modified its folding, making it susceptible to proteolytic processing by cellular proteases and, consequently, leading to syncytium formation and a minor increase in infectious titer yield. Nevertheless, considering the small fold increase for RD114A^{pro} and no increased titers for the rest of engineered R-peptide cleavage sites in RD114A and 4070A, the results indicate that proteolytic processing of these envelope glycoproteins was not hampering lentiviral vector production with T26S HIV-1 protease.

Regarding GaLV10A1, engineered R-peptide cleavage sites allowed rescuing infectious viral titers produced with T26S HIV-1 protease to similar levels of those obtained with the WT HIV-1 protease (**Figure 3.10**). Moreover, engineered R-peptide cleavage sites increased infectious particles production compared to the original retroviral cleavage sequence with both WT and T26S HIV-1 protease to similar values. This supports that insufficiently proteolytic processing of GaLV10A1 was hampering viral vector infectivity when using T26S HIV-1 protease. The highest infectious titer was achieved with GaLV10A1$^{\Delta R}$ (around 6×10^6 I.P./ml). These titers are very competitive considering those typically obtained with the highest titer envelope glycoprotein used in LV production, VSV-G. Although increasing infectious particles by 36.7-fold, GaLV10A1$^{\Delta R}$ is not suitable for the establishment of a continuous stable cell line for LV production, due to induced syncytium formation in HEK 293T cells expressing this envelope glycoprotein (**Figure 3.12**). Yet, it would be possible to develop a conditional packaging cell line, where an inducible promoter would control GaLV10A1$^{\Delta R}$ expression. Additionally, GaLV10A1$^{\Delta R}$ can be used in transient transfection production of lentiviral vectors replacing the commonly used pantropic VSV-G, offering a more specific tropism for *in vivo* gene therapy applications, especially to hematopoietic stem cells [50]. GaLV10A1^{giflet} was found to be the best candidate for stable cell line development, with a 19.8-fold increase for the T26S HIV-1 protease. GaLV10A1^{giflet} is also of interest for the development of packaging cell lines using WT HIV-1 protease, since a 2.5-fold increase was observed when compared to the non modified GaLV10A1. Nevertheless, better results are expected with packaging cell lines expressing T26S HIV-1 protease, since its putative

reduced cytotoxic activity would possibly allow constitutive HIV-1 *gag-pol* expression at a high level compared to WT HIV-1 protease.

Several studies have shown that deletion of the R-peptide from the cytoplasmic tail of *Gammaretrovirus* envelope glycoproteins induces syncytium formation [67,70]. Surprisingly, this event was not observed in HEK 293T cells expressing 4070A$^{\Delta R}$ envelope glycoprotein, unlike RD114A$^{\Delta R}$ and GaLV10A1$^{\Delta R}$ (**Figure 3.12**). Therefore, gene expression of cell surface receptors specific to 4070A, RD114A and GaLV10A1 envelope glycoprotein (*SLC20A2*, *SLC5A1* and *SLC20A1*, respectively) was assessed (**Figure 3.13**)**.** Gene expression quantification suggested that 4070A did not induce syncytium formation due to low expression of its cell surface receptor, *SLC20A2*. This observation strongly encourages the use of the high-titer GaLV10A1$^{\Delta R}$ for the establishment of a novel stable packaging cell line for continuous LV production using cell substrates with *SLC20A1* knock out. This could be achieved using genome editing tools, namely the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein Cas9 (CRISPR-Cas9) technology. Because of its simplicity and high efficiency, CRISPR-Cas9 has became the most commonly used tool for genome editing and has been proven possible to modify endogenous genes in a wide range of cells and organisms [74,75]. Therefore, this genome editing tool will be used in the follow-up of this work to knock out the cell surface receptor of GaLV10A1 in HEK 293T, in order to establish a novel stable packaging cell line for continuous LV production, using the newly engineered high-titer GaLV10A1$^{\Delta R}$ and the T26S HIV-1 protease.

The establishment of stable producer cell lines with T26S HIV-1 protease is ongoing. Currently, we have successfully developed stable cell lines expressing all the viral components required for lentiviral vector production, with the exception of the envelope glycoprotein. The highest mCherry (transgene) yielding clones population will be isolated and transfected with the plasmids encoding envelope glycoproteins, with the exception of those that induced syncytium formation. The resulting stable producer cells will allow the evaluation of lentiviral vector titers with the engineered envelope glycoproteins conjugated with the T26S HIV-1 protease in a stable continuous production approach. Considering the higher titer of 4070A compared to all envelope glycoproteins tested in transient production (**Figure 3.8 and 3.10**), the same result is expected in a stable production system. Yet, several studies have shown that GaLV envelope glycoproteins transduce more efficiently certain human cell types than 4070A [76–78], despite showing a lower titer. This observation is of particular interest for *in vivo* gene transfer applications.

In this work, the potential of T26S HIV-1 protease was evaluated and validated for lentiviral vector production. Additionally, engineered envelope glycoproteins were established which, combined with the possibility of genome editing of producer cells, open new possibilities for the establishment of continuous LV production systems. Moreover, lentiviral vectors pseudotyped with the engineered GaLV10A1 will offer a specific tropism to hematopoietic stem cells, which are attractive targets for lentiviral gene transfer *in vivo* gene therapy applications. Thus, this work contributes directly to the development of a novel packaging cell line for the constitutive production of lentiviral vectors for gene therapy, by evaluating for the first time the

use of a less toxic HIV-1 protease and by providing novel chimeric envelope glycoproteins providing increased titers.

5. Bibliography

1. Mountain, A. Gene therapy: The first decade. *Trends Biotechnol.* **18,** 119–28 (2000).
2. Edelstein, M. The Journal of Gene Medicine Clinical Trial Site. (2016). Available at: http://www.wiley.com/legacy/wileychi/genmed/clinical/. (Accessed: 12th May 2016)
3. Wirth, T., Parker, N. & Ylä-herttuala, S. History of gene therapy. *Gene* **525,** 162–9 (2013).
4. Visiogain. Gene Therapy R&D and Revenue Forecasts 2016-2026: Prospects for Drugs Treating Cancer, Cardiovascular Disorders, Eye Conditions, Rare Diseases and Other Needs. (2016).
5. Research and Markets*, Global Gene Therapy Market Forecast to 2020*. (2013).
6. Vannucci, L., Lai, M., Chiuppesi, F., Ceccherini-Nelli, L. & Pistello, M. Viral vectors: a look back and ahead on gene transfer technology. *New Microbiol.* **36,** 1–22 (2013).
7. Thomas, C. E., Ehrhardt, A. & Kay, M. A. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* **4,** 346–58 (2003).
8. Sheridan, C. Gene therapy finds its niche. *Nat. Biotechnol.* **29,** 121–8 (2011).
9. Pearson, S., Jia, H. & Kandachi, K. China approves first gene therapy. *Nat. Biotechnol.* **22,** 3–4 (2004).
10. Borysiewicz, L. K. *et al.* A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* **347,** 1523–7 (1996).
11. Walsh, S. R. & Dolin, R. Vaccinia viruses: vaccines against smallpox and vectors against infectious diseases and tumors. *Expert Rev. Vaccines* **10,** 1221–40 (2011).
12. Chayavichitsilp, P., Buckwalter, J. V., Krakowski, A. C. & Friedlander, S. F. Herpes simplex. *Pediatr. Rev.* **30,** 119–29 (2009).
13. Ledford, H. Cancer-fighting viruses win approval. *Nature* **526,** 622–3 (2015).
14. Flotte, T. R., Afione, S. A. & Zeitlin, P. L. Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am. J. Respir. Cell Mol. Biol.* **11,** 517–21 (1994).
15. Daya, S. & Berns, K. I. Gene therapy using adeno-associated virus vectors. *Clin. Microbiol. Rev.* **21,** 583–93 (2008).
16. Moran, N. First gene therapy nears landmark European market authorization. *Nat. Biotechnol.* **30,** 807–9 (2012).
17. Rodrigues, A. F., Alves, P. M. & Coroadinha, A. S. Production of Retroviral and Lentiviral Gene Therapy Vectors : Challenges in the Manufacturing of Lipid Enveloped Virus. *Viral Gene Therapy* (ed. Xu, K.), InTech (2011). Available at: http://www.intechopen.com/books/howtoreference/viral-gene-therapy/production-of-retroviral-and-lentiviral-gene-therapy-vectors-challenges-in-the-manufacturing-of-lipi . (Accessed: 30th June 2016)
18. Coroadinha, A. S. *et al.* Production of retroviral vectors: review. *Curr. Gene Ther.* **10,** 456–73 (2010).
19. Hacein-Bey-Abina, S. *et al.* A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **348,** 255–6 (2003).
20. Mullard, A. EMA greenlights second gene therapy. *Nat. Rev. Drug. Discov.* **15,** 299 (2016).
21. Naldini, L. *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–7 (1996).
22. Montini, E. *et al.* The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J. Clin. Invest.* **119,** 964–75 (2009).
23. Fauci, A. & Desroiers, R. Pathogenesis of HIV and SIV. *Retroviruses* (eds. Coffin, J. M., Hughes, S. H. & Varmus, H. E.). Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press (1997). Available at: http://www.ncbi.nlm.nih.gov/books/NBK19359/. (Accessed: 7th August 2016).
24. Centers for Disease Control. Pneumocystis Pneumonia - Los Angeles. *Morb. Mortal. Wkly. Rep.* **30,** 1-3 (1981).
25. Gottlieb, M. S. *et al.* Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N. Engl. J. Med.* **305,** 1425–31 (1981).
26. Barré-Sinoussi, F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220,** 868–71 (1983).
27. Gallo, R. C. *et al.* Isolation of human T-cell leukemia virus in acquired immune deficiency

syndrome (AIDS). *Science* **220,** 865–7 (1983).
28. International Committee on Taxonomy of Viruses (ICTV). *Virus Taxonomy* (2015). Available at: http://www.ictvonline.org/virustaxonomy.asp . (Accessed: 7th August 2016).
29. Coffin, J. M., Hughes, S. H. & Varmus, H. E., editors. The Place of Retroviruses in Biology. *Retroviruses*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press (1997). Available at: http://www.ncbi.nlm.nih.gov/books/NBK19382/ . (Accessed: 7th August 2016).
30. Goff, S. P. Retroviridae. *Fields Virology: Sixth Edition* **2** (eds. Knipe, D. M. & Howley, P. M.), Lippincott Williams & Wilkins, a Wolters Kluwer business, 1424–73 (2013).
31. Freed, E. O. & Martin, M. A. Human Immunodeficiency Viruses: Replication. *Fields Virology: Sixth Edition* **2** (eds. Knipe, D. M. & Howley, P. M.), Lippincott Williams & Wilkins, a Wolters Kluwer business, 1502-60 (2013).
32. Tomás, H. A., Rodrigues, A. F., Alves, P. M. & Coroadinha, A. S. Lentiviral Gene Therapy Vectors : Challenges and Future Directions. *Gene Therapy - Tools and Potential Applications* (ed. Martin, F.). InTech (2013). Available at: http://www.intechopen.com/books/gene-therapy-tools-and-potential-applications/lentiviral-gene-therapy-vectors-challenges-and-future-directions . (Accessed 25th September 2015)
33. Frankel, A. D. & Young, J. A. T. HIV-1 : Fifteen Proteins and an RNA. *Annu. Rev. Biochem.* **67,** 1–25 (1998).
34. Jones, K. A. & Peterlin, M. B. Control of RNA Initiation and Elongation at the HIV-1 Promoter. *Annu. Rev. Biochem.* **63,** 717–43 (1994).
35. Pollard, V. W. & Malim, M. H. The HIV-1 Rev protein. *Annu. Rev. Microbiol.* **52,** 491–532 (1998).
36. Lever, A., Gottlinger, H., Haseltine, W. & Sodroski, J. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63,** 4085–7 (1989).
37. Barré-Sinoussi, F., Ross, A. L. & Delfraissy, J. F. Past, present and future: 30 years of HIV research. *Nat. Rev. Microbiol.* **11,** 877–83 (2013).
38. Picanço-Castro, V., Russo-Carbolante, E. M. S. & Covas, D. T. Advances in lentiviral vectors: A patent review. *Recent Pat. DNA Gene Seq.* **6,** 82–90 (2012).
39. Pauwels, K. *et al.* State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. *Curr. Gene Ther.* **9,** 459–74 (2009).
40. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. & Trono, D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* **15,** 871–5 (1997).
41. Dull, T. *et al.* A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72,** 8463–71 (1998).
42. Mátrai, J., Chuah, M. K. L. & VandenDriessche, T. Recent advances in lentiviral vector development and applications. *Mol. Ther.* **18,** 477–90 (2010).
43. Felder III, J. M. & Sutton, R. E. Lentiviral Vectors. *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies - Third Edition* (ed. Templeton, N. S.), Taylor & Francis Group, 207-43 (2009).
44. DePolo, N. J. *et al.* VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Mol. Ther.* **2,** 218–22 (2000).
45. Sandrin, V. *et al.* Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood* **100,** 823–32 (2002).
46. Zhang, X. Y., La Russa, V. F. & Reiser, J. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. *J. Virol.* **78,** 1219–29 (2004).
47. Ikeda, Y. *et al.* Continuous high-titer HIV-1 vector production. *Nat. Biotechnol.* **21,** 569–72 (2003).
48. Bell, A. J., Fegen, D., Ward, M. & Bank, A. RD114 envelope proteins provide an effective and versatile approach to pseudotype lentiviral vectors. *Exp. Biol. Med. (Maywood)* **235,** 1269–76 (2010).
49. Stitz, J. *et al.* Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* **273,** 16–20 (2000).
50. Cronin, J., Zhang, X. Y. & Reiser, J. Altering the tropism of lentiviral vectors through pseudotyping. *Curr. Gene Ther.* **5,** 387–98 (2005).

51. Schweizer, M. & Merten, O. W. Large-scale production means for the manufacturing of lentiviral vectors. *Curr. Gene Ther.* **10,** 474–86 (2010).
52. Merten, O. W., Hebben, M. & Bovolenta, C. Production of lentiviral vectors. *Mol. Ther. Methods Clin. Dev.* **3,** 16017 (2016).
53. Gougeon, M. L. Apoptosis as an HIV strategy to escape immune attack. *Nat. Rev. Immunol.* **3,** 392–404 (2003).
54. Nie, Z. *et al.* HIV-1 protease processes procaspase 8 to cause mitochondrial release of cytochrome c, caspase cleavage and nuclear fragmentation. *Cell Death Differ.* **9,** 1172–84 (2002).
55. Nie, Z. *et al.* Human immunodeficiency virus type 1 protease cleaves procaspase 8 in vivo. *J. Virol.* **81,** 6947–56 (2007).
56. Hoffmann, M. *et al.* Fusion-active glycoprotein G mediates the cytotoxicity of vesicular stomatitis virus M mutants lacking host shut-off activity. *J. Gen. Virol.* **91,** 2782–93 (2010).
57. Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5547–51 (1992).
58. Sanber, K. S. *et al.* Construction of stable packaging cell lines for clinical lentiviral vector production. *Sci. Rep.* **5,** 9021 (2015).
59. Stornaiuolo, A. *et al.* RD2-MolPack-Chim3, a packaging cell line for stable production of lentiviral vectors for anti-HIV gene therapy. *Hum. Gene Ther. Methods* **24,** 228–40 (2013).
60. Marin, V. *et al.* RD-MolPack technology for the constitutive production of self-inactivating lentiviral vectors pseudotyped with the nontoxic RD114-TR envelope. *Mol. Ther. Methods Clin. Dev.* **3,** 16033 (2016).
61. Strang, B. L., Ikeda, Y., Cosset, F.-L., Collins, M. K. L. & Takeuchi, Y. Characterization of HIV-1 vectors with gammaretrovirus envelope glycoproteins produced from stable packaging cells. *Gene Ther.* **11,** 591–8 (2004).
62. Coroadinha, A. S. *et al.* The use of recombinase mediated cassette exchange in retroviral vector producer cell lines: predictability and efficiency by transgene exchange. *J. Biotechnol.* **124,** 457–68 (2006).
63. Konvalinka, J. *et al.* An active-site mutation in the human immunodeficiency virus type 1 proteinase (PR) causes reduced PR activity and loss of PR-mediated cytotoxicity without apparent effect on virus maturation and infectivity. *J. Virol.* **69,** 7180–6 (1995).
64. Kohl, N. E. *et al.* Active human immunodeficiency virus protease is required for viral infectivity. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4686–90 (1988).
65. Tomás, H. Desenvolvimento de linhas celulares produtoras de vectores retrovirais para terapia génica. Universidade Nova de Lisboa (2010). Available at: https://run.unl.pt/bitstream/10362/10576/1/Tomas_2010.pdf . (Accessed: 15th May 2016)
66. Beck, Z. Q., Hervio, L., Dawson, P. E., Elder, J. H. & Madison, E. L. Identification of efficiently cleaved substrates for HIV-1 protease using a phage display library and use in inhibitor development. *Virology* **274,** 391–401 (2000).
67. Christodoulopoulos, I. & Cannon, P. M. Sequences in the Cytoplasmic Tail of the Gibbon Ape Leukemia Virus Envelope Protein That Prevent Its Incorporation into Lentivirus Vectors. *J. Virol.* ***75*,** 4129-38 (2001)
68. Tedbury, P. R. & Freed, E. O. The cytoplasmic tail of retroviral envelope glycoproteins. *Prog. Mol. Biol. Transl. Sci.* **129,** 253-84 (2015).
69. Bouard, D. *et al.* An acidic cluster of the cytoplasmic tail of the RD114 virus glycoprotein controls assembly of retroviral envelopes. *Traffic.* **8,** 835–47 (2007).
70. Rein, A., Mirro, J., Haynes, J. G., Ernst, S. M. & Nagashima, K. Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. *J. Virol.* **68,** 1773–81 (1994).
71. Overbaugh, J., Miller, A. D. & Eiden, M. V. Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycophosphatidylinositol-anchored and secreted proteins. *Microbiol. Mol. Biol. Rev.* **65,** 371–89 (2001).
72. Naldini, L. Medicine. A comeback for gene therapy. *Science* **326,** 805–6 (2009).
73. Carrondo, M. J. T., Merten, O. W., Haury, M., Alves, P. M. & Coroadinha, A. S. Impact of retroviral vector components stoichiometry on packaging cell lines: effects on productivity and vector quality. *Hum. Gene Ther.* **19,** 199–210 (2008).
74. Ma, Y., Zhang, L. & Huang, X. Genome modification by CRISPR/Cas9. *FEBS J.* **281,** 5186–93 (2014).

75. Wiles, M. V., Qin, W., Cheng, A. W. & Wang, H. CRISPR–Cas9-mediated genome editing and guide RNA design. *Mamm. Genome* **26,** 501–10 (2015).
76. von Kalle, C. *et al.* Increased gene transfer into human hematopoietic progenitor cells by extended in vitro exposure to a pseudotyped retroviral vector. *Blood* **84,** 2890–7 (1994).
77. Chuah, M. K., Brems, H., Vanslembrouck, V., Collen, D. & Vandendriessche, T. Bone marrow stromal cells as targets for gene therapy of hemophilia A. *Hum Gene Ther.* **9,** 353–65 (1998).
78. Lam, J. S., Reeves, M. E., Cowherd, R., Rosenberg, S. A. & Hwu, P. Improved gene transfer into human lymphocytes using retroviruses with the gibbon ape leukemia virus envelope. *Hum Gene Ther.* **7,** 1415–22 (1996).

Annexes

Table A.1 – Primers and templates for plasmids construction.

Construct	Insert			Vector	
	Fragment	Source	Primers or Restriction Enzymes	Parental	Primers or Restriction Enzymes
pCMV-4070A	*4070a*	*pMonoZeo-4070A*	F - TTTTGGCAAAGAATTCATGGCGCGTTCAACG	*phGaLV10A1*	*EcoRI* *KasI*
			R - GTCAACACTAGGCGCCTATGGCTCGTACTC		
pCMV-RD114A	*RD114a*	*pLTR-RD114A*	F - TTTTGGCAAAGAATTCATGAAACTCCCAACA		
			R - GTCAACACTAGGCGCCTCATGGCTCGTACTC		
pCMV-GaLV10A1	-	-	-		F - TTTGGCAAAGAATTCATGGTATTGCTGCCTGG
					R - GAATTCTTTGCCAAAATGATG
pCMV-4070A$^{\Delta R}$	-	-	-	*pCMV-4070A*	F - GTGGTCCAGGCTCTATAGGCGCCTAGTGTTGACAATTAATC
					R - TAGAGCCTGGACCACTGATATCC
pCMV-4070A^{pro}	-	-	-		F - CAAAATTACCCTATAGTGCAACAATATCACCAGCTGAAGC
					R - TATAGGGTAATTTTGGCTCACTGATATCCTGTCTTTAACAAATTG
pCMV-4070A^{giflet}	-	-	-		F_1 - CTGGAAACCAGCCTGCAATATCACCAGCTGAAGCC
					R_1 - CAGGCTGGTTTCCAGAGCCTGGACCACTGATATCC
					F_2 - GGCAGCGGCATCTTCCTGGAAACCAGCCTGCAATATC
					R_2 - GAAGATGCCGCTGCCCACTGATATCCTGTCTTTAACAAATTG
pCMV-RD114A$^{\Delta R}$	-	-	-	*pCMV-RD114A*	F - GTGGTCCAGGCTCTGTGAGGCGCCTAGTGTTGAC
					R - CAGAGCCTGGACCACTGAG
pCMV-RD114A^{pro}	-	-	-		F - CAAAATTACCCTATAGTGCAGCAATATCACCAGCTAAAAC
					R - TATAGGGTAATTTTGGCTCACTGAGATCCTGTCTTTAAC
pCMV-RD114A^{giflet}	-	-	-		F_1 - CTGGAAACCAGCCTGCAATATCACCAGCTAAAACCC
					R_1 - CAGGCTGGTTTCCAGAGCCTGGACCACTGAGATC
					F_2 - GGCAGCGGCATCTTCCTGGAAACCAGCCTGCAATATC
					R_2 - GAAGATGCCGCTGCCCACTGAGATCCTGTCTTTAACAAATTG
pCMV-GaLV10A1$^{\Delta R}$	-	-	-	*pCMV-GaLV10A1*	F - GTAGTCCAGGCTTTATAGGGCGCCTAGTGTTGAC
					R - TAAAGCCTGGACTACTGAGATCC
pCMV-GaLV10A1^{pro}	-	-	-		F - CAAAATTACCCTATAGTGCAACAATACCACCAGCTAAAGC
					R - TATAGGGTAATTTTGGCTTACTGAGATCCTGTCTTTAACAAATTG
pCMV-GaLV10A1^{giflet}	-	-	-		F_1 - CTGGAAACCAGCCTGCAATACCACCAGCTAAAGCC
					R_1 - CAGGCTGGTTTCCAGAGCCTGGACTACTGAGATCC
					F_2 - GGCAGCGGCATCTTCCTGGAAACCAGCCTGCAATACC
					R_2 - GAAGATGCCGCTGCCTACTGAGATCCTGTCTTTAACAAATTGAAC

Table A.1 – Primers and templates for plasmids construction (continued).

Construct	Insert			Vector	
	Fragment	Source	Primers or Restriction Enzymes	Parental	Primers or Restriction Enzymes
pTet-GFP	*egfp*	*pRRLSIN.cPPT.PGK-GFP.WPRE*	F - TTTGGCAAAGAATTCATGGTGAGCAAGGGC	*phGaLV10A1*	F_1 - GAATTCTAGTGTTGACAATTA
			R - TCAACACTAGAATTCTTACTTGTACAGCTC		R_1 - GAATTCTTTGCCAAAATG
	PGK promoter		F - AAGTAAGAATTCTAGCCACGGGGTTGGGGT		F_2 - CTCACTATAGGAGGGCC
			R - CCCTCCTATAGTGAGCCTGGGGAGAGAGGT		R_2 - CTAGAATTCTTACTTGTACAGC
	BiTet Promoter	*pTargLox-polyATetVSV-G-FerHprom*	*BamHI*		F_3 - GAGTTCGAATGCTAGATTTTGGCAAAGAATTCATGG
					R_3 - AACTAAGTAAGGATCCTAGGTGCACTCTCAGTACAATC
pTet-HIV1 PR^{WT}/PGK-Zeo	*HIV-1 PR*	*pMDLg/pRRE*	F - TGGTTTAAACGAATTCATGCCTCAGATCACTCTTTGGCAGC R - CCCGTGGCTAGAATTCTTATTAAAAATTTAAAGTGCAGCCAATCTGAGTC	*pTet-GFP*	*EcoRI*
pTet-HIV1 PR^{T26S}/PGK-Zeo	*HIV-1 PR^{T26S}*	*pMDLg/pRRE^{T26s}*			
pTet-HIV1 PR^{D25N}/PGK-Zeo	*HIV-1 PR^{D25N}*	*pMDLg/pRRE^{D25N}*			

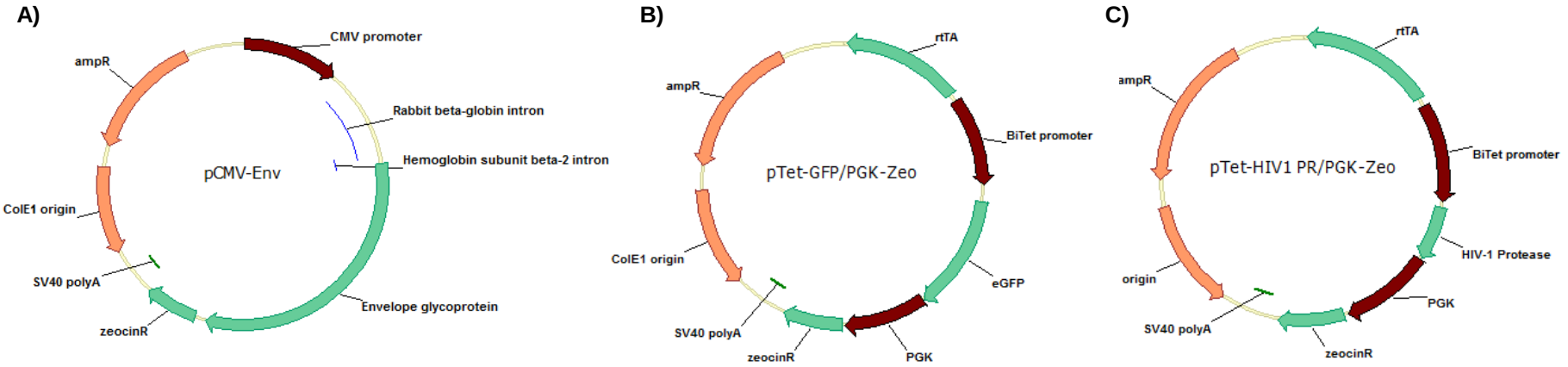

Figure A.1 – Plasmids constructs. A) Plasmid used to encode several envelope glycoproteins. **B)** Plasmid with inducible expression cassette encoding eGFP. **C)** Plasmid with inducible expression cassette encoding HIV-1 protease (WT, T26S and D25N).

Table A.2 – Primers for RT-qPCR.

Gene	Primers (5' → 3' sequence)
SLC20A2	F - CCCCAGAGGACAGTGAGAAG R - GGAAATGGAACAGGAGGTGA
SLC1A5	F - TACATTCTGTGCTGCCTGCT R - ATGAAACGGCTGATGTGCTT
SLC20A1	F - GGCAGAGATGGGTCTAGGTG R - TTGGTGTTGCCACTTTTGAA
HIV-1 protease	F - TTTGCCAGGAAGATGGAAAC R - TGCAGCCAATCTGAGTCAAC
RPL22	F - CTGCCAATTTTGAGCAGTTT R - CTTTGCTGTTAGCAACTACGC

Table A.3 – Crosing thresholds (CT) obtained from the RT-qPCR of the inducible expression cassette for HIV-1 proteases.

HIV-1 protease	**WT**		**T26S**		**D25N**	
Induction	-	+	-	+	-	+
CT*	28.61	23.90	26.66	24.78	29.05	25.45
RPL22* CT	19.84	19.97	20.13	20.00	19.94	20.12

* Average CT of 2 technical replicates