Enhancement of lentiviral vector production through alteration of virus-cell interactions

Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Jean-François Gélinas

Gene Medicine Research Group

Radcliffe Department of Medicine

and

Keble College

University of Oxford

Supervisors: Prof Deborah R. Gill and Prof Stephen C. Hyde

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Abstract

Gene therapy is the introduction or alteration of genetic material with the intention to treat disease. To support this aim, viruses have been modified, with elements linked to viral pathogenicity removed from their genome and replaced by the genetic material to be delivered. Gene therapy vectors based on lentiviruses have many advantages, such as the ability to transduce non-dividing cells and to target specific cell types via pseudotyping. They have been successfully used in *ex vivo* clinical trials for several haematopoietic stem cell disorders. Lentiviral vectors, however, suffer from substantially lower titres than the more popular adeno-associated virus (AAV)-based vectors and therefore have limited applicability for *in vivo* gene therapy which requires much greater quantities of virus.

The main aim of this thesis was to investigate strategies to improve lentiviral vector productivity during manufacture, in order to increase the likelihood of lentiviruses being adopted for disease treatment.

Initial experiments were based on the lentiviral vector manufacturing process currently being developed by the United Kingdom Cystic Fibrosis Gene Therapy Consortium for the generation of highly concentrated, purified lentivirus for clinical use.

Supplementation of FreeStyle 293 Expression Medium used during upstream processing was attempted, but none of the assessed supplements led to significant increases in lentiviral vector production. Investigation into intrinsic immunity to viral infection indicated that over-expression of the protein kinase RNA-activated (PKR) led to lower production titres, but over-expression of its inhibitors was not successful at increasing titres. The focus then shifted to reducing, or 'knocking-down', inhibitory factors present in the host cells, which could adversely affect viral titres. Investigation of the published HIV-1 literature revealed a possible 152 candidate inhibitory factors described as having a negative impact on HIV-1 replication in the late stages of the life cycle of the virus. A novel siRNA screen was developed to assess the effect of 'knock-down' of inhibitory factors on lentiviral vector titre. Application of the screen to 89 candidate inhibitory factors identified nine genes which, when knocked-down, resulted in increased lentiviral vector production by more than 40%.

Further work will be necessary to understand the role of the inhibitory factors in lentiviral vector production, but novel cell lines in which genes encoding these factors have been permanently deleted from producer cells could lead to higher titres, reducing costs in the manufacture of lentiviral vectors and making *in vivo* gene therapy more feasible from a health economics perspective.

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented herein were obtained by myself and all sources of published or unpublished information have been specifically acknowledged by means of a reference or personal communication notation, respectively.

List of Abbreviations

2-AP	2-aminopurine
293SF-LVP	HEK 293SF-PacLV #29-6-14 cell line
293SF-Titr	HEK 293SF-CymR-rtTA2s-M2 cell line
AAV	adeno-associated virus
ADA-SCID	adenosine deaminase deficiency form of SCID
ADAR1	adenosine deaminase, RNA-specific 1 (encoded by the gene *ADAR*)
ADAR1-p150	IFN-inducible full-length cytoplasmic 150 kDa ADAR1 protein
ADAR1-p150DCat	ADAR1-p150 without its catalytic domain
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	analysis of variance
bp	base pairs
BSA	Bovine Serum Albumin
CAG	modified chicken β-actin promoter with human cytomegalovirus immediate early enhancer
CAR	Chimeric Antigen Receptor
Cas9	CRISPR-associated protein 9
CCR5	CC-chemokine receptor 5
CDLC	Chemically Defined Lipid
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
cGMP	current Good Manufacturing Practice
CLC	Cholesterol Lipid Concentrate
CpG	cytidine-guanosine dinucleotide
CMV	human cytomegalovirus immediate early enhancer and promoter
CMV-	CMV without intron
cPPT	central polypurine tract
CRISPR	clustered regularly interspaced short palindromic repeats
CuO	cumate operator
CXCR4	CXC-chemokine receptor 4
CymR	cumate repressor
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DPBS	Dulbecco's phosphate-buffered saline
dsRBD	dsRNA-binding domain
dsRBP	dsRNA-binding protein
dsRNA	double-stranded RNA
EGFP	enhanced green fluorescent protein
EGFPLux	EGFP Lux fusion protein
EIAV	equine infectious anaemia virus
EIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2
eIF2α	alpha subunit of the translation eukaryotic initiation factor 2
ELISA	enzyme-linked immunosorbent assay
Env	envelope glycoprotein of HIV
F/HN	Sendai virus fusion and haemagglutinin-neuraminidase
FIV	feline immunodeficiency virus
FreeStyle 293	FreeStyle 293 Expression Medium

FTU	flow cytometry transducing unit
GFP	green fluorescent protein
hCEF	CpG-free CMV/elongation factor 1 alpha
HEK 293	Human Embryonic Kidney 293 cell line
HIV	human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
IFN	interferon
IRES	internal ribosome entry site
ISG	IFN-stimulated genes
ISG15	IFN-stimulated gene 15
LB	lysogeny broth
lncRNA	long non-coding RNA
LTR	long terminal repeat
Lux	firefly luciferase
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
mβCD	methyl-beta-cyclodextrin
NAC	N-Acetyl-L-Cysteine
NK	natural killer
P	phosphorylated
p24	HIV-1 capsid
pA	polyadenylation
PACT	PKR Activator (encoded by the gene *PRKRA*)
PBS	primer binding site
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
pGM169/GL67A	CFTR-encoding plasmid DNA complexed with a cationic liposome
PEI	polyethylenimine
PERT	product-enhanced reverse transcriptase
PFA	paraformaldehyde
P:I	particle to infectivity ratio
PKR	IFN-induced dsRNA-activated protein kinase (encoded by the gene *EIF2AK2*)
PLK1	polo-like kinase 1
polybrene	hexadimethrine bromide
ppm	particle per million
PPT	polypurine tract
Ptet-LTR	tetracycline-regulated LTR
rAAV	recombinant adeno-associated virus
rcf	relative centrifugal force
rHIV	recombinant HIV
RISC	RNA-induced silencing complex
RLU	relative light unit
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RRE	Rev response element
RSV	Rous sarcoma virus promoter
RT	reverse transcriptase

RT-qPCR	RT-quantitative PCR
rtTA2^{S}-M2	reverse tetracycline transactivator
SCID	severe combined immunodeficiency
SCID-X1	X-linked SCID
SD	standard deviation
SEM	standard error of the mean
shRNA	short hairpin RNA
SIN	self-inactivating
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SIV_{mac}	SIV of macaques
SV40	simian virus 40
TAR	trans-activation response element
Tet-On	inducible tetracycline-regulated gene expression system
TR5	tetracycline-regulated promoter
TRBP1	TAR RNA binding protein 1 (encoded by the gene *TARBP2*)
tRNA	transfer RNA
TSG101	tumour susceptibility 101
UK	United Kingdom
UKCFGTC	UK CF Gene Therapy Consortium
USA	United States of America
VAI RNA	adenovirus virus-associated RNA I
VSV	vesicular stomatitis virus
VSV-g	VSV G glycoprotein
v/v	volume/volume
Wnt	wingless/int
WPRE	posttranscriptional regulatory element of woodchuck hepatitis virus
w/v	weight/volume
ΔU3	deletion of the U3 region
ψ	packaging signal

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Chapter 1 – Introduction

1.1 Gene therapy

Gene Therapy is the introduction or alteration of genetic material with the intention to treat disease. Applications include the treatment of monogenic disorders, but also blocking cell proliferation in cancer with cell cycle regulatory proteins, secretion of growth factors, cytokines and antibodies, and expression of a novel antigen receptor on T cells to retarget them towards tumour or viral antigens (Reviewed in Giacca, 2010). Although a relatively new field, within a few decades, gene therapy has now progressed from a laboratory concept to clinical application (Reviewed in Wirth et al., 2013).

Several gene therapy products have been approved for clinical use. The first was the adenovirus-based Gendicine, manufactured by Shenzhen SiBiono GeneTech, in China for the treatment of head and neck cancer in 2003 (Pearson et al., 2004; Peng, 2005). This was followed in 2005 by an adenovirus-based oncolytic virus, Oncorine, manufactured by Shanghai Sunway Biotech, also approved in China for the treatment of head and neck cancer (Garber, 2006). In 2012, Glybera, a recombinant adeno-associated-virus (rAAV) vector manufactured by UniQure, was licenced in Europe for severe lipoprotein lipase deficiency (Yla-Herttuala, 2012). In 2016, Stimvelis, a retroviral vector brought forward by GSK received European marketing authorisation for the *ex vivo* treatment of the adenosine deaminase deficiency form of severe combined immunodeficiency (SCID) (ADA-SCID) (Schimmer & Breazzano, 2016).

1.1.1 Non-viral gene transfer agents

These products utilise only a few of the many approaches that have been developed to deliver therapeutic genetic information to target cells. A fundamental obstacle to successful gene transfer is delivery through the cell membrane and one approach to solve this particular problem is to induce transient damage in the cell membrane allowing the passage of the external genetic material. This can be achieved through physical approaches such as electroporation, magnetofection and sonoporation (Reviewed in Mehier-Humbert & Guy, 2005). Additional extracellular barriers such as glycoproteins and mucus layers exist in some tissues, which can prevent the genetic material from reaching its destination (Reviewed in Jones et al., 2013; Kim et al., 2016). Once inside the cells, barriers include nucleases, escape from endosomal compartments, inefficient cytosolic transport or nuclear localisation (Reviewed in Wiethoff & Middaugh, 2003).

The hydrophilic nature and large size of naked deoxyribonucleic acid (DNA), such as plasmid DNA molecules, often requires it to be combined with natural or synthetic carriers for protection and increased efficiency of delivery. These include combination with peptides, cationic lipids and polymers (Reviewed in Midoux et al., 2009). These complexes can enter the cell through a variety of cellular processes such as endocytosis, phagocytosis and pinocytosis.

Safety is a primary concern in gene therapy. In the beginning of gene therapy experimentation, there were a number of complications and concerns (Reviewed in Sheridan, 2011). Problems caused by gene transfer vectors include: toxicity, immune response against the vector or transduced cells, insertional mutagenesis and contamination of the germ line. Non-viral gene delivery has many advantages such as

a relative stability for pharmaceutical formulation and a reduced risk of toxicity, immunogenicity or oncogenicity compared to viral vectors (Reviewed in Yin et al., 2014). Furthermore, non-viral vectors depleted of unmethylated cytosine-guanosine dinucleotides (CpG) (Yew et al., 2002; Hyde et al., 2008) lead to longer expression (Bazzani et al., 2016) and can be repeatedly administered without generation of a limiting immune response (Hyde et al., 2000), which is the case with vectors containing viral proteins. They also allow large-scale production at a low cost. Finally, plasmid DNA vectors have little restriction on the capacity for incorporating genetic material. This allows for larger transgenes as well as expression regulators to be included.

However, the main issue with non-viral vectors is the duration of transgene expression. If the plasmid does not replicate or integrate into the genome and remains episomal, successive cell divisions will lead to the loss of plasmid in daughter cells. Furthermore, transgene expression from an episomal form can become silenced even in terminally differentiated cells (Löser et al., 1998). Hence, in part because non-viral vectors suffer from lower efficiency than viral vectors, as of August 2016, 69.1% of gene therapy clinical trials have used viral vectors (Ginn et al., 2013; Journal of Gene Medicine, 2016).

1.1.2 Viral gene transfer agents

The use of viral vectors for gene delivery capitalises on the co-evolution of viruses and mammalian host cells (Reviewed in Villarreal, 2005) which naturally makes them efficient gene transfer agents. Generally, elements linked to viral pathogenicity are removed from the virus genome and replaced by the transgene of interest. The genome still, nonetheless, contains the signals needed *in cis* for vector packaging and critical steps that occur in the target cell.

Viruses' life cycles being fully integrated into the cells' own life cycle; viral vectors are essentially 'manufactured' in cells. For safety reasons, the replicative phase of the viral life cycle, where viral particles are produced, must be limited to a single round in *ex vivo* producer cells. One notable exception to this is oncolytic viruses where viral replication, and therefore cell lysis, is necessary for the therapeutic effect. Viral vector production is typically achieved in cultured mammalian cells where the missing viral genes, allowing virus assembly and release, are supplied *in trans* by transient transfection, using a packaging cell line or provided by a helper virus. This separation renders the resultant vector incapable of making the proteins required for replication and, consequently, precludes the production of replication-competent virus, which can contaminate vector stocks. Therefore, except for oncolytic viruses, the infectious phase, from virus entry to genetic material transfer, is strictly non-replicative in order not to cause pathologies.

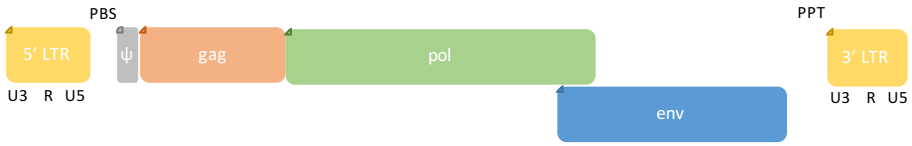
A number of naturally occurring viruses have been adapted as viral vectors for gene therapy (Reviewed in Giacca & Zacchigna, 2012). Intrinsic properties make some vectors more appropriate for certain disease targets than others. For example, the vectors' transgene capacity is important. Some vectors are simply too small and will not accommodate larger transgenes. Furthermore, the length of expression, whether transient or long-term, is affected by the choice of vector. The main viral vectors used for gene therapy are based on adenoviruses, adeno-associated-virus (AAV), as well as retroviruses (including lentiviruses) which are reviewed here. Other viruses frequently used as vectors include herpesviruses, poxviruses and the vaccinia virus (Journal of Gene Medicine, 2016).

1.2 Retroviruses

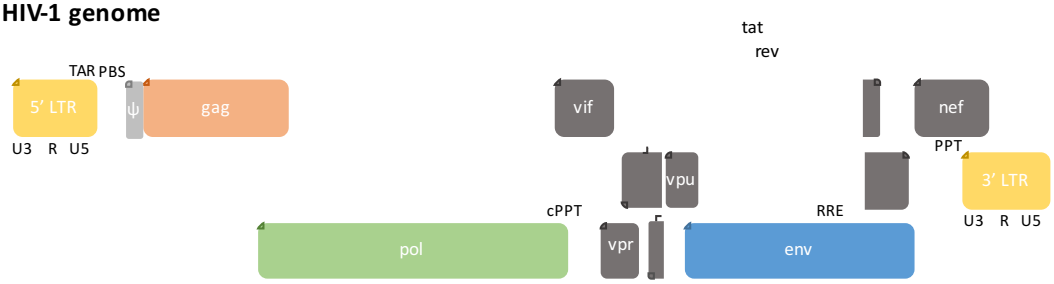
Retroviruses are a popular choice of vector for gene therapy. They are 80-100 nM, enveloped viruses of the *Retroviridae* family. Retroviruses are linked to a number of diseases including malignancies and immune deficiencies (Reviewed in Rosenberg & Jolicoeur, 1997). They were named retroviruses because of the reverse transcriptase enzyme (Baltimore, 1970; Temin & Mizutani, 1970) which converts their two genome copies of single-stranded positive sense ribonucleic acid (RNA) into DNA inside the host cell. The usual retroviral genome is 9-11 kilobase pairs long and, in its integrated proviral DNA form (Figure 1.1), is flanked by two long terminal repeats (LTRs) required for virus replication, integration, and expression. These consist of three regions, U3, R and U5. The U3 region contains a promoter which drives expression of viral transcripts. The transcription starts at the first nucleotide of the 5' R region. The U3 or R regions also contain a polyadenylation (pA) signal which directs cleavage and polyadenylation at the end of the 3' R region. Other important sequences include the packaging signal (ψ) which is required for RNA packaging into virions (Lever et al., 1989), splice donor and acceptor sites which allow the expression of different proteins from alternatively spliced mRNAs transcribed from the same promoter, and the polypurine tract (PPT), an initiation site during reverse transcription (Charneau et al., 1992). While there are different shorter splice variants, the full-length messenger RNA (mRNA) acts as the genome packaged into progeny virions.

This genome is enclosed into a number of layers as represented in Figure 1.2. The internal structure is made of various proteins (nucleocapsid, capsid (p24) and matrix proteins) derived from the *gag* gene and the external envelope which is derived from the host cell's plasma membrane. The envelope glycoprotein (Env), composed of surface and transmembrane domains encoded by the gene *env*, is inserted in this

Retroviral genome



HIV-1 genome



HIV-2 genome

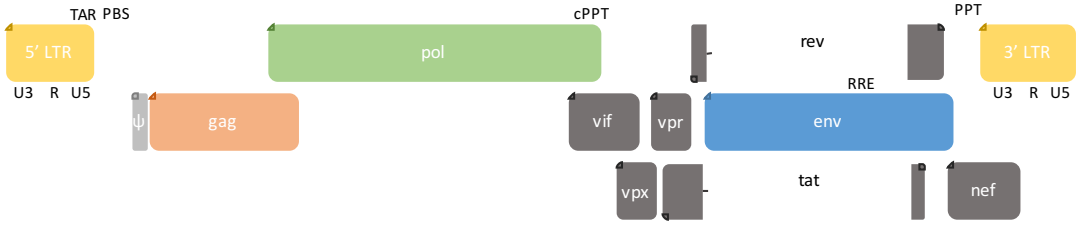


Figure 1.1 Prototypic retroviral, human immunodeficiency virus (HIV)-1 and -2 proviral genomes. Schematic representation of the proviral genomes of a prototypic retrovirus, and the lentiviruses HIV-1 and HIV-2 showing constituents in the three different reading frames. The proviral genome is flanked by two long terminal repeats (LTR) required for virus replication, integration, and expression. This consists of three regions: U3, R and U5. The 5' LTR of HIVs contains the transactivation response element (TAR), which is important for transcription enhancement by Tat. The primer binding site (PBS) allows binding of a transfer RNA (tRNA) primer for the initiation of reverse transcription. The packaging signal (ψ) is required for RNA packaging into virions. The polypurine tract (PPT), and central PPT (cPPT) for lentiviruses, are initiation sites during reverse transcription. The *gag* gene encodes the various proteins that constitute the internal structure of the virus including the nucleocapsid, capsid and matrix proteins (Figure 1.2). The *pol* gene encodes the enzymes reverse transcriptase, integrase and protease essential at various steps of the life cycle. The envelope protein, encoded by the gene *env*, is inserted in the external envelope derived from the host cell's plasma membrane and allows specific cell targeting. In HIVs, it also includes in its sequence the Rev response element (RRE) important for export of unspliced and incompletely spliced messenger RNA (mRNA) with the assistance of Rev. In HIVs, there are also six additional regulatory and accessory proteins involved in viral replication and counteracting cellular restriction factors. HIV-1 encodes Nef, Rev, Tat, Vif, Vpr and Vpu while HIV-2 encodes Vpx rather than Vpu.

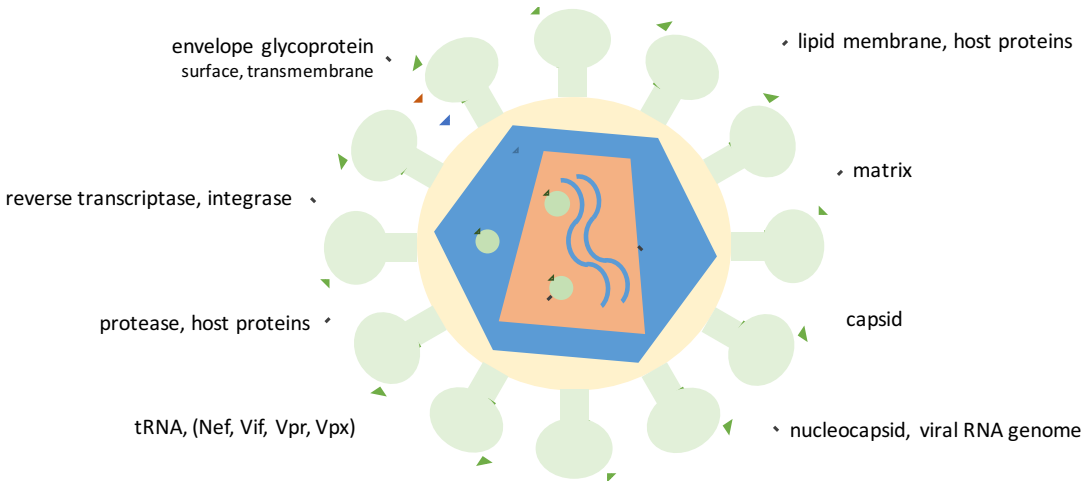


Figure 1.2 Prototypic retroviral particle. This schematic representation of the prototypic retroviral particle shows the viral RNA genome protected by an internal structure made of various proteins (nucleocapsid, capsid and matrix proteins). These are enclosed in an external envelope which is derived from the host cell's plasma membrane and contains both host proteins and the envelope glycoprotein (composed of a surface and a transmembrane domain). The particle also contains the enzymes reverse transcriptase, integrase and protease and a transfer RNA (tRNA) as well as host proteins. Lentiviral particles additionally contain regulatory and accessory proteins such as Nef, Vif, Vpr and Vpx in the case of the Human Immunodeficiency Viruses (HIVs).

membrane and allows retroviruses to target and bind to specific cells. In addition to its genome, the retroviral particle contains a transfer RNA (tRNA) primer for reverse transcription (Harada et al., 1975) which binds at the primer binding site (PBS) (Cordell et al., 1976; Eiden et al., 1976). It also contains the enzymes reverse transcriptase (RT), integrase and protease which are produced from the *pol* gene.

Retroviruses are divided into a number of subfamilies, with several that have been developed as a gene therapy vectors. These include gammaretroviruses, (Shimotohno & Temin, 1981; Wei et al., 1981), spumaviruses (Schmidt & Rethwilm, 1995) and lentiviruses (discussed next). The key characteristics of these subfamilies (Reviewed in Baum et al., 2006) are described in Table 1.1.

1.2.1 Lentiviruses

Lentivirus is a genus of the *Retroviridae* family which's members are characterised by a long incubation period. They were amongst the first viruses characterised with the Equine Infectious Anaemia Virus (EIAV), a lentivirus causing disease in horses, described in 1904 (Vallée & Carré, 1904). The most studied virus of this family, the Human Immunodeficiency Virus (HIV) type 1 (HIV-1), was isolated in 1983 (Barre-Sinoussi et al., 1983) and it, along with HIV type 2 (HIV-2), causes the Acquired Immune Deficiency Syndrome (AIDS) (Gallo et al., 1984). Other lentiviruses infect other primates (Simian Immunodeficiency Virus (SIV)), ungulates (such as EIAV) and cats (such as the Feline Immunodeficiency Virus (FIV)).

The life cycle of lentiviruses is presented in Figure 1.3. A number of host cell proteins participate in each of the steps of the viral life cycle (Reviewed in Goff, 2007) and some host proteins are packaged into virions (Reviewed in Ott, 2008) and, as such,

Table 1.1 Key characteristics of retroviruses used as gene therapy vectors

Genus	Example species	Key characteristics
Gammaretrovirus	Mouse leukaemia virus	Transduce dividing cells Genome integration
Lentivirus	Human immunodeficiency virus type 1 (HIV-1)	Transduce dividing and non-dividing cells Accessory proteins involved in infectivity Genome integration
Spumavirus	Human foamy virus	Non-pathogenic Accessory proteins involved in infectivity Reverse transcription occurs before entry Low preference for active gene integration

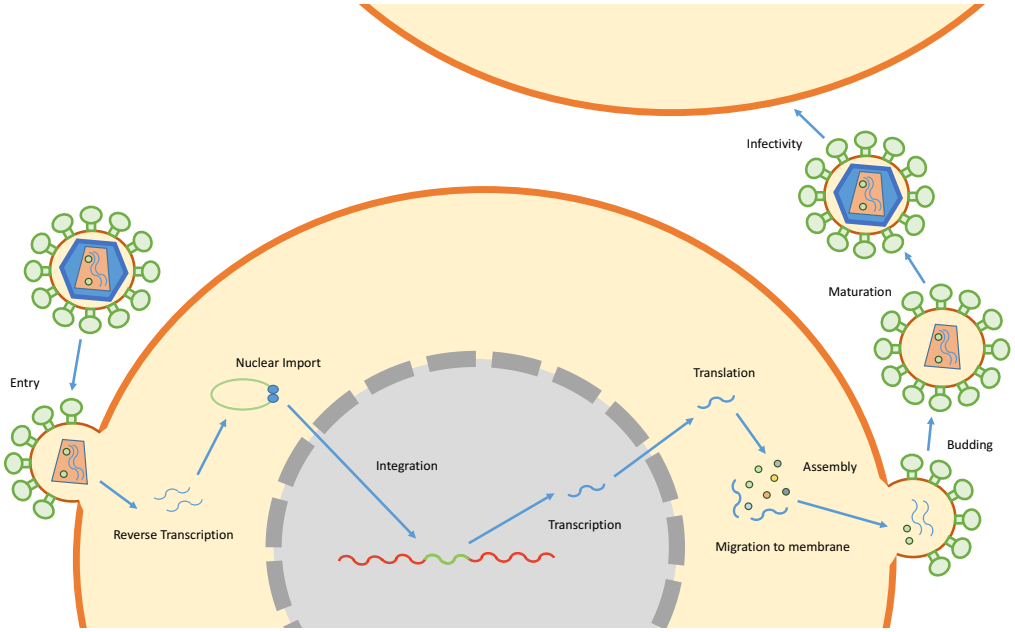


Figure 1.3 HIV-1 Life Cycle. *Entry:* HIV-1 replication starts by attachment of the viral envelope glycoprotein (Env) to the cell membrane receptor (CD4) and co-receptor, either CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4). Fusion of the viral and cell membrane then occurs allowing entry of the viral capsid into the cell. *Reverse transcription:* Using the viral enzyme reverse transcriptase, the viral RNA genome is reverse transcribed to double-stranded DNA. *Nuclear import:* The dsRNA genome is then imported into the nucleus and integrated into the cell's genome with the help of the viral protein integrase. *Transcription:* This integrated genome allows transcription of viral RNA. *Translation:* Some RNA is spliced and transcribed into the various structural (gag, pol and env), regulatory (rev and tat) and accessory proteins (nef, vif, vpr, vpu). *Migration to membrane:* These proteins and unspliced viral RNA genomes migrate to the membrane and assemble into new virions. *Budding:* Immature virions bud from the cell taking a portion of its membrane as well as host proteins. *Maturation:* The virions then mature through cleavage of the viral polyproteins by the viral protease. *Infectivity:* The resulting mature virions can then infect new cells.

play additional roles in the viral life cycle or act as viral inhibitory factors (Chapter 5 and (Franke et al., 1994; Gurer et al., 2002; Priet et al., 2003)).

1.2.1.1 Regulatory and accessory proteins

Lentiviruses also encode two regulatory proteins, Rev and Tat, as well as up to four additional regulatory proteins which have important functions in the viral life cycle.

Rev acts through the RRE, an RNA stem-loop structure in *env* (Figure 1.1) and is involved in the export of unspliced and incompletely spliced mRNA (Malim et al., 1989). Without Rev, only fully spliced transcripts are exported which produce Nef, Rev and Tat. Rev allows transcription of the other genes, as well as the export of the unspliced genome to be packaged into virions.

Tat, the trans-activator of transcription, increases the level of viral DNA transcription (Kao et al., 1987). It acts via an RNA stem-loop structure, trans-activation response element (TAR), situated 3′ to the 5’ LTR transcription start site (Figure 1.1). It also is involved in the major histocompatibility complex (MHC) class I expression down-regulation which promotes immune evasion (Howcroft et al., 1993).

HIVs also encode six accessory proteins: HIV-1 encodes Nef, Rev, Tat, Vif, Vpr and Vpu, while HIV-2 encodes the same except for Vpx instead of Vpu (Figure 1.1). The functions of these are described in Table 1.2. Most of the accessory proteins have as one of their function the inhibition of a cellular restriction factor. These genes act as an intrinsic layer to immunity against viruses and are explored in more details in Chapter 5.

Table 1.2 Key characteristics of Human Immunodeficiency Virus (HIV)-1 and -2 accessory proteins.

Virus	Accessory protein	Function	References	Restriction factors	References
HIV-1 & HIV-2	Nef	CD4 expression down-regulation	(Garcia & Miller, 1991)		
		Enhances infectivity	(Chowers et al., 1994)	SERINC3, SERINC5	(Rosa et al., 2015) (Usami et al., 2015)
		Major histocompatibility complex (MHC) class I expression down-regulation	(Le Gall et al., 1998)		
		Increases cholesterol synthesis and uptake and its transport to lipid rafts and virions	(Zheng et al., 2003) (Van 'T Wout et al., 2005b)		
HIV-1 & HIV-2	Vif	Enhances infectivity	(Fisher et al., 1987) (Strebel et al., 1987)	APOBEC3G	(Sheehy et al., 2002).
HIV-1 & HIV-2	Vpr	Nuclear import of the pre-integration complex (HIV-1 only)	(Heinzinger et al., 1994)		
		Cell cycle arrest	(Rogel et al., 1995)		
		Prevents activation of the innate immune system	(Laguette et al., 2014)		
		Prevents Env degradation by promoting its folding	(Mashiba et al., 2014) (Zhang et al., 2016)		
HIV-1	Vpu	Enhances virion release	(Strebel et al., 1988)	BST2	(Neil et al., 2008) (Van Damme et al., 2008)
		Degradation of CD4 allowing proper Env expression at the cell surface	(Willey et al., 1992)		
HIV-2	Vpx	Enhances infectivity	(Guyader et al., 1989)	SAMHD1	(Hrecka et al., 2011) (Laguette et al., 2011)
		Nuclear import of the pre-integration complex	(Fletcher et al., 1996)		

1.3 Development of retroviral vectors

Retroviruses were among the first viral vectors used for gene therapy (Shimotohno & Temin, 1981; Wei *et al.*, 1981). Retroviruses can integrate into the host genome, which is advantageous if the target disease is chronic and would require persistent transgene expression. Another significant advantage of the retroviral vector system is that the resultant virus particles can be encapsidated in the envelope of alternative viruses, a process termed pseudotyping (Reviewed in Temperton et al., 2015). This approach allows a pseudotyped vector to infect cells ordinarily resistant to infection with the original retroviral envelope protein. It also allows tropism selection of the engineered vector to be either broad or to target specific cells. This was first demonstrated when cells doubly infected with a retrovirus and the vesicular stomatitis virus (VSV) produced VSV-pseudotyped retroviral particles (Zavada, 1972). VSV is a member of the *Rhabdoviridae* family, which includes the rabies virus. It was first identified in livestock cases of vesicular stomatitis, a vesicle-inducing disease similar to foot-and-mouth disease (Cotton, 1926). However, unlike foot-and-mouth disease, VSV is infectious in humans (Hanson et al., 1950). Retroviral vectors are, for this reason, commonly pseudotyped with the VSV G glycoprotein (VSV-g) (Burns et al., 1993). The VSV-g receptor, the low-density lipoprotein receptor, appears to be ubiquitously expressed and VSV-g pseudotyped vectors have a broad tropism, infecting many cell types in different species (Finkelshtein et al., 2013). It also allows the vector to withstand ultracentrifugation shearing forces encountered during purification (Burns *et al.*, 1993).

1.3.1 Development of lentiviral vectors

As it is the case for retroviral vectors, lentiviral vectors can also be easily pseudotyped. The VSV-g pseudotyped lentiviral vectors (Akkina et al., 1996; Naldini et al., 1996b; Reiser et al., 1996) efficiently transduce organs such as the liver and muscle (Kafri et

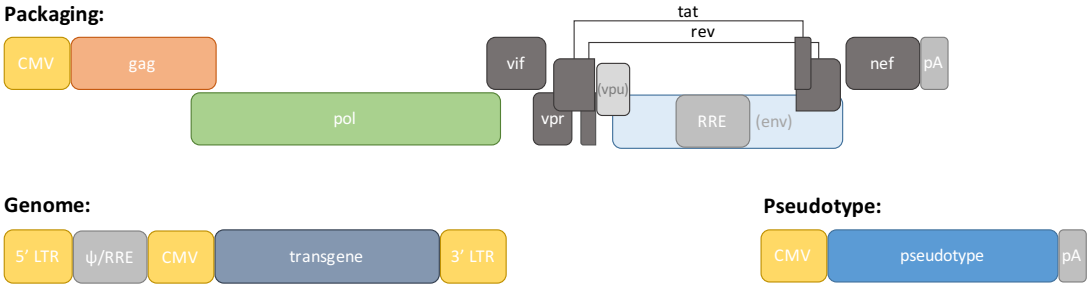
al., 1997), cells of the retina (Miyoshi et al., 1997) and neurons (Blomer et al., 1997). Nonetheless, for some cell targets, VSV-g pseudotyping does not allow efficient transduction, presumably due to the absence or low frequency of the VSV-g receptor on the vector-accessible portion of the cell surface. As a result, other pseudotypes have been developed to target these cells (Reviewed in Cronin et al., 2005). For example, pancreatic islet cells are more efficiently transduced with lentiviral vectors pseudotyped with the envelope glycoprotein of the lymphocytic choriomeningitis virus compared with VSV-g pseudotyped virus (Kobinger et al., 2004). Differentiated airway epithelium cells *in vivo* are largely refractory to transduction with VSV-g pseudotyped HIV-based vectors (Goldman et al., 1997), consequently, to efficiently target the lung, lentiviral vectors using pseudotypes from Ebola virus (Kobinger et al., 2001), Marburg virus (Sinn et al., 2003), Sendai virus (Kobayashi et al., 2003a), baculovirus (Sinn et al., 2005) or influenza virus (Mckay et al., 2006) have been developed.

The popularity of lentiviruses as vectors for gene therapy is partly due to their ability to carry large transgenes and transduce non-dividing cells (Weinberg et al., 1991). This latter feature, not a property of the retroviral family in general, is facilitated, amongst other mechanisms, via a nuclear localisation sequence located within the matrix protein sequence of *gag*, which dramatically enhances nuclear import of the viral pre-integration complex (Bukrinsky et al., 1993). Lentiviral vectors also have the ability to establish long-term transgene expression (Naldini *et al.*, 1996b). Various lentiviral vectors have been developed as gene therapy vectors, including: EIAV (Olsen, 1998; Mitrophanous et al., 1999), FIV (Poeschla et al., 1998b), HIV-1 (Naldini *et al.*, 1996b), HIV-2 (Poeschla et al., 1998a), HIV/SIV hybrid (White et al., 1999), SIV (Nakajima et al., 2000; Negre et al., 2000; Schnell et al., 2000) and a few others (Reviewed in Cronin *et al.*, 2005).

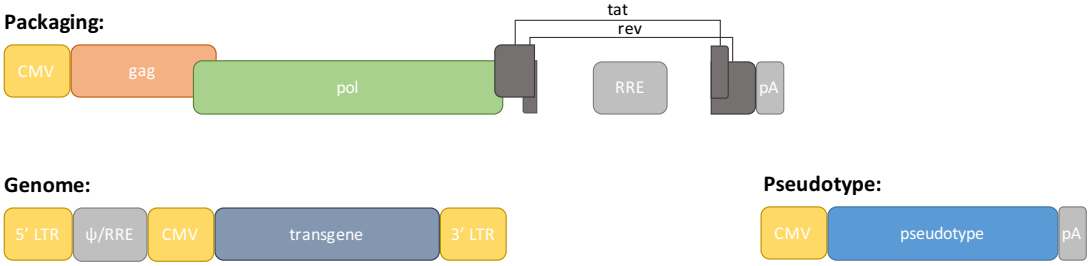
HIV-1-based vectors are based on cloned proviruses (Shaw et al., 1984; Adachi et al., 1986) that have been subsequently optimised for safety. For the first-generation of lentiviral vectors, the different elements of the viral genome were separated into different plasmids (Naldini *et al.*, 1996b), following the path used to generate other retroviral vectors (Soneoka et al., 1995). In lentiviral vectors, a three-plasmid system was designed for vector production following transient transfection of mammalian cells (Figure 1.4). The first plasmid, termed the packaging construct, consists of the proviral DNA, with LTRs substituted by a human cytomegalovirus immediate early enhancer and promoter (CMV) (Foecking & Hofstetter, 1986) and the pA site from the insulin gene, to drive expression of all viral proteins required for vector production. However, this plasmid is defective for the production of the viral envelope and the accessory protein Vpu. The second plasmid, the genome plasmid, contains the transgene, under the control of an internal promoter, as well as the elements required for packaging, reverse transcription and integration. A full-length viral mRNA transcript is not encoded by the final virion, as it can only be generated in the presence of Tat and Rev (Section 1.2.1). These are only present during the production phase when the packaging plasmid is present. Thus, this division of the vector components between multiple plasmids essentially eliminates the likelihood of generating a replication-competent vector. Finally, the third plasmid, named pseudotype plasmid, is used to replace expression of the HIV-1 Env with a heterologous envelope protein.

In the second-generation vectors (Zufferey et al., 1997), *env* and the genes encoding the accessory proteins (Nef, Vif, Vpr and Vpu) were deleted (Figure 1.4). In the third-generation vectors (Dull et al., 1998; Kim et al., 1998), a chimeric 5' LTR was introduced in which the U3 region was replaced by a constitutive promoter, either CMV or from the Rous sarcoma virus (RSV) (Gorman et al., 1982), as done previously for

First generation lentiviral vector plasmids



Second generation lentiviral vector plasmids



Third generation lentiviral vector plasmids

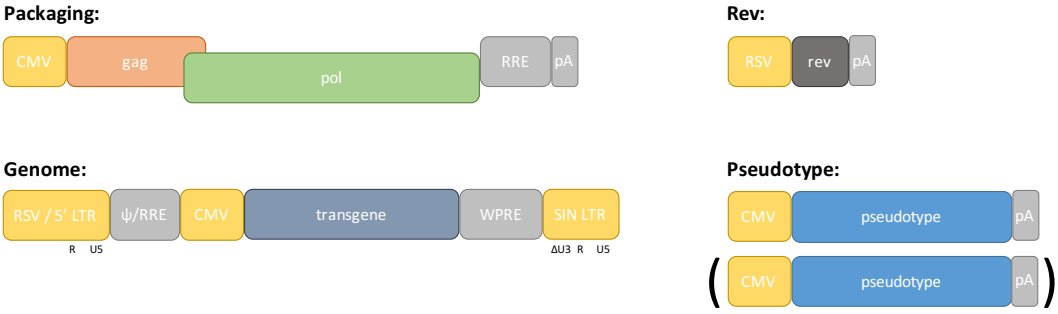


Figure 1.4 Plasmids needed to generate first, second and third-generation lentiviral vectors. Schematic representation of the plasmids needed to generate first, second and third-generation lentiviral vectors. The first-generation vectors (Naldini *et al.*, 1996b) consist of a three-plasmid system separating the different elements of the viral genome: (1) packaging plasmid, consisting of the proviral DNA, but defective for Env and Vpu (in parentheses and lighter shades); (2) genome plasmid, containing the transgene, its promoter and packaging elements; (3) pseudotype plasmid, allowing different tropisms. The second-generation (Zufferey *et al.*, 1997) is similar to the first, but *env* and the virulence genes *nef*, *vif*, *vpr* and *vpu* are deleted from the packaging plasmid. In the third-generation, the addition of a constitutive promoter (human cytomegalovirus immediate early enhancer and promoter (CMV) or the Rous sarcoma virus promoter (RSV)) instead of U3 in the 5' LTR allows the removal of the *tat* gene, while *rev* is provided on an independent (fourth) plasmid. The vector is self-inactivating (SIN) by a deletion of key nucleotides (ΔU3) in the 3′ long terminal repeat (LTR) of the genome plasmid (Miyoshi et al., 1998; Zufferey et al., 1998). The post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) is present to enhance gene expression (Zufferey et al., 1999). Some pseudotypes, such as that based on Sendai virus fusion and haemagglutinin-neuraminidase (F/HN) (Kobayashi *et al.*, 2003a), require two pseudotyping proteins and may require an additional pseudotyping plasmid (indicated by the use of parentheses) constituting a five-plasmid based system. Additional details are provided in Section 1.3.

other retroviral vectors (Soneoka *et al.*, 1995). In this case, it allowed high-level expression of vector RNA and, consequently, the removal of the *tat* gene, its transactivation of the 5' LTR no longer being required. In the case of the other regulatory protein, the *rev* gene was placed on a separate plasmid, but its presence was still required for the accumulation of vector transcripts that can be packaged. It has since been shown to be possible to alleviate this need for *rev* through codon-optimisation of the gag-pol gene which increases the amount of gag-pol mRNA present and ensures Rev-independence (Kotsopoulou et al., 2000).

Similar to retroviral vectors (Yu et al., 1986), third-generation lentiviral vectors are engineered to be self-inactivating (SIN) by a deletion of the promoter-enhancer region in the U3 region (ΔU3) of the 3′ LTR (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998). This section contains elements that regulate the HIV-1 LTR promoter activity such as transcription factors binding sites and the TATA box. The proviral 5′ LTR is copied from this modified 3′ LTR during reverse transcription transferring the deletion. This abolishs LTR promoter activity without greatly affecting titres or transgene expression. Biosafety is consequently improved by reducing the possibility of replication-competent viruses and recombination with wild-type HIV. Testing for replication-competent lentiviruses, while a very unlikely occurrence, is nevertheless a regulatory requirement since the first lentiviral vector clinical trial (Reviewed in Manilla et al., 2005). SIN LTRs also prevent downstream transcription from the 3′ LTR into the cell's genomic DNA (Herman & Coffin, 1987), which could lead to activation of downstream endogenous oncogenes. Gene expression was further increased by the addition of the post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) (Donello et al., 1998) in the untranslated region 3' LTR of the genome plasmid which increases the levels of nuclear transcripts (Zufferey *et al.*, 1999).

Like their lentivirus progenitors, these lentiviral vectors integrate into the host cell. Viral sequences are therefore maintained through cell division preventing dilution of the therapeutic effect. To reduce the risk of insertional mutagenesis, integrase defective lentiviral vectors have been developed (Philippe et al., 2006; Yanez-Munoz et al., 2006), where the integrase enzyme has been mutated to impair its activity. The viral genome resulting from integrase defective vectors hence remains episomal in the transduced cells and will be lost in rapidly dividing cells, resulting in transient expression of the transgene (Nordin et al., 2016). Although viral protein-mediated integration is lacking, residual integrase-independent integration can still occur, albeit at a much reduced rate similar to that of naked DNA (Reviewed in Banasik & Mccray, 2010). Additionally, some of these vectors also suffer from lower titres or transgene expression (Reviewed in Banasik & Mccray, 2010).

1.4 Clinical applications of retroviral vectors

The first approved gene transfer application in humans was approved in 1989 and used a retroviral vector whereby an antibiotic-resistance gene was introduced into tumour-infiltrating lymphocytes by a retroviral vector to allow tracking (Rosenberg et al., 1990). As of August 2016, there have been 597 clinical trials that used retroviral vectors (24.3% (145/597) of which used lentiviruses) (Journal of Gene Medicine, 2016), the majority of them (358/597 (60.0%)) targeting cancers. There are two main categories of clinical applications of retroviral vectors for gene therapy: (1) *ex vivo* produced cell therapy products, where stem/progenitor, or long-lived, cells (typically bone marrow stem cells or T cells) are harvested from a patient, transduced with retroviral vectors, and are subsequently returned to the patient, an approach that typically targets cancers, and (2) directly *in vivo*-administered products, where

retroviral vectors are customarily administered directly to a target organ, an approach that typically targets monogenetic diseases.

The first approved clinical trial utilising a therapeutic gene (rather than a marker such as an antibiotic resistance gene) for gene therapy used a retroviral vector and was directed at individuals suffering from ADA-SCID (Anderson et al., 1990), a genetic disorder where the development of functional T cells and B cells is disrupted. Two patients were treated and, after 2 years of treatment and 2 years of follow-up, their T cells normalised, with persistent adenosine deaminase expression, as did many cellular and humoral immune responses. This was the first demonstration of safety and efficacy in a retroviral vector clinical trial (Blaese et al., 1995).

Another set of gene therapy trials treated 20 infants with X-linked SCID (SCID-X1), a disorder characterised by an early block in T and natural killer (NK) cell differentiation. It is caused by mutations in the γ-chain cytokine receptor subunit of interleukin receptors, preventing growth, survival, and differentiation of progenitor cells. This leads to death from infection in the first year of life unless treated by allogeneic haematopoietic stem-cell transplantation. In these trials, patients were treated with autologous CD34+ bone marrow cells transduced with a first-generation Moloney murine leukaemia virus vector expressing interleukin-2 receptor γ-chain complementary DNA (cDNA) and containing LTRs with viral enhancer sequences. This treatment successfully resulted in reconstitution of T cells and in disease-free survival similar to that obtained with stem cell transplant (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002). However, it also resulted in vector-induced leukaemia through enhancer-mediated mutagenesis in 25% of patients (Hacein-Bey-Abina et al., 2003a). This was shown to be due to the integration of the vector into the host cell

genome leading to insertional mutagenesis. In the above-mentioned trials, this led to vector-mediated up-regulation of the LMO2 or CCND2 proto-oncogenes, which led to an uncontrolled clonal expansion of mature T-cells (Hacein-Bey-Abina et al., 2003b; Howe et al., 2008). Since then, insertional mutagenesis remains the chief safety concern for integrating vectors.

SIN retroviral vectors for SCID-X1 have been developed (Thornhill et al., 2008) to address this issue and are being assessed in clinical trials (Hacein-Bey-Abina et al., 2014). Ultimately, building on successes and challenges in the SCID gene therapy field, the first gene therapy product using a retroviral vector to receive European marketing authorisation was Stimvelis by GSK for ADA-SCID in 2016 (European Medicines Agency, 2016).

1.4.1 Clinical applications of lentiviral vectors

Aside from safety concerns relating to insertion mutagenesis, other issues with retroviral vectors include: low titres, low transfection efficiencies, particle instability and difficulty to be concentrated compared to non-enveloped viruses. In addition, retroviral particles only infect proliferating cells and are ineffective at transducing non-dividing post-mitotic cell targets (Lewis & Emerman, 1994).

Lentiviral vectors have been developed to help mitigate some of these problems. As described in Section 1.3.1, lentiviral vectors were developed early with SIN LTRs and are accordingly lacking the problematic 3' LTR U3 enhancer elements. Additionally, the insertion site selection of lentiviral vectors is different from that of γ-retroviral vectors. Both γ-retroviruses and lentiviruses integrate non-randomly in the host cell's genome, with a preference for active chromatin, promoters and regulatory regions.

However, γ-retroviral vectors target at high frequency genes involved in growth, development and differentiation of the target cell, which may induce insertional tumours or pre-neoplastic clonal expansion. This would be due to differences in both the LTR enhancer and the integrase enzyme of the vectors. These, in γ-retroviral vectors only, promote interaction with active host cell promoters and enhancers enriched in transcription factor binding sites (Felice et al., 2009; Montini et al., 2009). This has been demonstrated in two clinical trials for Wiskott–Aldrich syndrome where in contrast to γ-retroviral vectors (Boztug et al., 2010), lentiviral-based vectors did not induce *in vivo* selection of clones carrying integrations near oncogenes in patients (Aiuti et al., 2013).

The first clinical trial using a lentiviral vector was conducted in 2003. It used a vector expressing an antisense HIV-1 *env* sequence in patients who had failed antiviral therapy and led to improved immune function (Levine et al., 2006). Early *in vivo* clinical trials have since been performed for Parkinson's disease (Palfi et al., 2014) and age-related macular degeneration (Reviewed in Pecen & Kaiser, 2015). However, lentiviral vectors have been mostly successful in *ex* vivo clinical trials such as when used to produce Chimeric Antigen Receptor (CAR)-modified T cell therapies; where impressive clinical therapy has been achieved for chronic lymphoid leukaemia (Kalos et al., 2011; Porter et al., 2011) and acute lymphoid leukaemia (Grupp et al., 2013) amongst other diseases. Other *ex vivo* clinical trials using haematopoietic stem cells transduced with lentiviral vectors showed positive results in X-linked adrenoleukodystrophy (Cartier et al., 2009), metachromatic leukodystrophy (Biffi et al., 2013), Wiskott–Aldrich syndrome (Aiuti *et al.*, 2013) and SCID-X1 (De Ravin et al., 2016).

The typical diseases for which gene therapy was first thought to be the route towards an easy cure were monogenetic diseases. Cystic Fibrosis (CF), an autosomal recessive life-shortening disease, was one of the first targets. It is a multi-organ disease affecting the mucosal surfaces of the lungs, intestine, pancreas and reproductive tract (Andersen, 1938). However, lung disease is the overwhelming cause of morbidity and mortality (Cystic_Fibrosis_Foundation, 2014). The United Kingdom (UK) Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) developed a non-viral gene therapy for CF lung disease. It consists of a Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-encoding plasmid complexed with a cationic liposome mixture (pGM169/GL67A) (Hyde *et al.*, 2008; Mclachlan et al., 2011) which was shown to be safe for aerosol delivery to the lung in clinical studies (Alton et al., 2015b). Moreover, in a Phase IIb trial involving 136 CF patients, this treatment led to a statistically significant improvement in lung function and a stabilisation of lung disease compared with placebo (Alton et al., 2015a). However, the treatment effect was modest, prompting the clinical evaluation of a lentiviral vector.

The current UKCFGTC lentiviral vector lead candidate uses a five-plasmid transfection to generate an SIV-based vector. It encodes a codon-optimised CpG-free CFTR cDNA under the transcriptional control of a novel CpG-free CMV/elongation factor 1 alpha (hCEF) promoter (Hyde *et al.*, 2008). This vector is pseudotyped with the Sendai virus (murine parainfluenza virus type 1) fusion and haemagglutinin-neuraminidase (F/HN) envelope proteins (Kobayashi *et al.*, 2003a), to facilitate delivery to lung cells (Mitomo et al., 2010; Alton et al., 2017). In Sendai virus, the haemagglutinin-neuraminidase is responsible for haemagglutination and receptor binding and has neuraminidase activity (Scheid et al., 1972). The glycoprotein F is required for virus-induced haemolysis, cell fusion and the initiation of infection. It is produced as an inactive

precursor which necessitates proteolytic cleavage by trypsin to activate infectivity (Homma, 1971). This lentiviral vector product is now being considered for current Good Manufacturing Practice (cGMP) vector production and clinical evaluation.

1.5 Lentiviral vector production

A fundamental bottleneck in the translation of lentiviral vectors for disease treatment, particularly for *in vivo* applications, is the need to manufacture sufficient quantities of highly concentrated and purified cGMP grade vector. In order to remain competitive with other gene therapy systems, lentiviral vector production titres need to be increased significantly – even a doubling of virus titre would significantly reduce overall costs.

The current lentiviral vector production process (Figure 1.5) is broadly inspired by previously developed techniques for the production of other retroviral vectors and is divided into upstream processing (transient transfection or producer cell activation to harvest) and downstream processing (which can include clarification, concentration and/or purification steps).

1.5.1 Upstream processing

1.5.1.1 Producer cells

Almost universally, cells of the Human Embryonic Kidney 293 (HEK 293) lineage are the preferred cells for the production of lentiviral vectors. The HEK 293 cell line is a permanent line of primary human embryonal kidney transformed by exposing cells to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977). The cells express the transforming gene of adenovirus 5. This has been shown to strongly transactivate

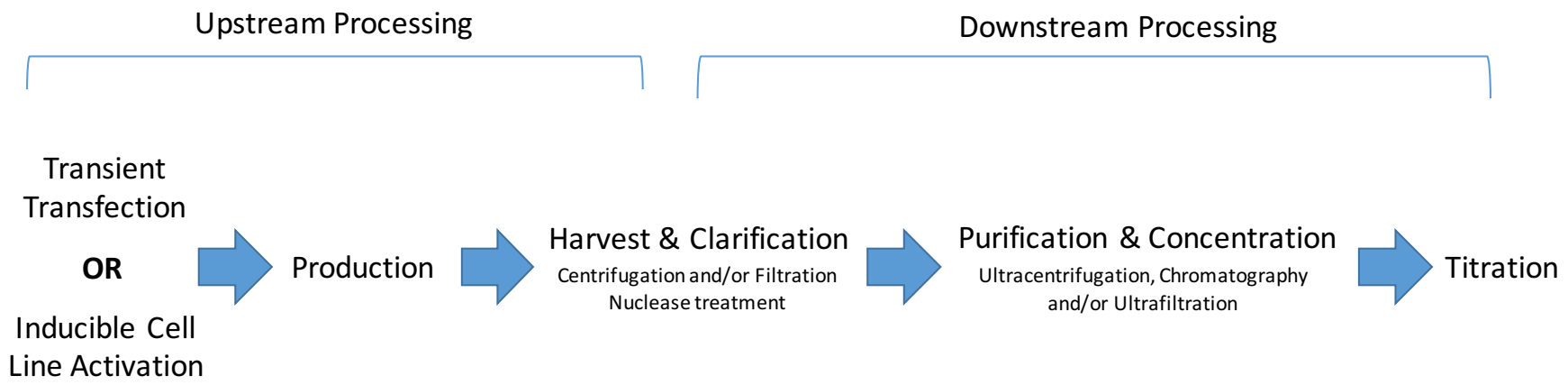


Figure 1.5 Schematic showing the main upstream and downstream processing steps to produce and purify lentiviral vectors. Upstream processing comprises lentiviral vector production, initiated either by transient transfection of producer plasmids (Section 1.3.1) or inducible cell line activation, and subsequent harvest of the viral particles. Downstream processing depends on the purity and concentration of virus required. Clarification allows removal of the cells by centrifugation and/or filtration. A nuclease treatment allows the removal of residual plasmid DNA. If concentrated and purified virus is required, techniques such as ultracentrifugation, chromatography and/or ultrafiltration can be used. The final product is then quantified through titration. Additional details are provided in Section 1.5.

the human cytomegalovirus promoter (Gorman et al., 1989) used in lentiviral vector plasmids (Figure 1.4). HEK 293 cells also allow high transfection yields with most gene transfer agents and can easily be adapted to suspension culture (Stillman & Gluzman, 1985) and serum-free media (Cote et al., 1998). The HEK 293T variant expresses the simian virus 40 (SV40) large tumour antigen (DuBridge et al., 1987) allowing episomal amplification of plasmids containing the SV40 origin of replication. This allows transiently transfected plasmids to persist in the cells throughout the production phase (Van Craenenbroeck et al., 2000), but could be a contaminant in the final product. The main cell line used in lentiviral vector production is the HEK 293T/17. It was selected as a highly performing clone of the HEK 293T for the generation of high-titre retroviruses by transient transfection (Pear et al., 1993). HEK 293T have also been shown to produce higher lentiviral vector titres than HEK 293 when using transient transfection (Ausubel et al., 2012).

The choice of cell culture medium to grow these cells can have a dramatic effect on final lentiviral vector titre, especially if the medium contains serum or other supplements (Section 3.1). Additionally, different vessels can be used to produce vectors from the microliter scale to the multi-litre scale. Two modes of production influence the choice of vessel. Production in adherent cells can be done in disposable plastic plates, flasks or larger cell factories. Alternatively, production can occur in suspension in vessels such as disposable plastic plates or Erlenmeyer flasks on orbital shakers or large scale bioreactors or stirrer tanks.

1.5.1.2 Transient transfection or activation of inducible cell line

The use of transient transfection (Figure 1.5) to introduce the plasmids required for vector production (Figure 1.4) is a versatile system as, none of the components being

fixed, it is easy to make plasmid substitutions to generate a variety of vectors. While many transfection reagent options exist, few are cost-effective at the multi-litre scale. Lipid-based transfection reagents are widely used for *in vitro* application as they consistently result in higher transfection efficiencies in a wide variety of cell types (Felgner et al., 1987), but would be too costly at larger scales. Therefore, two main reagents are used to perform the transient transfection: calcium phosphate and polyethylenimine (PEI).

Calcium phosphate is an inorganic salt that is used to introduce DNA into mammalian cells (Graham & Van Der Eb, 1973). DNA-calcium phosphate precipitates are absorbed by cells when added to cell cultures. It is frequently used to produce lentiviral vectors as it is both inexpensive and very efficient. There is nevertheless a challenge in terms of scalability as the reaction is very sensitive to small variations in pH that affect the initiation and growth of the precipitates (Graham & Van Der Eb, 1973) and requires the presence of serum or albumin to reduce cytotoxicity (Girard et al., 2001).

PEI is an organic macromolecule with a very high cationic-charge density potential, with buffering capacity at a wide range of pH such as those encountered in the complex multi-stage process of transfection. This property was demonstrated to be fundamental in making PEI a highly efficient compound for the delivery of oligonucleotides and plasmids both *in vitro* and *in vivo* (Boussif et al., 1995). Through electrostatic interaction, PEI binds to DNA and forms small complexes that cells can endocytose and they eventually localise to the nucleus (Godbey et al., 1999b). Available in a wide range of molecular weights, isoforms of PEI are either linear or branched, affecting the efficiency of its gene transfer activity (Reviewed in Godbey et al., 1999a). Apart from its low cost, other advantages include its relatively low cytotoxicity when bound to DNA

(Boussif *et al.*, 1995) and its ability to support efficient transient gene expression in mammalian cells grown in serum-free suspension culture (Schlaeger & Christensen, 1999).

To reduce toxicity, the media, containing the transfection reagent and plasmid DNA, is usually changed, or the total culture volume highly increased, after up to a day of incubation with the cells. Sodium butyrate is usually used at this stage as it is a known enhancer of transfection efficiency and transgene expression (Gorman et al., 1983), of HIV-1 LTR activity (Bohan et al., 1987), as well as of retroviral (Olsen & Sechelski, 1995; Soneoka *et al.*, 1995) and lentiviral vector production (Gasmi et al., 1999; Sakoda et al., 1999). The proposed mechanism for this enhancement is through the inhibition of the enzyme histone deacetylase which leads to a hyper-acetylation of histones in treated cells and increased transcription (Candido et al., 1978; Sealy & Chalkley, 1978).

In an inducible cell line system, most or all the genetic information necessary to produce the components of the lentiviral vector are already present in the cell line. A *packaging* cell line contains all the elements except for the genome, which is transiently transfected at the start of production, while a *producer* cell line has all the necessary components including the genome. Most packaging and producer cell lines have some, or all, of the lentiviral genetic material under inducible transcriptional control, using promoters where the addition of a substance to the cell culture media activates or represses switches allowing vector production (Section 6.1.2). This can be for biosafety reasons, preventing vector production in cell culture outside the production phase. Crucially, inducible promoters are also used because some components, such as expression of Rev (Kaplan & Swanstrom, 1991) and the VSV-g pseudotype (Ory et

al., 1996), have been shown to be toxic to the producer cells. Such producer/packaging systems lack the flexibility to make changes to vector composition, but by their fixed nature can lead to more reproducible vector production, which is desirable for clinical trials.

1.5.2 Downstream processing

Once the vector production is complete, the viral particles need to be harvested and downstream processing is initiated. These steps are usually fully scalable and, consequently, not cost-limiting and are, by comparison, less of a focus for major improvement than the upstream production itself.

During the harvest process, a nuclease is often added to reduce plasmid DNA carry-over into the final product. This degrades most residual nucleic acids such as contaminating host DNA, RNA and residual plasmid DNA into fragments < 500 base pairs (bp) (Ausubel *et al.*, 2012) minimising carry-over into the final product. Other treatments can also be included at this step. For example, as previously mentioned, the fusion component of the Sendai virus F/HN pseudotype is produced as an inactive precursor which necessitates proteolytic cleavage by trypsin to activate infectivity (Homma, 1971). While porcine trypsin can be used for this process, alternative animal-free recombinant options are also available for increased safety.

1.5.2.1 Clarification

The first step in the downstream processing is clarification, which is the removal of the producer cells and large particles from the supernatant. For small scale preparations (< 1 L) and samples for *in vitro* work, this can be accomplished by low-speed centrifugation followed by microfiltration using a 0.45 µm filter. The centrifugation step

removes the larger particles and avoids clogging of the filter. For larger scale preparations (> 1 L), in order to prevent filter pore clogging due to the larger volume going through, and therefore reduction of the actual pore size which could affect titres, a series of filters with decreasing pore size can be used (Reeves & Cornetta, 2000). This efficient strategy can alleviate the need for centrifugation with minimum vector loss. Once harvested, the vector preparation can either be used directly or further purified and concentrated if required (Figure 1.5).

1.5.2.2 Purification

The purpose of purification is to remove contaminants such as cell culture reagents, serum proteins and producer cell-derived DNA, RNA and proteins from the vector supernatant. This reduces the risk of local inflammatory and immune responses following *in vivo* delivery (Scherr et al., 2002; Baekelandt et al., 2003) and increases the *ex vivo* transduction efficiency (Yamada et al., 2003).

Density gradient ultracentrifugation allows the separation of particles according to their buoyancy density or rate of sedimentation in a gradient media such as sucrose, caesium chloride or iodixanol. It offers the potential of separating viral particles from defective vectors and cell membrane vesicles. It is, nonetheless, time-consuming and poorly scalable for large scale applications (Reviewed in Segura et al., 2006).

Chromatography is a purification technique used to separate a mixture by passing it in solution or suspension through a medium in which the components move at different rates and can, therefore, be separated. The chromatography column containing beads which are coated with functional groups that capture viral particles and allows the rest of the solution and, therefore, impurities pass through. Captured viral particles are then

displaced from the column using desorption agents and collected in purified fractions. A number of adsorptive chromatography procedures have been described, including ion-exchange chromatography, affinity chromatography and size-exclusion chromatography, allowing the purification of vectors based on their chemical surface properties or the molecular composition of their envelope (Reviewed in Segura *et al.*, 2006). These techniques cannot, however, discriminate between viral particles and defective vectors or cell membrane vesicles.

1.5.2.3 Concentration

Clinical-grade lentiviral vectors need to be concentrated from their large production volume to the actual dose volume to be delivered to patients. The concentration step can be either performed before purification to reduce the volume supplied to the purification apparatus, as a final step to concentrate virus after purification, or both before and after purification.

Centrifugation is a commonly used method for concentrating viruses. Ultracentrifugation or very long, low-speed, centrifugation can pellet viruses which can then be resuspended in small volumes to get a highly concentrated preparation. Nonetheless, the shear stress, extended processing time and the concentration of high molecular weight inhibitors of transduction (Le Doux et al., 1996) can reduce the number of active viral particles. Furthermore, centrifugation can co-purify subcellular contaminants and cellular genomic DNA which can only be removed by a purification step (Transfiguracion et al., 2003). Alternatively, additives can be included to precipitate the viral particles. This allows shorter low-speed centrifugation to be sufficient to pellet viruses from large volumes (Reviewed in Segura *et al.*, 2006).

An alternative to centrifugation is ultrafiltration. It allows concentration of large volumes of supernatant in shorter times where the virus particles are enriched in the retentate while water and small molecular weight molecules are removed in the permeate. At small scale, centrifugal filtration devices can be used (Reiser *et al.*, 1996), while tangential-flow filtration is better suited for larger volumes (Paul et al., 1993). Tangential-flow filtration uses hollow fibre filters and the feed flow travels tangentially across the surface of the filter, rather than perpendicularly towards the filter like in dead-end filtration, which reduces clogging.

1.5.2.4 Storage

If not used immediately, viral vectors can be stored frozen at -80°C to protect the virus from thermal inactivation and allow potency to be maintained over several months (Wikstrom et al., 2004). While cryoprotectants such as DMSO and glycerol are used in long-term cell cryostorage, these were shown not to play an essential role in retroviral vector preparations (Lee et al., 1996b). In any case, the number of freeze-and-thaw cycles should be kept to a minimum as the vectors will lose up to half of their activity after two to four freeze-and-thaw cycles (Burns *et al.*, 1993; Bowles et al., 1996). Lyophilisation is also an option (Levy & Fieldsteel, 1982) although it could lead to a decrease in titre (Lee *et al.*, 1996b).

1.5.3 Titration

A successful viral gene therapy vector must deliver its transgene in sufficient quantity while not being toxic to the host. To this effect, viral vectors are typically quantified (titre) based on two attributes. The first, the non-functional titre, indicates the total number of viral particles in a preparation. Examples of methods to determine such titre are listed in Table 1.3. This titre does not, however, indicate the potency of the vectors,

as a high number of viral particles are defective (Kirkwood & Bangham, 1994). Overestimation of the actual functional vector titre hence occurs through quantification of p24, reverse transcriptase or viral RNA that originates from non-functional vector particles. There are a number of possible explanations for this phenomenon: defective genomes could be present in virions, the virions might not enter the target cell, the genome fails to integrate, the genome is degraded in the cytoplasm of the target cell or the virions fail to produce detectable transgene expression (Reviewed in Geraerts et al., 2006).

A second titre, the functional titre, indicates the number of transducing particles. This is the number of particles that can enter cells and transfer the transgene. It is determined by techniques such as those listed in Table 1.3, following a limiting dilution of the vector in order to obtain one viral transduction event per cell. At this step, hexadimethrine bromide (polybrene) is often added (Naldini *et al.*, 1996b) as it is known to enhance transduction efficiency of viruses (Toyoshima & Vogt, 1969), including retroviruses (Manning et al., 1971), by enhancing receptor-independent virus adsorption on target cell membranes (Davis et al., 2002). Another polycation, diethylaminoethyl (DEAE)-dextran has also been shown similarly to enhance the efficiency of transduction of both lentiviruses (Denning et al., 2013) and other retroviruses (Manning *et al.*, 1971).

Finally, the particle to infectivity ratio (P:I) is derived from the ratio of the non-functional titre to the functional titre. It can widely differ between different laboratories as a result of the many different techniques used and variation in assay performance. It nevertheless allows internal evaluation of the purity and efficacy of viral preparations.

Table 1.3 Examples of titration techniques for non-functional and functional titre.

Non-functional titre		
Technique	**Detects**	**Source**
enzyme-linked immunosorbent assay (ELISA)	capsid (p24)	(Naldini et al., 1996a)
real-time polymerase chain reaction (PCR)	partial reverse transcripts in virions (Trono, 1992)	(Scherr et al., 2001)
reverse transcription (RT) quantitative PCR (qPCR) (RT-qPCR)	viral RNA	(Martin-Rendon et al., 2002)
RNA dot blot analysis	viral RNA	(Onodera et al., 1997)

Functional titre		
Technique	**Detects**	**Source**
antibiotic selection	colony forming units	(Cone & Mulligan, 1984)
β-galactosidase assay	transgene expression	(Naldini *et al.*, 1996b)
flow cytometry	fluorescent transgene expression	(Dull *et al.*, 1998)
luciferase assay	transgene expression	(Naldini *et al.*, 1996a)
qPCR	integrated proviral DNA copies per cell	(Sastry et al., 2002)
qPCR-based product-enhanced reverse transcriptase (PERT) assay	RT activity	(Vermeire et al., 2012)
RT assay	RT activity	(Tonini et al., 2004)
RT-qPCR	lentiviral mRNA copies	(Lizee et al., 2003)
semi-quantitative Northern blot	Proviral RNA expression	(Miyoshi *et al.*, 1998) (Zufferey *et al.*, 1998)
semi-quantitative Southern blot	integrated proviral DNA	(Miyoshi *et al.*, 1998)
semi-quantitative Western blot	transgene expression	(Lizee *et al.*, 2003)

1.6 Aims

The majority of lentiviral vector applications, such as *ex vivo* therapies, have to date required relatively small quantities of viral vector, whereas large quantities will be required to further exploit this vector system *in vivo*. As outlined above, the main bottleneck is the manufacturing scale-up of lentiviral vectors at a cGMP standard, suitably pure for use in patients and in sufficient quantities. The main aim of this thesis is to investigate strategies to improve the lentiviral vector productivity during manufacture, by focusing on upstream processing. This requires an understanding of the lentivirus replication cycle in the host cell, together with an appreciation of the crucial parameters required for efficient lentiviral vector production.

The studies described in Chapter 3 investigate media supplementation as a first step to enhance vector production yields. Chapter 4 focuses on using knowledge of intrinsic cellular immunity to HIV-1 and overexpression of related cellular proteins. Chapter 5 comprises of an investigation of the HIV-1 literature to identify potential cellular factors acting in the late phase of its life cycle. Chapter 6 describes the development of a novel assay using gene inhibition with small interfering RNA (siRNA) molecules and studies in Chapter 7 demonstrate the utility of this assay to assess the effect of several of these cellular factors on virus production. Together, these studies provide the basis for the development of new producer cell lines to maximise the productivity of lentiviral vector for clinical use.

Chapter 2 – Materials & Methods

2.1 Materials

2.1.1 Reagents and solutions

Solutions were prepared using either Milli-Q reverse osmosed ultrapure water (Merck, Feltham, UK), nuclease-free water (Promega, Southampton, UK), endotoxin-free water for injection (Fannin, Wellingborough, UK) or ethanol absolute (VWR, Lutterworth, UK) as indicated. Before being used in cell culture, solutions were sterilised by 0.2 µm surfactant-free cellulose acetate filter (Thermo Scientific, Loughborough, UK). Solutions were stored at 4°C except for plasmid DNA which was stored at -20°C. Concentrations of solutions are indicated by percentages referring to volume/volume (v/v) or weight/volume (w/v) ratios at room temperature.

Lysogeny broth (LB) (Bertani, 1951) was prepared using 1% (w/v) Tryptone (Amresco, Solon, OH, United States of America (USA)), 1% (w/v) Sodium Chloride (Sigma, Gillingham, UK), 0.5% (w/v) Granulated Yeast Extract (Merck) in 4 L Milli-Q water. LB agar was prepared in a similar fashion, except that an additional 1.5% (w/v) of Select Agar (Life Technologies, Paisley, UK) was added. LB and LB agar solutions were sterilised by autoclaving for ≥ 20 minutes at 121 °C.

2.1.2 Centrifugation

Centrifugation was carried out using the 5415D microcentrifuge with the F45-24-11 or F45-36-8 rotors (Eppendorf, Westbury, NY, USA) for microtubes (Corning, Flintshire, UK), the J2-HS centrifuge with the JA-10 or JA-20 rotor (Beckman, High Wycombe, UK) for centrifuge tubes (Sarstedt, Leicester, UK) and pots (Beckman), or the Sorvall Legend RT with swing bucket or plate rotor attachments (Thermo Scientific) for conical tubes and

plates (Corning) respectively, at speeds specified in the text. Centrifugation using the J2-HS centrifuge were performed at 4°C while all other centrifugations were performed at room temperature.

2.1.3 Microscopy

Microscope slides were visualised using an Optiphot-2 optical microscope (Nikon, Kingston upon Thames, UK). Plates and flasks were visualised with a Leitz DM IRBE inverted optical microscope (Leica Microsystems, Milton Keynes, UK) with a Lumen 200 Fluorescence Illumination System (Prior Scientific, Rockland, MA, USA) for fluorescence microscopy. Microscopy images were captured using a Coolpix 4500 (Nikon) or LG US991 (LG Electronics, Huntsville, AL, USA) digital camera. High-throughput screening microscopy images were captured using the Operetta High-Content Imaging System (PerkinElmer, Llantrisant, UK).

2.2 Cells

Cells and their lineages used in these studies are described in Table 2.1. For experiments in Chapters 3, 4 and 6, cells of the HEK 293 lineage: HEK 293T/17 (Pear *et al.*, 1993) (ATCC, Manassas, VA, USA, CRL-11268) were used. Their lineage is presented in Table 2.1 and is further described in Section 1.5.1.1. These cells were previously adapted to suspension growth using sequential serum reduction (gift from L. Davies) and were grown in polycarbonate Erlenmeyer flasks (Corning). This adaptation to suspension had been done by transitioning to an increasing proportion of the new media by 25% per passage, with agitation starting when the transition is complete to generate the final cell line used here: suspension-adapted HEK 293T/17, simply referred to as HEK 293T from this point on. For suspension cultures, HEK 293T were maintained on an orbital platform with an orbital diameter of 1.9 cm (Thermo Scientific) at 135

Table 2.1 Cell lines used in the studies and their lineage

New cell line	Parental cell line	Modification	Reference	Used in
HEK 293 lineage				
HEK 293	Human embryonic kidney cells	Sheared fragments of adenovirus type 5 DNA	(Graham et al., 1977)	
HEK 293SF lineage				
HEK 293S	HEK 293	Transition to suspension	(Stillman & Gluzman, 1985)	
HEK 293SF	HEK 293S	Transition to serum-free media	(Cote *et al.*, 1998)	
293SF-CymR-rtTA2^{S}-M2 (293SF-Titr)	HEK 293SF	Doxycycline and cumate switches	(Broussau *et al.*, 2008)	Chapters 6 & 7
293SF-PacLV	293SF-Titr	Lentiviral packaging plasmids	(Broussau *et al.*, 2008)	
293SF-PacLV #29-6-14 (293SF-LVP)	293SF-PacLV	Lentiviral genome plasmid	(Broussau *et al.*, 2008)	Chapters 6 & 7
HEK 293T lineage				
HEK 293T	HEK 293	SV40 large T antigen	(Dubridge et al., 1987)	
HEK 293T/17	HEK 293T	Highly performant clone	(Pear *et al.*, 1993)	
HEK 293T (suspension)	HEK 293T/17	Transition to suspension and serum-free media	Previously established in the laboratory	Chapters 3, 4 & 6

revolutions per minute (rpm) in a humidified incubator (Thermo Scientific) at 8% CO_2 and 37°C in FreeStyle 293 Expression Medium (FreeStyle 293) (Life Technologies) without serum or antibiotics. Cell growth was monitored by determining live cell density using 0.2% trypan blue dye exclusion and a Neubauer improved haemocytometer (Sigma). Cells were passaged twice a week by diluting to 3.5 x 10^5 live cells per mL.

For siRNAs experiments, two cell lines were obtained from R. Gilbert (National Research Council of Canada, Montréal, Canada). The HEK 293-derived lentiviral vector producer cell line 293SF-PacLV #29-6-14 (293SF-LVP) was previously described (Broussau *et al.*, 2008), the lineage is indicated in Table 2.1 and further described in section 6.1.2. Production of the LVR2-GFP (rHIV.VSV-g CMV GFP) lentiviral vector (Table 2.2) is induced in these cells by the addition 1 µg/mL (w/v) doxycycline hyclate (Sigma) (from a 1 mg/mL stock in nuclease-free water) and 10 µg/mL (w/v) 4-isopropylbenzoic acid (cumate) (Sigma) (from a 10 mg/mL stock in ethanol absolute) to produce a third-generation SIN HIV-based lentiviral vector. The HEK 293-derived cell line 293SF-CymR-rtTA2^S-M2 (293SF-Titr) harbours a doxycycline switch and was used to titre the LVR2-GFP (rHIV.VSV-g CMV GFP) vector produced by the 293SF-LVP cells, which's trangene is under a doxycycline inducible promoter. These were also previously described (Broussau *et al.*, 2008) and their lineage is indicated in Table 2.1 and further described in section 6.1.2. These cells were cultured and passaged as described above for HEK 293T cells.

2.3 Plasmids

2.3.1 Lentiviral plasmids

Lentiviral vectors produced in the described experiments are listed in Table 2.2. For experiments in Chapters 3 and 4, vGM035 (rHIV.F/HN CMV- EGFPLux), a third-

Table 2.2 Viruses used in the studies and plasmids used to generate them

Virus	Genome	Gag/Pol	Rev	Pseudotype
vGM035 (rHIV.F/HN CMV- EGFPLux)	pGM290	pGM281	pRSV-Rev	pGM301 & pGM303 (F/HN)
LVR2-GFP (rHIV.VSV-g CMV GFP)	pLVR2- GFP	pMPG-CMV-Gag/polRRE	pTR5-CuO-Rev	pTR5-CuO-VSVg- IRES-GFPq (VSV-g)

Legend The plasmids used to generate vGM035 are described in Section 2.3.1 while the generation of the inducible cell line that generates LVR2-GFP is described in (Broussau *et al.*, 2008) and summarised in Section 6.1.2. Abbreviations: CMV: human cytomegalovirus immediate early enhancer and promoter, CMV-: human cytomegalovirus immediate early enhancer and promoter without intron, EGFPLux: enhanced green fluorescent protein and firefly luciferase fusion protein, F/HN: Sendai virus fusion and hemagglutinin-neuraminidase envelope pseudotype, GFP: green fluorescent protein, rHIV: recombinant human immunodeficiency virus, VSV-g: vesicular stomatitis virus G glycoprotein.

generation, HIV-based lentiviral vector expressing an enhanced green fluorescent protein (EGFP) - firefly luciferase (Lux) fusion protein (EGFPLux) under the control of a human cytomegalovirus immediate early enhancer and promoter without intron (CMV-) and pseudotyped with Sendai virus F/HN proteins, was produced by transient transfection of HEK 293T cells. This vector was generated using the HIV-1 vector genome plasmid pGM290 (a derivative of pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene #12252) where the phosphoglycerate kinase (PGK), green fluorescent protein (GFP) and WPRE sequences have been replaced with CMV from pCIKCFTR (Rose et al., 2002), EGFPLux from pEGFP-Luc (Promega), and the mut6 form of the WPRE sequence lacking an intact X protein coding sequence (Zanta-Boussif et al., 2009)). Additional HIV-1 and Sendai virus F/HN proteins were supplied by plasmids pGM281 (a monomeric form of pMDLg/pRRE (Addgene #12251), pRSV-Rev (Addgene #12253) as well as pGM301 and pGM303, derivatives of pCAGGS-Fct4 and pCAGGS-SIVct+HN (Kobayashi *et al.*, 2003a), respectively, where the ampicillin resistance gene has been substituted for a kanamycin resistance gene (Table 2.3). For virus production the plasmids pGM290, pGM281, pRSV-Rev, pGM301 and pGM303 were used in a mass ratio of 20:10:5:7:7 respectively.

2.3.2 Other plasmids

For PKR inhibitors experiments (Chapter 4), pcDNA1-PKR, (Meurs et al., 1995) pcDNA3-TRBP1, (Ong et al., 2005) pcDNA3.1-ADAR1p150-V5, (Clerzius et al., 2009) pCMV-PACT-FLAG (Laraki et al., 2008) and associated parental plasmids lacking expressed cDNA (Life Technologies) were obtained from A. Gatignol (Lady Davis Institute for Medical Research, Montréal, Canada). Each has been shown to lead to overexpression of a functional form of their respective transgene in HEK 293T cells (Clerzius et al., 2013). The pAdVAntage™ vector is commercially available (Promega).

Table 2.3 Plasmids used in the studies

Plasmid	Promoter	Gene	Source
pAdVAntage™		VAI RNA & VAII RNA	Promega
pcDNA1-empty	CMV	None	Life Technologies
pcDNA1-PKR	CMV	PKR	(Meurs *et al.*, 1995)
pcDNA3-empty	CMV	None	Life Technologies
pcDNA3-TRBP1	CMV	TRBP1	(Ong *et al.*, 2005)
pcDNA3.1-empty-V5	CMV	None (V5 tag)	Life Technologies
pcDNA3.1-ADAR1DCat-V5	CMV	ADAR1DCat (V5 tag)	(Clerzius *et al.*, 2009)
pCMV-empty-FLAG	CMV	None (FLAG tag)	Life Technologies
pCMV-PACT-FLAG	CMV	PACT (FLAG tag)	(Laraki *et al.*, 2008)
pGM281	CMV	Gag/Pol	derivative of Addgene
pGM290	CMV-	EGFPLux	derivative of Addgene
pGM301	CAG	Sendai virus F	derivative of (Kobayashi *et al.*, 2003a)
pGM303	CAG	Sendai virus HN	derivative of (Kobayashi *et al.*, 2003a)
pMD2-G	CMV	VSV-g	Addgene
pRSV-Rev	RSV	Rev	Addgene

Abbreviations: ADAR1DCat: 150 kDa adenosine deaminase, RNA-specific 1 (ADAR1) protein without its catalytic domain, CAG: modified chicken β-actin promoter with human cytomegalovirus immediate early enhancer (Miyazaki et al., 1989), CMV: human cytomegalovirus immediate early enhancer and promoter, CMV-: human cytomegalovirus immediate early enhancer and promoter without intron, EGFPLux: enhanced green fluorescent protein and firefly luciferase fusion protein, F: Sendai virus fusion envelope protein, FLAG: FLAG epitope tag, a synthetic 8-amino acid oligopeptide (Hopp et al., 1988), HN: Sendai virus hemagglutinin-neuraminidase envelope protein, PACT: PKR Activator, PKR: interferon-induced double-stranded RNA-activated protein kinase, RSV: Rous sarcoma virus, V5: V5 epitope tag, a 14-amino acid oligopeptide derived from simian virus 5 (Hanke et al., 1992), VAI RNA & VAII RNA: adenovirus virus-associated RNA I & II, VSV-g: vesicular stomatitis virus glycoprotein.

To amplify plasmids, One Shot Stbl3 Chemically Competent Cells (Life Technologies) were transformed as per the manufacturer's instructions and clones selected on LB agar plates with 100µg/mL (w/v) ampicillin sodium salt (Sigma) (from a 100 mg/mL (w/v) stock in Milli-Q water) or 50 µg/mL (w/v) kanamycin sulfate (Sigma) (from a 50 mg/mL (w/v) stock in Milli-Q water). Isolated colonies were cultured in the presence of the appropriate antibiotic for plasmid DNA extraction using the EndoFree Plasmid Mega Kit (QIAGEN, Manchester, UK), following the manufacturer's instructions, and resuspended in endotoxin-free water for injection. Plasmid concentration was assessed using a NanoDrop spectrophotometer 2000 (Thermo Scientific), which has a range of 0.002 to 15 mg/mL. A sample of 2 µL was used in triplicate reads, the purity confirmed with Optical Density 260/280 ratios close to 1.8 being acceptable.

2.4 Lentiviral vector production

2.4.1 General protocol

A summary of the steps required for lentiviral vector production is presented in Figure 2.1. The required number of 125 mL or 1000 mL flasks or wells of a 6-well plate (Corning) were seeded with HEK 293T at 1 x 10^6 ± 10% cells/mL. Subsequently, these cells were transiently transfected with a 'transfection mix' of 1.5 µg plasmid DNA per 1 x 10^6 cells using 25 kDa branched PEI (Sigma) (from a 0.1M stock in Milli-Q water) at an N:P ratio (ratio of moles of amine groups of cationic polymers to moles of phosphate groups of DNA) of 15:1 to initiate virus production. For experiments using transient transfection performed in flasks, 24 hours after transfection, the cells were centrifuged 6 minutes at 500 relative centrifugal force (rcf) and the supernatant replaced by fresh FreeStyle 293 to reduce PEI toxicity (media change). At this point, sodium butyrate (Sigma) was added at a concentration of 5 mM. After a total incubation of 72 hours, cells were harvested and pelleted by centrifugation, 6 minutes at 500 rcf. The supernatant was filtered using a

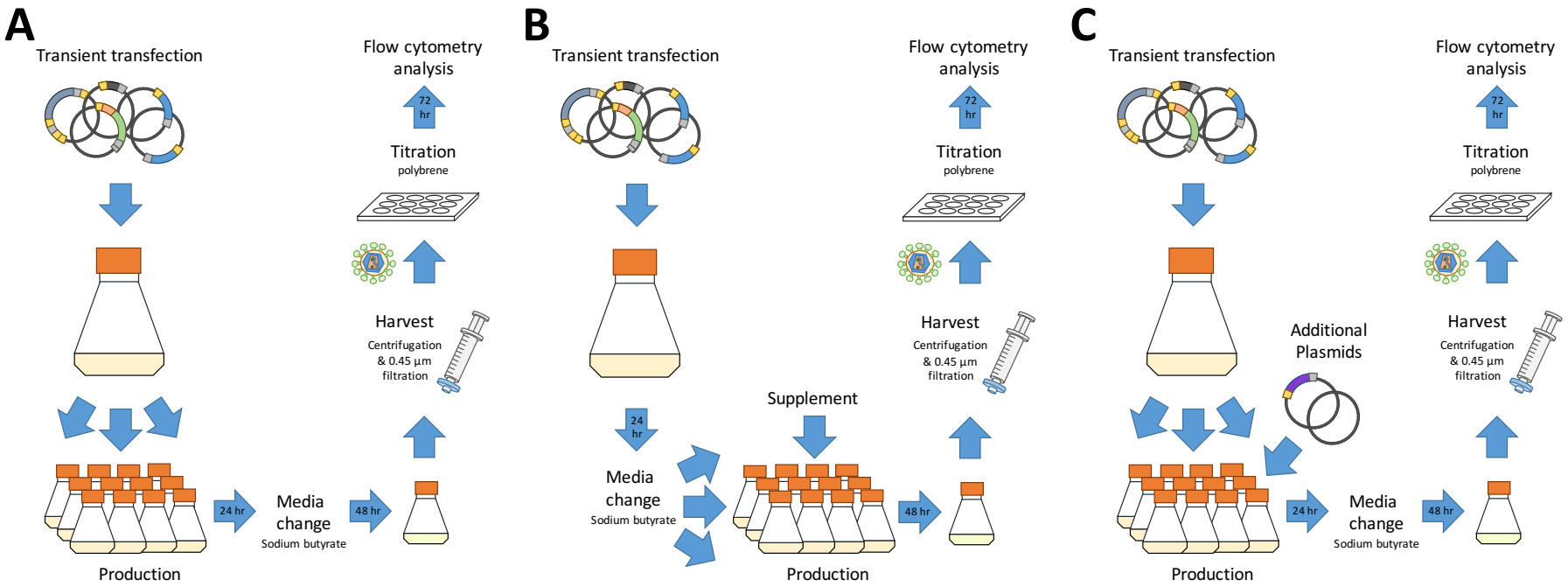

A
Transient transfection
Flow cytometry analysis
72 hr
Titration
polybrene
Harvest
Centrifugation & 0.45 µm filtration
24 hr
Media change
Sodium butyrate
48 hr
Production
B
Transient transfection
Flow cytometry analysis
72 hr
Titration
polybrene
Harvest
Centrifugation & 0.45 µm filtration
24 hr
Media change
Sodium butyrate
Supplement
48 hr
Production
C
Transient transfection
Flow cytometry analysis
72 hr
Titration
polybrene
Additional Plasmids
Harvest
Centrifugation & 0.45 µm filtration
24 hr
Media change
Sodium butyrate
48 hr
Production

Figure 2.1 Schematic representation of the different transient transfection, production and titration protocols for lentiviral vectors produced in flasks. In all cases, a large flask was seeded with Human Embryonic Kidney 293T cells (HEK 293T). These cells were transiently transfected with a 'transfection mix' of the five plasmids required for virus production complexed with polyethylenimine (PEI). In all cases except (B), the cells were then immediately split into the required number of replicates. In (C), the additional plasmids were added to each individual flask at this point. Twenty-four hours after transfection, the cells were centrifuged 6 minutes at 500 rcf and the supernatant replaced with fresh media, containing sodium butyrate, to reduce PEI toxicity (media change). In (B), the cells were split into the required number of replicates at this stage and the assayed supplement was added. After an additional 48 hours of incubation, the virus production was harvested, the cells pelleted by centrifugation and the supernatant filtered using a 0.45 µm syringe filter. A sample of the filtrate was used for titration in a 12 well plate seeded with HEK 293T in media containing hexadimethrine bromide (polybrene), the rest was frozen at -80°C. After a 72 hour incubation, the titre was determined by flow cytometry.

0.45 µm syringe filter with a Supor membrane (Pall, Portsmouth, UK) and either used immediately for titration and/or other studies or stored at -80°C.

2.4.2 Supplementation studies

A summary of the steps for supplementation studies (Chapter 3 and 4) is presented in Figure 2.1B. For these, cells were first transfected in a single 1000 mL flask, to ensure equivalent transfections for each condition. Twenty-four hours after transfection, the cells were centrifuged and, to reduce PEI toxicity, the supernatant replaced by 225 mL fresh FreeStyle 293 containing sodium butyrate at a concentration of 5mM. The cells were then split into the required number of 125 mL flasks containing 18 mL volume in each to evaluate the different supplements. The supplements tested were: Cholesterol Lipid Concentrate (CLC), Chemically Defined Lipid Concentrate (CDLC) (Life Technologies), Lipid Mixture 1 (Sigma), Gelatin Peptone N3, (Organotechnie, La Courneuve, France), N-Acetyl-L-Cysteine (NAC) (Sigma) and Pluronic F-68 (Life Technologies). Each was diluted to the required concentration in FreeStyle 293, filtered using a 0.2 µm surfactant-free cellulose acetate filter (Thermo Scientific) and 2 mL was added to three replicate flasks. For each experiment, three flasks were not supplemented and had only 2 mL of FreeStyle 293 added to act as the non-supplemented control. One experiment, evaluating 2-aminopurine (Sigma), was performed in a 6-well plate following the procedure described in 2.4.1, but skipping the media change and with supplementation 24 hours after transfection of the producer plasmids.

2.4.3 Studies involving additional plasmids

A summary of the steps for studies with additional plasmids (Chapter 4) is presented in Figure 2.1C. For these, cells were first transfected in a single 1000 mL flask, to ensure equivalent transfections for each condition. Smaller 125 mL flasks with 20 mL volume in

each were then generated to introduce the different additional plasmids transfected using Lipofectamine 2000 (Life Technologies) as per the manufacturer's protocol or PEI if specified. One experiment, evaluating pAdVAntage™, was performed in a 6-well plate following the procedure described in 2.4.1, but skipping the media change and with Lipofectamine 2000 transfection of pAdVAntage™ directly after that of the producer plasmids.

2.4.4 Lentiviral vector production for siRNA studies

The final siRNA screening protocol is summarised in Figure 2.2. The protocol was adapted from Section 2.4.1 and its development is described in detail in Chapter 6. A clear polystyrene 96-well flat bottom plate (Corning) was used in which the two external rows and columns (A, B, G and H / 1, 2, 11 and 12) were filled with 200 µL Dulbecco's phosphate-buffered saline (DPBS) to minimise evaporation in the central experimental wells. The 32 central experimental wells were seeded with 2 x 10^4 ± 10% of 293SF-LVP cells in 80 µL FreeStyle 293. Seeded plates were left undisturbed for a minimum of 10 minutes before being used to ensure uniform cell seeding. The ON-TARGET Plus siRNAs (GE Dharmacon, Little Chalfont, UK) at 1 µM, in 45 µL FreeStyle 293, were incubated for 5 minutes with a mixture of 2.25 µL Lipofectamine 2000 in 42.75 µL FreeStyle 293. Of this mix, 20 µL was added to each appropriate well for a final siRNA concentration of 100 nM with 0.5 µL of Lipofectamine 2000 per well. Three days later, lentiviral vector production was activated by adding 10 µL of a 0.2 µm-filtered 10 µg/mL doxycycline and 100 µg/mL cumate mix in FreeStyle 293 for a final concentration of 1 µg/mL doxycycline and 10 µg/mL cumate in each well (assuming 10 µL original volume loss by evaporation over three days). After three days of production, the supernatant of each well was transferred to a 96-well 0.2 µm filter plate (Corning) and centrifuged 6 minutes at 500 rcf to collect the filtrate in a clear 96-well-plate. In one instance, the plate

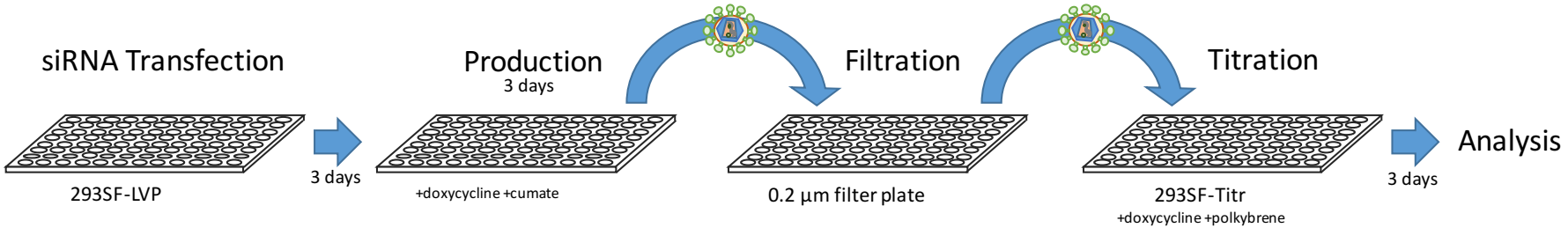


Figure 2.2 Schematic representation of the lentiviral vector production and titration protocols for siRNA experiments. On the first day of the experiment, 293SF-PacLV #29-6-14 (293SF-LVP) cells were seeded and each well is transfected with a different siRNA pool. After three days, virus production was initiated by the addition of doxycycline and cumate. After an additional three days, the production was harvested by filtration using a 0.2 µm 96-well filter plate. A sample of each well was then applied to the corresponding well of a titration plate seeded with 293SF-CymR-rtTA2s-M2 (293SF-Titr) cells in media containing doxycycline and hexadimethrine bromide (polybrene). After three days, the titration plate was analysed. A more detailed description in presented in Section 2.6.

used for production was read for single well relative fluorescence using an Envision (PerkinElmer) before the harvest step.

2.5 Lentiviral vector titration

2.5.1 Functional titre by flow cytometry

All lentiviral vectors produced in flasks or 6-well plates (Section 2.4.1) directed the expression of EGFP through the EGFPLux transgene (Table 2.2). The titre for these was determined by counting EGFP positive transduced cells by flow cytometry. Before titration, the virus being pseudotyped with Sendai virus F/HN proteins (vGM035; Table 2.2), was activated for 1 hour at 37°C with 7.5 µL of a 1:10 dilution in FreeStyle 293 of 0.05% (w/v) trypsin (Sigma) per 500 µL of virus stock and the reaction was then stopped by the addition of 50 µL defined trypsin inhibitor (Life Technologies). HEK 293T cells were seeded at 5 x 10^5 ± 10% cells per well in a 12-well plate (Corning) in FreeStyle 293 containing 8 µg/mL (w/v) polybrene (Sigma). The cells were subsequently transduced by the addition of diluted virus stocks – typically 1:5 diluted virus in FreeStyle 293, though other dilutions were used as necessary to ensure the proportion of transduced cells did not exceed ~20%. After 72 hours' incubation, EGFP production was confirmed by fluorescence microscopy and the cells were harvested in 5 mL polystyrene round-bottom tubes (Corning) and centrifuged 6 minutes at 500 rcf. Pellets were resuspended in 350 µL of flow cytometry buffer containing 10% (w/v) Bovine Serum Albumin (BSA), 10mM EDTA and 2% (v/v) paraformaldehyde (PFA) (Sigma) in DPBS. The number of EGFP positive cells was determined using a FACSCalibur (BD, Oxford, UK) cell sorter and its associated CellQuest Pro 6.0 (BD) software (Figure 2.3). Titres were obtained by gating for EGFP positive cells, compared to triplicate untreated samples average set at ≤ 0.05% EGFP positive cells, and converting the obtained percentage into flow cytometry

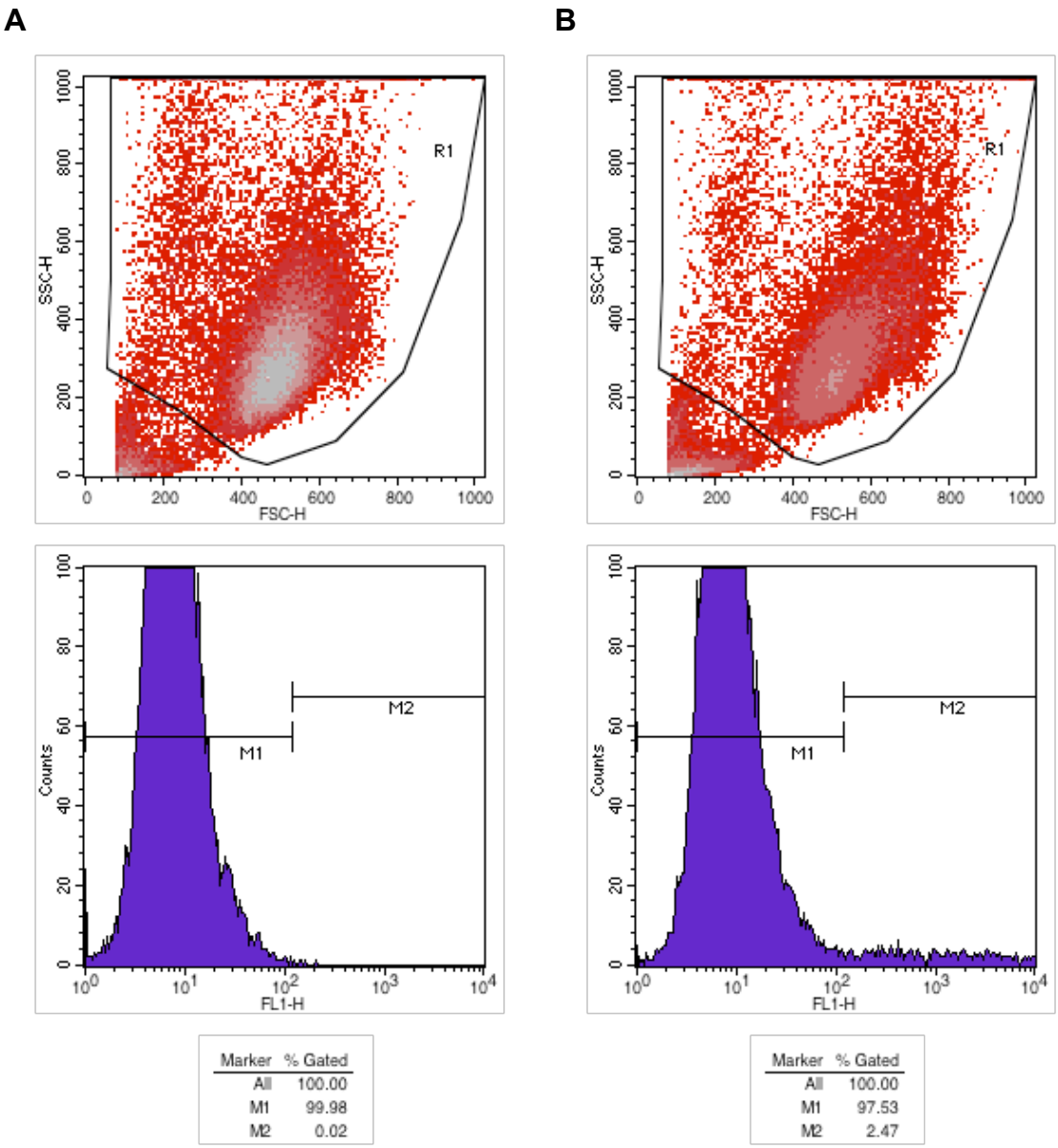


Figure 2.3 Sample of data used to measure functional titre by flow cytometry. Titres were obtained by gating for cells (top graph) as determined by forward and side scatter. The percentage of EGFP positive cells was established by comparison to an untreated sample (A) where a gate was set at ≤ 0.05% EGFP positive cells for the average of the triplicate samples. The obtained gated percentage for the evaluated sample (B) was converted into flow cytometry transducing units (FTU), a measure of functional titre, by multiplying by the number of cells per well and the sample dilution. For each experiment, titres were normalised with the control condition of each experiment set at 100%.

transducing units (FTU), a measure of functional titre, by multiplying by the number of cells per well and the sample dilution. For each experiment, titres were normalised with the control condition of each experiment set at 100%.

2.5.2 Functional titre using the Operetta

This protocol was adapted from Section 2.5.1, and its development is described in Chapter 6. A black 96-well with a clear flat bottom plate (PerkinElmer) was used in which the two external rows and columns (A, B, G and H / 1, 2, 11 and 12) were filled with 200 µL DPBS to minimise evaporation in the central experimental wells. The 32 central experimental wells were seeded with 5 x 10^3 ± 10% of 293SF-Titr cells in 80 µL of FreeStyle 293 containing 8 µg/mL (w/v) polybrene. Seeded plates were left undisturbed for a minimum of 10 minutes before being used to ensure uniform cell seeding. Either 15, 30 or 45 µL of production filtrate was applied to these cells for transduction as indicated in the text. Three days later, the plates were sealed with a Ranin Clear Heat Seal (Ranin, Luton, UK) using the ALPS 50V plate sealer (Thermo Scientific) and the plates read on the Operetta High-Content Imaging System, at the Target Discovery Institute (Oxford, UK). Cell images from 15 fields in each well were taken and the number of EGFP positive cells per well was counted using the Harmony high-content imaging and analysis software with the PhenoLOGIC plug-in (PerkinElmer). For each plate, titres were normalised with the control condition of each experiment set at 100%.

2.7 Statistical analyses

Where possible, statistically significant differences between group means were determined by Student t-tests, Mann-Whitney tests or one-way analysis of variance (ANOVA) using Prism 7, (GraphPad, La Jolla, CA, USA). ANOVA results were reported as F(k-1, N-k) = F value, p = p value, where k is the number of groups and N the number

of data points. This was followed, if appropriate, by Dunnett's post-test to compare each of a number of treatments with a single control. Correlation between sets of data was analysed using the Pearson correlation coefficient with a 95% confidence interval.

A calculated p value of > 0.05 was deemed non-significant (indicated with 'ns' on figures). Calculated p values of < 0.05 were deemed a significant difference and indicated with one star (*) on figures. More statistically significant p values of < 0.01, and < 0.001 were indicated with two stars (**) and three stars (***), respectively. Error bars in figures represent the standard error of the mean (SEM). Statistical power was evaluated using G*Power 3.1 (Faul et al., 2007) (University of Düsseldorf, Düsseldorf, Germany).

2.8 Nomenclature

Human genes are identified with their HUGO Gene Nomenclature Committee official symbol (Gray et al., 2015). Alternative names, such as resulting protein names or former names, used in cited papers are indicated in parentheses in tables as well as at their first mention in the text for clarity.

Chapter 3 – Lentivirus vector production optimisation through media supplementation

3.1 Introduction

3.1.1 Serum-free media

Production of viral gene therapy products often involves the use of bovine serum in cell culture. This supplementation is performed in order to provide a source of essential nutrients to the cells and increase virus production yields. The use of serum in cell culture poses ethical considerations (Reviewed in Even et al., 2006) as well as a potential risk of transfer of viruses or prions into the final product (World Health Organisation, 1997). While extensive contaminant testing could address the safety issue, it can be mitigated by careful tracking of the reagents' sources (i.e. country of origin, batch number, etc.). It does, however, remain a risk, which must be assessed and reported (Food and Drug Administration, 2008). Furthermore, immune responses caused by the use of bovine serum have been observed in bone marrow transplant, (Macy et al., 1989) cell therapy (Selvaggi et al., 1997; Mackensen et al., 2000) and gene therapy (Tuschong et al., 2002) clinical trials. As a result, there is regulatory pressure to reduce or remove animal serum from production processes (World Health Organisation, 1997). Moreover, if serum continues to be used, supply limitation of certified material is another issue which could arise, with demand rising as more products reach the stage of large-scale manufacture (Brindley et al., 2012). While human serum in cell culture (Kurita et al., 2008) can be used as an alternative for small-scale manufacturing, it is unlikely to be a viable option, in terms of both the supply and cost, for long-term large-scale manufacturing.

Another option is serum-free production. Lentiviral vectors have been produced in the absence of serum, reducing the immunogenicity of the final product without affecting final transduction efficiency (Baekelandt *et al.*, 2003). In turn, efforts to reduce the reliance on serum, and to provide more completely defined mammalian cell growth conditions, have led to the development of specialised media that are both serum-free and protein-free. The UKCFGTC, after extensive testing and validation, has selected FreeStyle 293 (Section 2.2) for its lentivirus production (L Davies, personal communication) and, for simplicity, this media was also used here for suspension cultures. FreeStyle 293 is a chemically defined, animal origin-free, protein-free medium developed to support growth and transfection of FreeStyle 293-F cells, a fast-growing clonal isolate of HEK 293 that has been adapted to suspension culture in serum-free media. FreeStyle 293 was previously demonstrated in media selection experiments to support high transfection efficiency with a range of transfection reagents (L. Davies, personal communication). In addition, FreeStyle 293 is manufactured at a cGMP-compliant facility and is marketed as suitable for cGMP production of vectors. It contains the GlutaMAX™ supplement, marketed as minimising toxic ammonia build-up and improving cell viability; however, the rest of its formulation is proprietary. It is, therefore, not obvious to assess if constituents usually present in serum added as a supplement might be lacking for optimised lentiviral vector production.

3.1.2 Lentiviral vector production needs

Lentiviruses are enveloped viruses, therefore as newly developed virions bud from producer cells they capture a portion of the cell membrane (Figure 1.1), which then constitutes a key component of the viral particle. Viral and host cell membranes are of different composition, the HIV envelope is, for example, more ordered than the host cell membrane from which it originated (Aloia et al., 1993). HIV-1 buds from areas of the

plasma membrane with high levels of lipid rafts (Nguyen & Hildreth, 2000) which are organised areas enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins (Simons & Ikonen, 1997). It has been hypothesised that these components are preferentially incorporated in the viral envelope as a result of preferential sorting of HIV-1 Gag to lipid rafts (Nguyen & Hildreth, 2000). More specifically, the molar ratio of cholesterol to phospholipid is about 2.5 times higher in virion envelopes compared to the host cell membrane (Aloia *et al.*, 1993) and the levels of several other raft lipids and proteins are elevated in virions compared with their progenitor cells.

3.1.2.1 Cholesterol and HIV-1

Cholesterol levels in the cell are usually reduced upon viral infection and interferon (IFN) β treatment (Blanc et al., 2011). However, primary cells and cell lines infected with HIV-1 increase the expression of several endogenous enzymes of the cholesterol biosynthesis pathway. This includes HMGCR, the enzyme responsible for the rate-limiting step in cholesterol biosynthesis, and SREBF-2, the transcription factor regulating the cholesterol biosynthesis pathway (van 't Wout et al., 2005a). The increase in the expression of cholesterol biosynthesis enzymes ultimately results in an increase in cholesterol production (van 't Wout et al., 2005a). This effect was, however, only observed in cells infected with a virus containing a functional Nef protein (Section 1.2.1.1), suggesting that Nef is responsible for the regulation of this pathway and the increased cholesterol biosynthesis (van 't Wout et al., 2005a). As previously described, second- and third-generation lentiviral vectors do not contain Nef (Zufferey *et al.*, 1997). The effect of exogenous cholesterol on these vectors is, therefore, unknown.

3.1.3 Aim

The hypothesis driving this chapter is that the exodus of proteins and lipids that occurs during virus budding could deplete the producer cells' reserves, and ultimately viability, leading to a lower rate of virus production. To address this, the effect of the addition of different media supplements to the producer cell growth medium is explored in this chapter with a view to increasing lentiviral vector production titres.

3.2 Results

3.2.1 Experimental setup

The vGM035 (rHIV.F/HN CMV- EGFPLux) vector (Table 2.2) was used throughout the experiments described below. An HIV-1-based vector was chosen because it is relatable to the HIV-1 literature concerning growth conditions and the EGFPLux transgene because it allows simple quantification of titres through flow cytometry. The Sendai virus F/HN pseudotype is being evaluated by the UKCFGTC and has not yet been described in terms of the use of media supplementation for enhanced yields.

In order to measure the effect of different supplements on the production of vGM035 (rHIV.F/HN CMV- EGFPLux), an experimental method that could result in reproducible lentivirus yields was required. Preliminary studies indicated that the transient transfection step was a source of potential variability between experiments. Hence, two strategies were investigated: a multiple independent transfections strategy, where 12 flasks of cells were transfected independently, and a single-flask transfection strategy, where cells in a single, large flask were transfected and then subsequently split into 12 smaller flasks.

For the multiple independent transfections strategy, 20 mL of HEK 293T cells at 1 x 10^6 cells/mL were seeded in 12 separate 125 mL flasks. Twelve independently prepared

transfection mixtures (each comprising 30 μg of plasmid DNA) were used to transfect the flasks. The media was changed after 24 hours and the vGM035 (rHIV.F/HN CMV-EGFPLux) virus (Section 2.4.1) generated was harvested after a further 48 hours' incubation. The viral titre for each of the 12 independent cultures, as determined by flow cytometry (Section 2.5.1), is shown in Figure 3.1A.

For the single-flask transfection strategy, a single 'transfection mix' (comprising 375 μg of the vector plasmid DNA mixture) was prepared and used to transfect 250 mL of HEK 293T cells at 1 x 10^6 cells/mL seeded into one 1000 mL flask. Twenty-four hours after transfection, the media was changed by centrifugation (Section 2.4.1) and the culture divided between 12 separate 125 mL flasks, 20 mL per flask. The vGM035 (rHIV.F/HN CMV- EGFPLux) virus generated was harvested after a further 48 hours' incubation. The virus titre for each flask was determined using flow cytometry (Section 2.5.1) and results are shown in Figure 3.1B. This approach more closely resembles that adopted when using a stable producer cell line where all cultures are derived from a single, typically clonal, source. Figure 2.1A summarises the steps of this protocol.

As shown in Figure 3.1A and B, the absolute titres of vGM035 (rHIV.F/HN CMV-EGFPLux) generated by these two approaches vary largely between replicates. To be able to compare the performance of different supplements, titres were normalised to the average titre of the non-supplemented flasks in each experiment. Comparing the normalised titration results from the multiple independent transfections strategy (mean of twelve flasks: 100, standard deviation (SD): 93.5) with those from the single-flask transfection strategy (mean of twelve flasks: 100, SD: 51.4) indicated that there was less variation observed using the single-flask transfection strategy. Using these data to model future studies incorporating three replicates, statistical power analysis demonstrated the

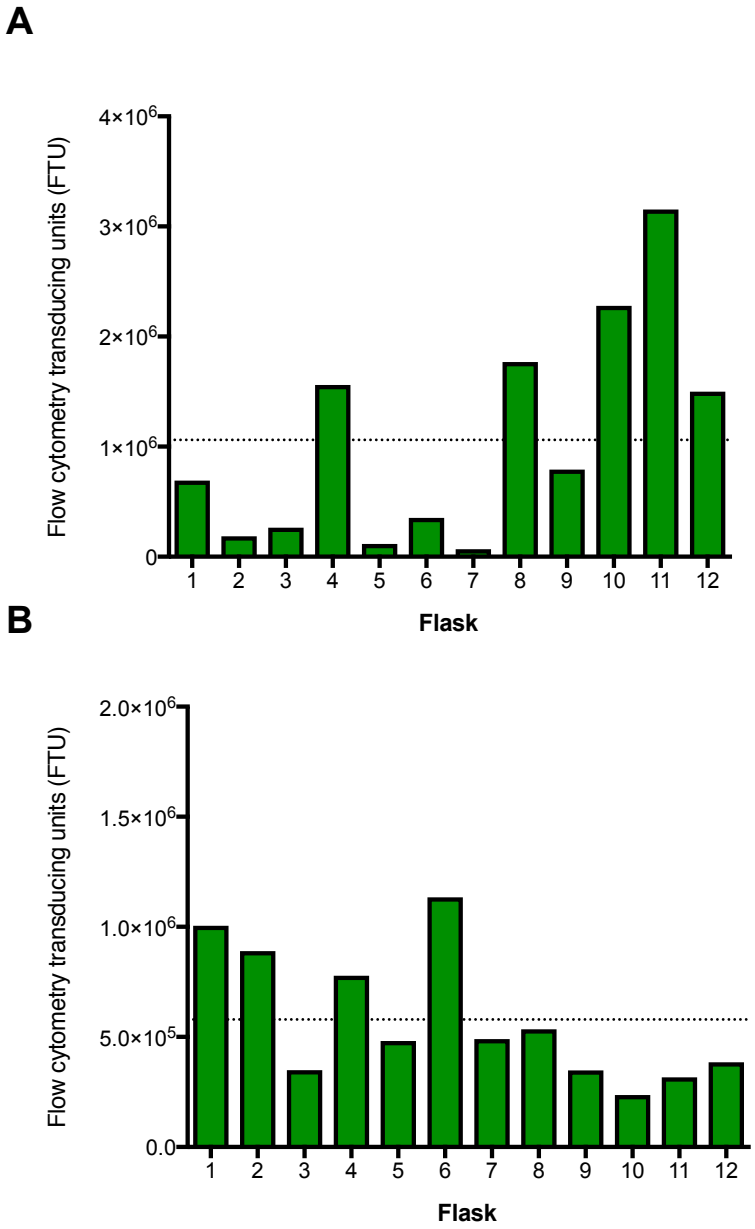

A
4×10^6
3×10^6
2×10^6
1×10^6
0
Flow cytometry transducing units (FTU)
1 2 3 4 5 6 7 8 9 10 11 12
Flask
B
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
Flow cytometry transducing units (FTU)
1 2 3 4 5 6 7 8 9 10 11 12
Flask

Figure 3.1 Lentiviral vector production yields for the multiple independent transfections strategy flask and the single-flask transfection strategy. Individual vGM035 (rHIV.F/HN CMV- EGFPLux) yields from flasks containing non-supplemented medium from A) multiple independent transfections strategy where 12 independent 125 mL flasks containing 20mL HEK 293T cells at 1 x 10^6 cells/mL were transfected with independently prepared transfection mixes containing the five plasmids necessary to produce vGM035 (rHIV.F/HN CMV- EGFPLux), or B) single-flask transfection strategy where 250 mL HEK 293T cells at 1 x 10^6 cells/mL in a 1000 mL flask were transfected with the five plasmids necessary to produce vGM035 (rHIV.F/HN CMV- EGFPLux) and subsequently divided into 12 separate 20 mL cultures into 125 mL flasks. Titres were measured by flow cytometry (Section 2.5.1) and shown in flow cytometry titration units (FTU). The dashed lines show the average titre for all flasks for each strategy.

superiority of the single-flask transfection strategy (Section 2.6). Using the multiple independent transfections approach a minimum of a 3.33-fold increase in titre would be necessary to observe a statistical difference with 80% power using triplicates, whereas the multiple independent transfections approach, the single flask approach could readily distinguish a 2.28-fold increase in titre with the same power. Given the reduced variability, and the anticipated lower threshold for reaching statistical differences when comparing experimental groups, the single-flask transfection strategy was selected for all subsequent experiments here and in Chapter 4.

3.2.2 Effect of lipid supplementation on lentiviral vector yields

3.2.2.1 Cholesterol Lipid Concentrate (CLC)

To address the possibility of lipid depletion in cells producing lentiviral vectors, several lipid supplements have been investigated with the aim of increasing lentiviral vector productivity and/or infectivity. Of these, cholesterol has been shown previously to significantly improve production (1.89-fold), titres (11.71-fold) and infectivity (6.15-fold) of a VSV-g-pseudotyped lentiviral vector (Chen et al., 2009).

CLC is a commercial, animal-free, cholesterol-based proprietary formulation sold as a 250x concentrate suitable for cGMP manufacturing. The addition of the recommended amount of 1X CLC to HEK 293T cells, prior to transfection, was investigated previously in the laboratory and resulted in decreased viral yield up to 2-fold (L. Davies, personal communication). One potential explanation for this unexpected adverse effect of cholesterol supplementation was that it had a negative effect on HEK 293T cell transfection with the producer plasmid mixture. To mitigate the impact of this potential effect, a study was performed where CLC supplementation was carried out during vGM035 production 24 hours post-transfection.

Post-transfection CLC supplementation was investigated using the single-flask transfection strategy described above (Section 3.2.1). The steps for supplementation studies are presented in Figure 2.1B. A single 1000 mL flask containing 1 x 10^6 cells/mL in 250 mL media was transfected with the plasmid mixture required to produce vGM035. Twenty-four hours after transfection, the flasks were centrifuged for media change (Section 2.4) and the pelleted cells resuspended in 225 mL FreeStyle 293 with sodium butyrate. The culture was then divided between 12 separate 125 mL flasks with (18 mL per flask). Triplicate flasks were supplemented with 2 mL of CLC diluted in FreeStyle 293 for final concentrations of 0.5X, 1X or 2X CLC in a final volume of 20 mL. Three flasks, where 2 mL of FreeStyle 293 without CLC were added, served as a triplicate negative control. Virus produced under these conditions was harvested 48 hours after supplementation and titrated as described in Section 2.4. It is relevant to note that some of the supplement in the production media is carried over to the titration plates. It is not, however, expected to have a significant effect as the volume of production supernatant used for titration represents onlyb about 0.5% of the volume in the titration wells. Contrary to supplementation with CLC before transfection, here, there was no significant change in titre with any of the CLC concentrations evaluated post-transfection (Figure 3.2A), although a trend for inhibition of virus production was noted with 2x CLC.

Methyl-beta-cyclodextrin (mβCD) is used as a complexing agent in the CLC formulation to enhance solubility in water-based tissue culture media (Christian et al., 1997). Interestingly, mβCD can selectively extract cholesterol from the plasma membrane (Kilsdonk et al., 1995; Klein et al., 1995), which can affect cellular processes such as endocytosis (Rodal et al., 1999) as well as cell sensitivity to HIV-1 infection (Liao et al., 2001; Viard et al., 2002). A number of studies have explored the inhibitory impact of mβCD on HIV-1 yields (Ono & Freed, 2001) and infectivity (Campbell et al., 2002; Guyader et al., 2002). This impact appears less when pseudotyping vectors with VSV-g

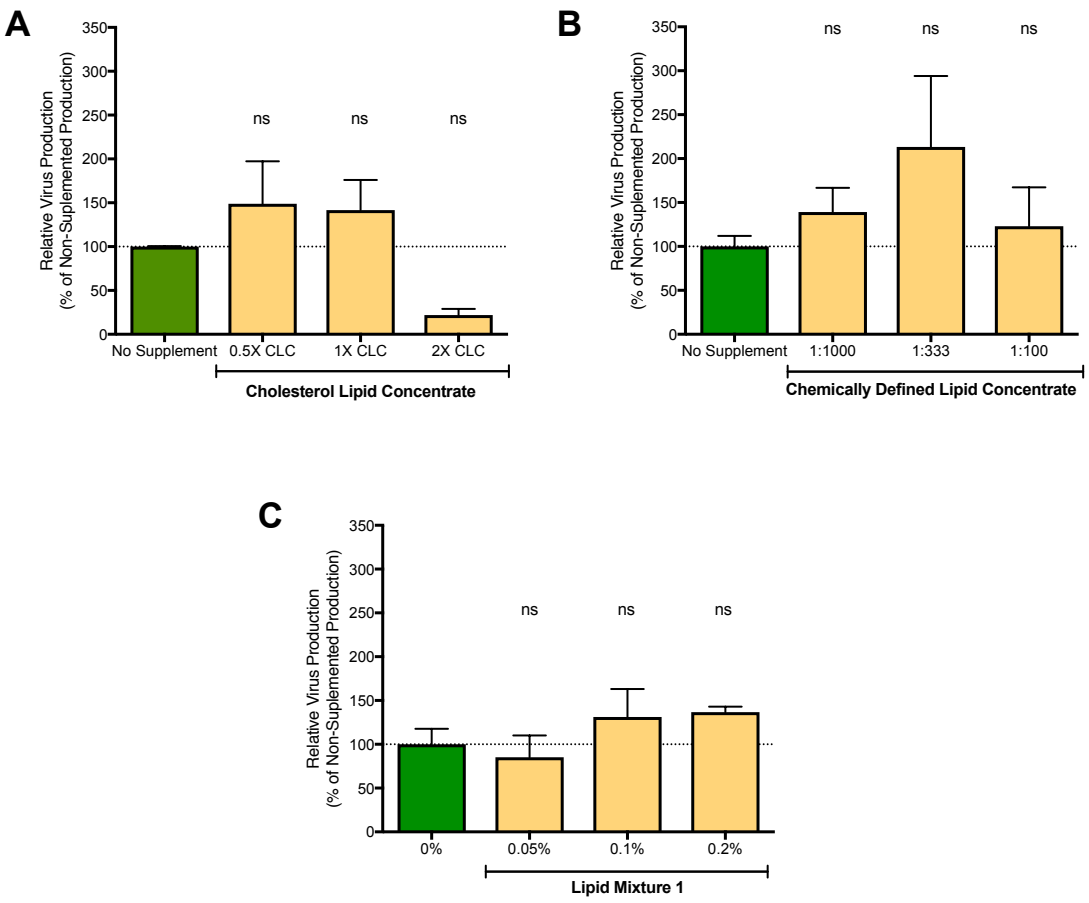


Figure 3.2 Lentiviral vector titres following lipid medium supplementation. vGM035 (rHIV.F/HN CMV- EGFPLux) yields in non-supplemented medium (dark green) or medium supplemented (yellow) with A) Cholesterol Lipid Concentrate, B) Chemically Defined Lipid Concentrate or C) Lipid Mixture 1. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to non-supplemented values. Bars represent the mean of triplicate production studies ± SEM. There were no statistically significant differences between group means as determined by one-way ANOVA: A) $F(3,8) = 3.798$, $p = 0.0582$, B) $F(3,8) = 1.025$, $p = 0.4315$, C) $F(3,8) = 1.230$, $p = 0.3606$). A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the charts.

(Guyader *et al.*, 2002), although the effect, if any, on a Sendai virus F/HN pseudotype (as used here) is unknown. A possible solution to this problem would be to exclude mβCD from the cholesterol supplement, however, the CLC manufacturer was unable to produce either mβCD-free CLC or CLC-supplemented medium (Life Technologies, personal communication). It was, therefore, determined that CLC could not be used as a possible supplement at this stage.

3.2.2.2 Chemically Defined Lipid Concentrate (CDLC)

Due to the possible issues of mβCD-containing supplements, an alternative mβCD-free supplement containing cholesterol, CDLC, was evaluated. CDLC is a concentrated lipid emulsion of saturated and unsaturated fatty acids and surfactants, designed to reduce or replace foetal bovine serum in cell culture media. The impact of CDLC addition on vGM035 production using the single-flask transfection strategy was evaluated. A single 1000 mL flask containing 1 x 10^6 cells/mL in 250 mL media was transfected with the plasmid mixture required to produce vGM035. Twenty-four hours after transfection, the flasks were centrifuged for media change (Section 2.4) and the cells resuspended in 225 mL FreeStyle 293 with sodium butyrate. The culture was then divided between 12 separate 125 mL flasks (18 mL per flask). CDLC is supplied as a concentrate (Section 2.4) and the manufacturer's guidelines recommended evaluating dilutions ranging from 1:100 to 1:1000. Triplicate flasks were supplemented with 2 mL of CDLC diluted in FreeStyle 293 for final dilutions of 1:1000, 1:333 or 1:100 of CDLC in a final volume of 20 mL. Three flasks, where 2 mL of FreeStyle 293 were added without CDLC, served as a triplicate negative control. Virus produced under these conditions was harvested 48 hours after supplementation and titrated as described in Section 2.4. Although a trend for increased virus production in the presence of CDLC was observed, there was no significant change in titres with any of the CDLC concentrations evaluated (Figure 3.2B).

3.2.2.3 Lipid Mixture 1

A lentiviral vector producer cell line used extensively in Chapter 6 and 7, 293SF-LVP (Table 2.1), was recommended to be cultured in a low-calcium, serum-free media containing the commercial cell culture supplement Lipid Mixture 1 (Broussau *et al.*, 2008). According to the manufacturer, Lipid Mixture 1 contains non-animal derived fatty acids (2 µg/mL arachidonic and 10 µg/mL each linoleic, linolenic, myristic, oleic, palmitic and stearic), 0.22 mg/mL cholesterol from New Zealand sheep′s wool, 2.2 mg/mL Tween-80, 70 µg/mL tocopherol acetate and 100 mg/mL Pluronic F-68 solubilised in cell culture water. Lipid Mixture 1 has been shown to increase 25 kDa linear PEI transient transfection efficiency and, when used at 0.1% v/v, to not affect cell growth in HEK 293-derived cells (Pham et al., 2003). The impact of Lipid Mixture 1 on lentiviral vector production yields was, therefore, assessed here. As described in Section 3.2.1, a single, large flask of transfected cells was prepared and, 24 hours later, split into 12 flasks after the media change, at which point sodium butyrate was added. Triplicate flasks were supplemented with half (0.05%), once (0.1%) or twice (0.2%) the reported beneficial concentration of Lipid Mixture 1, or with FreeStyle 293 only, as a negative control. Virus produced under these conditions was harvested 48 hours after supplementation and titrated as described in Section 2.4. Figure 3.2C shows that none of the concentrations had a significant impact on virus titre.

3.2.3 Effect of protein supplementation on lentiviral vector yields

Proteins are another component of the cell membrane that could be depleted by virion budding. The producer cell line used in Chapter 6 and 7, 293SF-LVP (Table 2.1), was originally grown in a low-calcium serum-free media containing the animal-derived Gelatin Peptone N3 supplement (Broussau *et al.*, 2008). Gelatin Peptone N3 is manufactured by a controlled enzymatic hydrolysis of gelatine and has been shown to have a positive

effect on cell growth and transfection efficiency in HEK 293 derived cells at a concentration of 0.5% v/v (Pham *et al.*, 2003). The impact of Gelatin Peptone N3 on production yields was, therefore, assessed. As above, a single, large flask of transfected cells was prepared and, 24 hours later, split into 12 flasks after the media change, at which point sodium butyrate was added. Triplicate flasks were supplemented with half (0.25%), once (0.5%) or twice (1%) the optimal reported concentration, or with FreeStyle 293 only, as a negative control. Virus produced under these conditions was harvested 48 hours after supplementation and titrated as described in Section 2.4. Figure 3.3 shows that none of the concentrations of Gelatin Peptone N3 tested had a significant impact on virus titre, although a trend for inhibition of virus production was noted with 1% supplementation.

3.2.4 Effect of N-Acetyl-L-Cysteine (NAC) supplementation on lentiviral vector yields

Medium supplements that are not membrane components, but that can still affect cell viability, were also assayed. NAC is a widely used mucolytic agent and paracetamol overdose management medication with a general anti-apoptotic effect (Mayer & Noble, 1994) and as a result is also used as a media supplement in the manufacture of some cell therapy products (B. Levine, personal communication). NAC is also known to have effects on HIV-1, inhibiting HIV-1 LTR-directed gene expression (Roederer et al., 1990), suggesting an inhibitory effect on HIV-1 transcription. NAC was consequently assayed as an anti-HIV-1 drug, showing no benefit in asymptomatic patients (Akerlund et al., 1996), but possible benefit if taken before initiating anti-retroviral therapy (Spada et al., 2002). In HIV-1 replication studies in cell culture, however, a dose-dependent effect of NAC has been observed on HIV-1 yields (Pani et al., 1993). NAC doses of 0.12 and 0.25 mM decreased the infectious HIV-1 yield up to two-fold and cell viability to 75% of

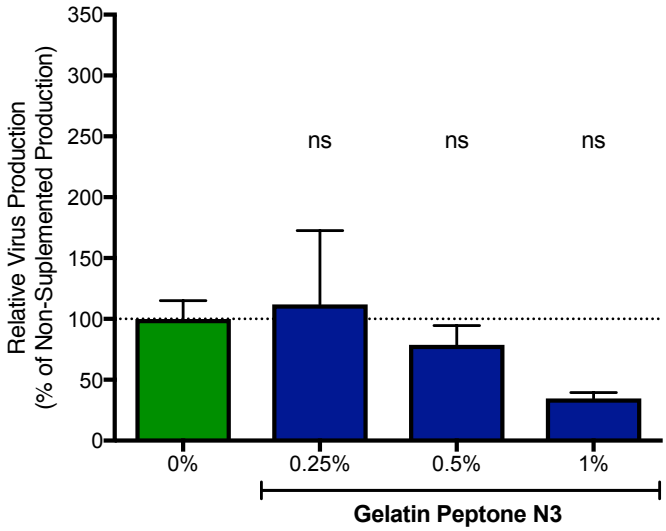


Figure 3.3. Lentiviral vector titres following Gelatin Peptone N3 medium supplementation. vGM035 (rHIV.F/HN CMV- EGFPLux) yields were measured in non-supplemented medium (dark green) or medium supplemented (dark blue) with Gelatin Peptone N3. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to non-supplemented values. Bars represent the mean of triplicate production studies ± SEM. There were no statistically significant differences between group means as determined by one-way ANOVA ($F(3,8) = 1.113$, $p = 0.3993$). A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart.

the level of non-supplemented controls. By contrast, NAC doses of 0.5-2 mM increased HIV-1 yield up to two-fold, while increasing cell viability to 120% of the level of non-supplemented controls, demonstrating a correlation with NAC's modulation of cell growth. Finally, in chronically infected T lymphocytes, high NAC concentrations (4-16 mM) increased the cell proliferative rate increasing virus multiplication 4- to 6-fold, although, at such concentrations, NAC also had an inhibitory effect in acutely infected cells, interfering with early events in the life cycle and reducing titres up to 4-fold (Pani *et al.*, 1993).

The impact of NAC on lentiviral vector production yields was, therefore, assessed. As described in Section 3.2.1, a single, large flask of transfected cells was prepared and, 24 hours later, split into 12 flasks after the media change for supplementation, at which point sodium butyrate was added. In an attempt to cover the reported range of concentrations affecting HIV-1 yields and cell proliferation (Pani *et al.*, 1993) three doses were assayed: 0.25 mM (reported to decrease HIV-1 yields), 1mM (reported to increase HIV-1 yields) and 4 mM (reported to increase cell proliferation, but to decrease titres in acutely infected cells) as well as a FreeStyle 293 only control. Virus produced under these conditions was harvested 48 hours later and titrated as described in Section 2.4. The results showed that, under the conditions used here, the two lower concentrations did not have a significant impact on virus production yield, contrary to previous reports using different conditions. However, the higher NAC dose had a significant ($p < 0.05$) two-fold adverse impact on virus titre (Figure 3.4A) and consistent with NAC's reported HIV-1 interference capacity at high doses in acutely infected cells (Pani *et al.*, 1993).

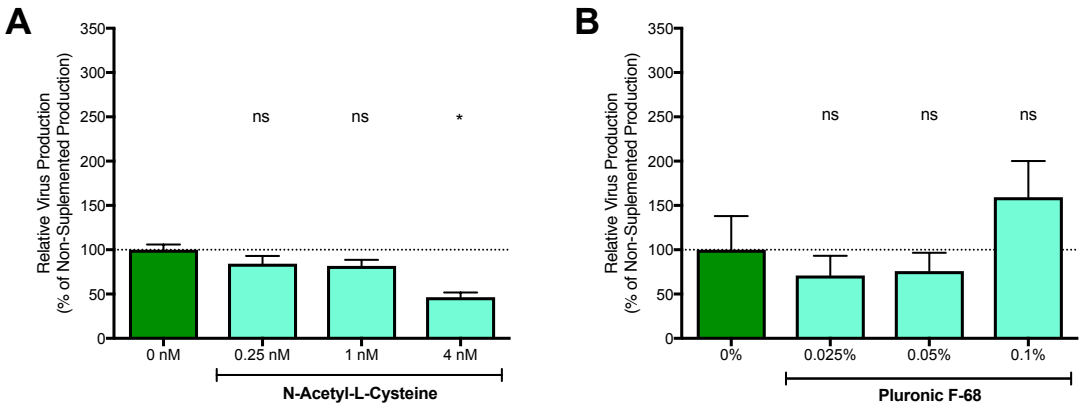


Figure 3.4. Lentiviral vector titres following N-acetyl-L-cysteine (NAC) and Pluronic F-68 medium supplementation. vGM035 (rHIV.F/HN CMV- EGFPLux) yields were measured in non-supplemented medium (dark green) or medium supplemented (turquoise) with increasing concentrations of A) NAC or B) Pluronic F-68. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to non-supplemented values. Bars represent the mean of triplicate production studies ± SEM. Statistically significant differences between group means were determined by one-way ANOVA: A) $F(3,8) = 11.04$, $p = 0.0032$, B) $F(3,8) = 1.621$, $p = 0.2598$, followed, if appropriate, by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on figures. Calculated p values of < 0.05 were deemed a significant difference as indicated with one star (*) on charts.

3.2.5 Effect of polaxamer supplementation on lentiviral vector yields

Polaxamers, such as Pluronic F-68, are non-ionic surfactants, comprising of triblock copolymers made of hydrophilic polyethyleneoxyde and hydrophobic polypropylene oxide. Pluronic F-68 is widely used to protect cells from injury due to agitation and/or gas bubble sparging in bioreactors (Kilburn & Webb, 1968b). The cell protective effect of Pluronic F-68 has been attributed to two possible mechanisms: (i) it could reduce the cell-to-bubble attachment (Jordan et al., 1994) and damage caused by bubble bursting (Dey et al., 1997), (ii) it could also increase the resistance of the cells to shear stress by decreasing the plasma membrane fluidity in cells (Ramirez & Mutharasan, 1990).

Pluronic F-68 is known to be a component of FreeStyle 293, and the manufacturer's recommendations suggest supplementation with an additional 2.5-5 mL/L of 10% Pluronic F-68 (0.025-0.05%) in large-scale bioreactor suspension cultures to reduce shear stress in the culture. While it was not assumed that additional Pluronic F-68 would necessarily increase viral titres in flask conditions, it was critical to rule out a deleterious effect on titres in bioreactors. An experiment was therefore designed to assay vector production yields using medium supplemented with additional Pluronic F-68.

As described in Section 3.2.1, a single, large flask of transfected cells was prepared and, 24 hours later, split into 12 flasks after the media change for supplementation, at which point sodium butyrate was added. The manufacturer’s recommended concentration of Pluronic F-68 supplementation is 0.05%, therefore, triplicate flasks were supplemented with 0.5X (0.025%), 1X (0.05%) or 2X (0.1%) of the upper limit of the recommended concentration of additional Pluronic F-68, or with FreeStyle 293 only, as a negative control. Virus produced under these conditions was harvested 48 hours after supplementation and the virus titrated as described in Section 2.4. Vector titres were not

affected by any concentration of Pluronic F-68 added in the culture medium of the transfected cells (Figure 3.4B), although a trend for enhanced virus production was noted with 0.1% Pluronic F-68.

3.3 Discussion

A critical parameter affecting the cost of viral vector manufacturing is the final production titre, therefore, improvements to lentiviral vector production processes should ultimately increase efficiency and reduce overall costs. To this effect, serum-free media reduces the complexity, duration, and cost of downstream processing as well as risks of transfer of viruses or prions into the final product. Furthermore, suspension production increases yields (Lesch et al., 2011) and allows the use of controlled bioreactors enhancing reproducibility. This chapter aimed to address issues arising during lentiviral vector production in serum-free suspension culture, specifically, the potential for depletion of cell membrane lipid and protein components that could occur due to high levels of virus production as well as mitigating stresses due to bubble-bursting and shear forces generated during cell culture. A simple protocol for a well-controlled, small-scale (20 mL) lentiviral vector production was developed based on transient transfection of HEK 293T cells in suspension. Several steps were taken to reduce potential variability, including the use of a master mix to bulk transfect cells in a single, large flask, which was then split to prepare 12 identical flasks of transfected cells for experimentation (Section 3.1). This allowed the experiments described in this chapter to be powered at 80%, sufficient to detect a 2.28-fold increase in titre.

Using this protocol, several media supplements were evaluated for their effect on lentiviral vector titre. All supplements tested were added 24-hour after transient transfection with the component lentiviral vector plasmids to minimise interaction of the

supplement with the transfection mixture/process. Disappointingly, none of the media supplements tested significantly increased vector titre in these studies. CDLC supplementation lead to the largest increase in titre (2.13-fold), but the observed trend did not reach significance, both because it did not reach the 2.28-fold threshold determined by power calculations and because of great inter-replicate variability (± 0.81-fold). A study in quadruplicate might be sufficiently powered, although quintuplicates would be needed to confirm the significance of the result considering the large inter-replicate variability. Another possibility would be to aggregate marginal gains by combining supplements with multiple non-significant but positive trends such as a combination of 1:333 CDLC and 0.1% Pluronic F-68.

To prevent any interference with the transfection process, supplements were always added at the media change step (Section 2.4.1) It is possible, however, that a longer exposure of the cells to the supplements might be necessary to observe detectable effects in virus yield. Alternatively, it is also possible that the transfection step itself could be enhanced by the supplement as it has been reported for Lipid Mixture 1 and Gelatin Peptone N3 (Pham *et al.*, 2003).

In the case of lipid supplements, the issue of the presence of mβCD in the supplements was noted above. It is also relevant to note the reactivity of mβCD with polyethylene which lines the WAVE Bioreactor which is used for the UKCFGTC large-scale production (L Davies, personal communication). This issue was previously described (Okonkowski et al., 2007) following the unsuccessful growth of cholesterol-dependent cell lines in this bioreactor (Kadarusman et al., 2005). This would prevent the use of CLC in the WAVE Bioreactor as it might cause leaching of cholesterol from the cell membrane. A possible solution would be to use an alternative bioreactor coating. While other companies make

bioreactors, the cost of transitioning to a new production system would most probably offset any short-term benefit of CLC supplementation.

In the case of polaxamers, Pluronic F-68 is reported to protect cells from sparging and injury due to agitation in bioreactors (Kilburn & Webb, 1968b). It is listed by the manufacturer as a component of FreeStyle 293, but at an undisclosed concentration. It is further recommended to supplement with an additional 2.5-5 mL/L of 10% Pluronic F-68 in agitating bioreactors. As no adverse effect was observed on titres in the experiment described above, it was decided to implement 2.5 mL/L 10% Pluronic F-68 (0.025%) supplementation in the UKCFGTC's ongoing large-scale production of lentiviral vectors (L Davies, personal communication). Each large-scale (1L and 5L) production run being unique, expensive and with multiple changing parameters, it has not been possible to determine the significance of any beneficial effect observed following this supplementation. However, cell counts and viability did appear to have increased and, importantly, no dramatic deleterious effects have been observed following implementation in bioreactors (L. Davies, personal communication).

Another supplement, chloroquine, has been extensively studied in the literature in this context. Chloroquine is a lysosomotropic amine used as an effective and safe anti-malarial and anti-rheumatoid agent. Its accumulation inhibits enzymes present in lysosomes and therefore proteolytic processes (Reviewed in Solomon & Lee, 2009). The inhibition of lysosomal enzymatic activity has also been shown to inhibit the degradation of DNA transfected with calcium phosphate (Luthman & Magnusson, 1983). This effect was observed in a calcium phosphate transient transfection production of retroviral vectors where titres were doubled with chloroquine addition (Pear *et al.*, 1993). However, the enhanced gene expression and transfection efficiency are counterbalanced by extensive cell toxicity after relatively brief (> 4 hours) exposure (Ciftci & Levy, 2001). In

large-scale production, quickly removing a toxic reagent is difficult and having to perform such a step would preferably be avoided.

While chloroquine has also been used in lentiviral vector production (Reiser *et al.*, 1996; Mochizuki et al., 1998; Park et al., 2000; Beyer et al., 2002), a study of its effects on lentiviral vector yields detected no change in titres in a calcium phosphate transient transfection production (Karolewski et al., 2003). Furthermore, the transfection reagent might be critical in chloroquine's effect. Chloroquine addition was observed to reduce lentiviral vector titres when used in combination with PEI in DMEM without serum (Kuroda et al., 2009). It has been suggested that PEI has a similar 'DNA protection effect' as chloroquine in lysosomes (Boussif *et al.*, 1995), which might explain this discrepancy. For these reasons chloroquine was not tested as a supplement in this chapter.

Several protein, lipid and other supplements are commercially available that could ultimately benefit lentiviral vector production, but to thoroughly evaluate these would probably require a protocol with a higher practical throughput. Moreover, the supplements tested in this study could have a benefit in an alternate experimental production setup. This is exemplified by the discordant results found with NAC supplementation in the present experimental setup (Section 3.2.4) compared to those obtained in HIV-1 infection of lymphocytes. It is, therefore, possible that third-generation lentiviral vector production and the native HIV-1 life cycle have different requirements. Some supplements, such as caffeine, have been shown to increase lentiviral vector titres (Ellis et al., 2011), but have not been shown to be efficacious in the production system used in this study (L. Davies, personal communication). The use of the Sendai virus F/HN pseudotype might also result in different requirements compared with the more widely used VSV-g pseudotype. Both pseudotypes comprise proteins that are integrated

into the cell membrane, but the exact effect of their presence on membrane composition is unknown.

The FreeStyle 293 used for the experiments described here is a highly specialised medium developed for the optimal growth of HEK 293-derived cells in suspension and it is possible that only marginal benefit can be obtained by any additional media supplementation in this context. This could explain why supplements observed to improve production yields in other media have not been successful here. It may, therefore, be helpful to reassess the potential of these supplements to benefit production if alternative (perhaps non-optimal) media were used.

Finally, while variability was minimised using a single transfection rather than 12 independent transfections for each experiment, the variability of the experimental setup did not allow determination of small variations in titres (below 2.28-fold) as being significant. As described earlier in the case of CDLC (Section 3.2.2.2), a larger number of replicates could have addressed this problem. It is important to note that each experiment was performed only once as it was decided that only experiments that showed a benefit for lentiviral vector production in an initial experiment would be repeated in further independent experiments. An alternative setup involving a producer cell line (Figure 1.5) could also be employed to minimise variation, removing the variability introduced by the transfection step.

Another factor that might explain the lack of effect of supplementation in the experimental setup, compared with an observed impact in other studies, might be sub-optimal production yields in the present setup. Even with a producer cell line, if the maximal production of lentiviral vector particles is not reached, then depletion of membrane components may not yet be rate-limiting. Thus, it may be useful to reassess the possible

benefit of such supplementation during late-stage production optimisation. From these observations, subsequent experimental approaches shifted focus to host cell factors impacting lentiviral vector production.

Chapter 4 – PKR inhibition to suppress intrinsic cell response to viruses

4.1 Introduction

Studies described in Chapter 3 explored growth media supplementation as an avenue to enhance lentiviral vector production, but this approach was not successful at improving lentiviral vector titres. The current chapter describes attempts at a more direct approach, focussing on the intrinsic immune response to viral infection and exploiting host inhibitors of this activity to boost lentiviral vector production.

4.1.1 Intrinsic immune response to viral infections

When infected by a virus, a number of changes occur in the cell. Pattern recognition receptors recognise pathogen-associated molecular patterns (which can be lipids, lipoproteins, nucleic acids or proteins (Reviewed in Rustagi & Gale, 2014). Their recognition activates intracellular pathways leading to the expression of inflammatory cytokines, chemokines and IFN (Reviewed in Akira et al., 2006).

The IFN signalling pathway is one of the principal antiviral mechanisms. Cells in contact with IFN express IFN-stimulated genes (ISGs), such as the IFN-induced double-stranded RNA-activated protein kinase (PKR), a serine/threonine kinase with antiviral and anti-cellular growth activities (Meurs et al., 1990; Clemens et al., 1993). PKR is activated by binding to low levels of double-stranded RNA (dsRNA) through its dsRNA-binding domains (dsRBDs) (Sadler & Williams, 2007). Potential activators of PKR include all dsRNA longer than 30 nucleotides such as the highly structured TAR RNA element of HIV-1 (Figure 4.1) (Edery et al., 1989; Roy et al., 1990; Sengupta et al., 1990) and a number of cellular proteins (Reviewed in Burugu et al., 2014).

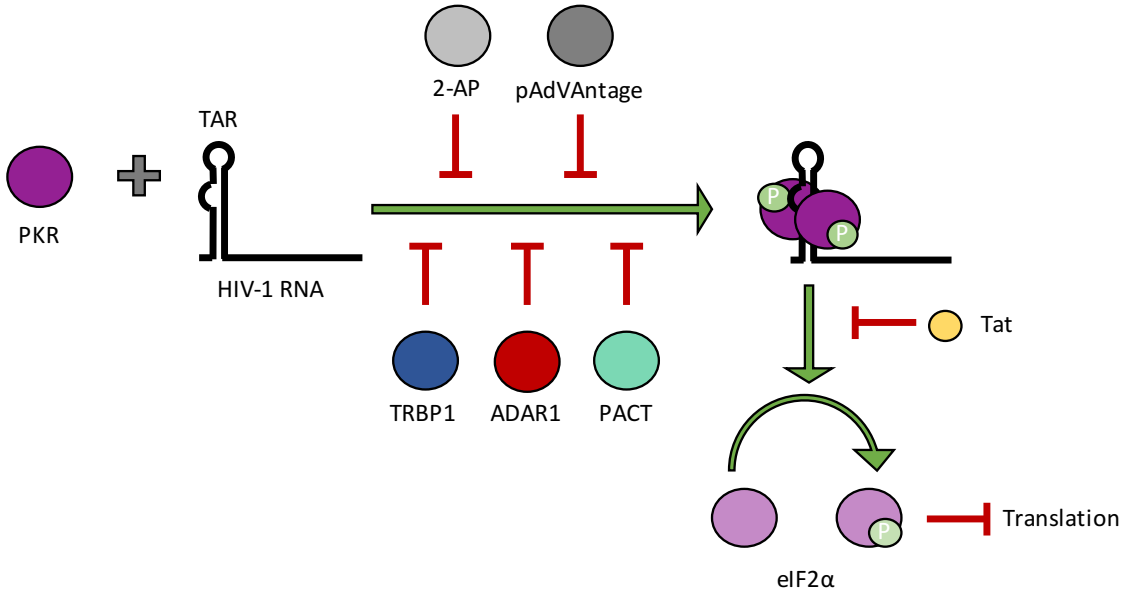


Figure 4.1. Schematic representation of the regulation of HIV-1 translation by the interferon-induced double-stranded RNA-activated protein kinase (PKR) and the action of selected PKR inhibitors. PKR dimerises and becomes activated (phosphorylated (P)) when in contact with double-stranded RNA such as the TAR RNA element of HIV-1's genome. Activated PKR phosphorylates the alpha subunit of the translation eukaryotic initiation factor 2 (eIF2α), which inhibits global protein translation. Cellular factors such as the TAR RNA binding protein 1 (TRBP1), adenosine deaminase, RNA-specific 1 (ADAR1) and the PKR activator (PACT) prevent or inhibit PKR phosphorylation, as does 2-aminopurine (2-AP) and adenovirus virus-associated RNA I produced by adenovirus or the pAdVAntage™ plasmid. The viral protein Tat is also involved in this process, at a later step, inhibiting PKR-mediated phosphorylation of eIF2α.

When PKR binds dsRNA, a conformational change occurs (Nanduri et al., 2000) unmasking the ATP-binding site of the kinase domain. This allows autophosphorylation of PKR on several sites (Taylor et al., 1996) and dimerization (Figure 4.1), which is essential for its kinase activity (Patel et al., 1995). PKR is encoded by the gene eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*) named as such because, when activated, it phosphorylates the alpha subunit of the translation eukaryotic initiation factor 2 (eIF2α). This negatively affects the translational initiation rate and efficiency for both cellular and viral mRNAs (Figure 4.1) (Gale et al., 1998). This process is tightly regulated by a number of cellular factors (Reviewed in Burugu et al., 2014) and strongly contributes to virus inhibition and making PKR activation a critical component of many antiviral and cell growths pathways (Reviewed in Garcia et al., 2006).

The antiviral effect of PKR is well documented in cell culture with HIV-1 (Reviewed in Burugu et al., 2014). Due to its antiviral effect, several viruses encode PKR inhibitors. In the case of HIV-1, it has been shown that Tat interferes with the correct eIF2α positioning at PKR's kinase domain becoming itself phosphorylated instead of eIF2α (Figure 4.1) (Brand et al., 1997). This phosphorylation, in turn, increases Tat's interaction with the TAR RNA element, enhancing transcription (Endo-Munoz et al., 2005). Tat is absent in third-generation lentiviral vectors, and the effect of PKR on the production of these vectors has not been studied.

4.1.2 PKR inhibition

Due to PKR's repressive effect on protein translation, a number of cellular factors are involved in its inhibition, in order to manage cell growth, through dsRNA sequestration or by preventing its phosphorylation. This type of restriction can, for example, be achieved through the TAR RNA binding protein 1 (TRBP1) (Garcia et al., 2007). Another

mechanism, protein-protein interaction, plays a role in PKR inhibition with dsRNA-binding proteins (dsRBP) such as TRBP1 (Daher et al., 2001), the adenosine deaminase, RNA-specific 1 (ADAR1) (Nie et al., 2007) and the PKR activator (PACT) (Clerzius *et al.*, 2013) which all bind to PKR through their dsRBDs.

Viruses, as targets of PKR's inhibition of translation, have evolved a number of mechanisms to limit PKR activation. These include the expression of competitive inhibitory RNAs or viral proteins that act either by direct PKR inhibition, dsRNA sequestration, rescuing translation through eIF2α dephosphorylation or acting as competitive substrates (Peters et al., 2002; Garcia *et al.*, 2006). HIV-1 uses a number of cellular mechanisms during infection to inhibit PKR activation (Reviewed in Clerzius et al., 2011). More precisely, HIV-1 recruits TRBP1 in the proximity of PKR through its TAR element (Daher *et al.*, 2001; Ong *et al.*, 2005; Christensen et al., 2007), and the overexpression of the dsRBPs TRBP1 (Benkirane et al., 1997; Daher *et al.*, 2001), ADAR1 (Phuphuakrat et al., 2008; Clerzius *et al.*, 2009; Doria et al., 2009) and PACT (Clerzius et al., 2013) have been shown to reverse the effect of overexpressed PKR and enhance HIV-1 production (Figure 4.1). These mechanisms which increase HIV-1 production could possibly be exploited for enhancement of lentiviral vector production.

4.1.2.1 TRBP1

TRBP1, encoded by the gene *TARBP2*, is a cellular dsRBP first identified for its strong binding of TAR RNA probes and was shown to stimulate HIV-1 LTR expression in both human and murine cells (Gatignol et al., 1991). Proposed functions for TRBP1 include: inhibition of PKR activation (Park et al., 1994; Benkirane *et al.*, 1997; Daher *et al.*, 2001; Ong *et al.*, 2005), regulation of cell proliferation (Lee et al., 2004; Lee et al., 2006), PKR-independent translational activation and control of mRNA translation in testis (Lee et al.,

1996a; Dorin et al., 2003), control of PACT activity (Laraki *et al.*, 2008; Daher et al., 2009) and regulation of miRNA function (Haase et al., 2005; Daniels et al., 2009).

TRBP1 interacts with PKR through both direct protein interaction and competitive binding for dsRNA substrates. It acts to counteract the inhibitory effects of PKR on HIV-1 LTR-driven expression and on viral replication (Benkirane *et al.*, 1997; Daher *et al.*, 2001). For example, in human astrocytic brain cells, which express low levels of TRBP1, HIV-1 induces a high activation of PKR resulting in low HIV-1 replication (Bannwarth et al., 2001; Ong *et al.*, 2005; Bannwarth et al., 2006). TRBP1 has been shown to increase HIV-1 LTR-driven expression in these cells as well as HIV-1 production, while a decrease of TRBP1 expression inhibited virus production (Christensen et al., 2007).

4.1.2.2 ADAR1

ADAR1, encoded by the gene *ADAR*, is an RNA-editing enzyme that catalyses the deamination of adenosine to inosine in nuclear and viral RNAs (Kim et al., 1994; O'connell et al., 1995; Melcher et al., 1996; Gerber et al., 1997; Lai et al., 1997). Inosine is recognised as a guanosine in translation (Basilio et al., 1962) changing the RNA's primary sequence information. Furthermore, inosine base pairs with cytidine, leading to I-U mismatches instead of the original A-U, changing the structure of the RNA, which affects biological processes involving either sequence- or structure-specific interactions (Reviewed in Bass, 2002).

ADAR1 acts both as an antiviral and a proviral agent in virus-infected cells (Reviewed in Gelinas et al., 2011; Samuel, 2011). Three isoforms of ADAR1 can be found in human cells: an IFN-inducible full-length cytoplasmic 150 kDa protein (ADAR1-p150) (Patterson et al., 1995) and two constitutively expressed 110 kDa (ADAR1-p110) (Patterson &

Samuel, 1995) and 80 kDa (ADAR1-p80) (Yang et al., 2003) proteins. ADAR1-p150, the IFN-inducible cytoplasmic long form of the protein, is expected to be part of the cell response against infections. However, ADAR1-mediated editing significantly enhances HIV-1 infectivity (Phuphuakrat *et al.*, 2008; Doria *et al.*, 2009) and the overexpression of ADAR1-p150 increases HIV-1 production in the absence of exogenous PKR and completely rescues PKR-induced inhibition of HIV-1 production (Clerzius *et al.*, 2009; Doria *et al.*, 2009). Conversely, two studies have described an antiviral role for ADAR1 in HIV-1 infection, where ADAR1 overexpression reduced both extracellular and intracellular p24 (Biswas et al., 2012) and ADAR1 knock-down led to an increased number of HIV-1 infectious units (Weiden et al., 2014).

4.1.2.3 PACT

PACT, encoded by the gene *PRKRA*, is a stress-inducible protein (Patel et al., 2000) that induces apoptosis by PKR activation (Patel & Sen, 1998). Such stress prevents TRBP1-PACT interactions and allows PACT activation of PKR. Consequently, PACT acts as a PKR activator in cells with low TRBP1 concentration or after stress induction (Daher *et al.*, 2009; Singh et al., 2011), whereas it acts as a PKR inhibitor in cells with high TRBP1 content (Daher et al., 2009). HIV-1 infection leads to interactions between PKR and PACT, and PACT overexpression was shown to be able to reverse PKR's inhibition of HIV-1 while PACT knock-down rescued PKR activation and HIV-1 inhibition (Clerzius et al., 2013).

4.1.3 Aim

In HIV-1 proviral transfection systems, PKR overexpression was shown to have an inhibitory effect on production (Reviewed in Burugu et al., 2014), which could be rescued by overexpression of the PKR inhibitors TRBP1 (Benkirane *et al.*, 1997; Daher *et al.*,

2001), ADAR1 (Phuphuakrat *et al.*, 2008; Clerzius *et al.*, 2009; Doria *et al.*, 2009) and PACT (Clerzius et al., 2013). This chapter investigates if a similar effect can be observed in a transient transfection producing a third-generation lentiviral vector and assays other reported PKR inhibitors in order to enhance lentivirus production.

4.2 Results

4.2.1 Effect of PKR on lentiviral vector production

To study the effects of PKR overexpression on lentiviral vector production, the plasmid pcDNA1-PKR and its parental plasmid pcDNA1-empty lacking PKR were obtained (Table 2.3). The parental plasmid was used to keep the amount of DNA constant in each transfection condition.

The lentiviral vector production study incorporating pcDNA1-PKR and/or pcDNA1-empty was conducted using a variation of the single-flask transfection strategy optimised in Chapter 3 (Section 3.2.1). A summary of the steps for all studies using additional plasmids after the initial transfection of the lentiviral vector producing plasmids is presented in Figure 2.1C. Briefly, an 11 mL 'transfection mix' comprising 375 μg of the producer plasmid DNA (Section 2.3.1) mixture to generate vGM035 (rHIV.F/HN CMV-EGFPLux) was prepared. It was used to transfect 220 mL of HEK 293T cells at 1 x 10^6 cells/mL seeded into one 1000 mL flask. At this point, the culture was divided between 12 separate 125 mL flasks, 19 mL per flask, and a series of second 'transfection mixes' was prepared sufficient, each, for three of these flasks. These second transfection mixes added 1 mL containing 10 μg of plasmid DNA (pcDNA1-PKR and/or pcDNA1-empty) complexed with 5 μL of Lipofectamine 2000 to each flask for a total production volume of 20 mL. This separate transfection step was used to introduce the different assayed conditions into a uniformly prepared set of transfected cells, and a different transfection

reagent was used to limit interference caused by the additional plasmids. The experimental conditions were varied such that the ratio of pcDNA1-PKR:pcDNA1-empty was either: 0:10, 2.5:7.5, 5:5, or 10:0 µg respectively. The condition incorporating 5µg of pcDNA1-PKR in 20 mL was chosen so as to match (w/v) previously published studies where 0.5 µg of the same plasmid was used in studies performed in 2 mL at the 6-well plate (approximately 34.5 mM diameter circular dishes) scale (Daher et al., 2001, Clerzius et al., 2009, Clerzius et al., 2013). Twenty-four hours after the transfection events, the media was changed by centrifugation and sodium butyrate was added. The virus was harvested after a further 48 hours' incubation. The virus titre for each flask was then determined using flow cytometry (Section 2.5.1).

In Figure 4.2, the results are presented normalised to the flasks transfected with 0:10µg of pcDNA1-PKR:pcDNA1-empty. In agreement with published HIV-1 studies, the results showed that incorporating increasing amounts of the PKR expression plasmid pcDNA1-PKR into the lentivirus production process led to a progressive reduction in titre – with the observed trend reaching statistical significance with sufficient power (Section 3.2.1) at the two highest doses. It is consequently apparent that the extensive modifications involved in the third-generation of lentiviral vectors do not seem to have altered the inhibitory effect of overexpressed PKR. Endogenous PKR is known to be expressed and functional in HEK 293T cells (Clerzius *et al.*, 2013) and could, therefore, be targeted by inhibitors. The effect of selected PKR inhibitors was accordingly assessed next.

4.2.2 Effect of TRBP1 on lentiviral vector production

To study the effects of TRBP1 overexpression on lentiviral vector production, the plasmids pcDNA3-TRBP1 and the parental plasmid pcDNA3-empty were used (Table 2.3). Lentiviral vector production studies incorporating pcDNA3-TRBP1 and/or pcDNA3-

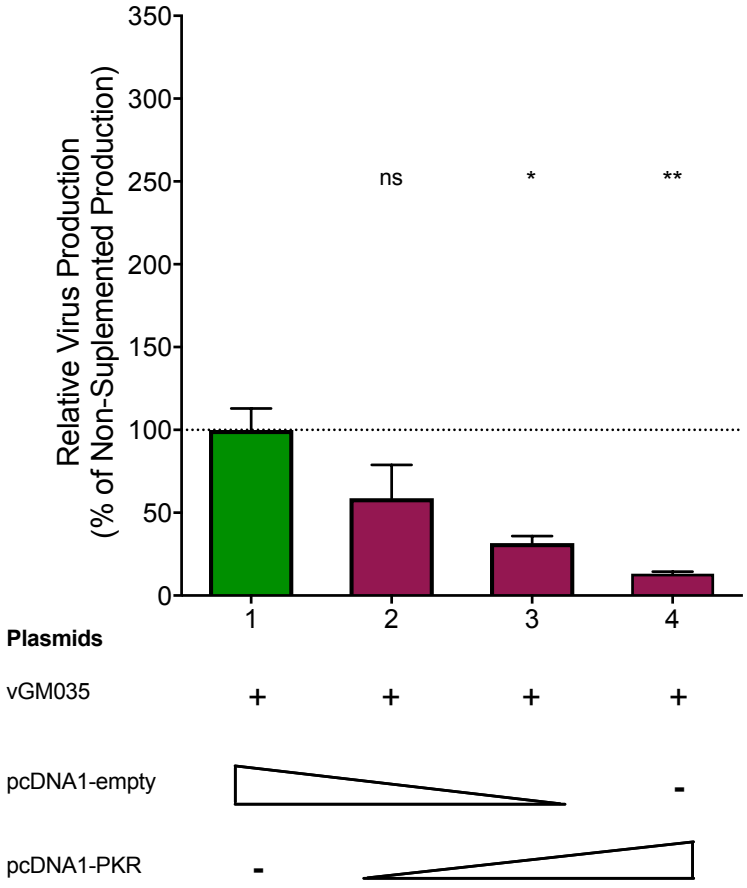


Figure 4.2 Effect of transfection of a PKR expression plasmid on lentiviral vector production. HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV-EGFPLux) producer plasmids using 25 kDa branched PEI (Section 2.4). An additional transfection using Lipofectamine 2000 (Section 2.4.3) was performed with varying ratios of pcDNA1-PKR and pcDNA1-empty such that total amount of additional transfected plasmid DNA remained constant at 10 µg. The ratio of pcDNA1-PKR:pcDNA1-empty was either: 0:10, 2.5:7.5, 5:5, or 10:0 – conditions 1 to 4 respectively. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to levels with empty plasmid alone ± SEM. Statistically significant differences between group means were determined by one-way ANOVA: $F(3,8) = 9.672$, $p = 0.0049$, followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on figures. Calculated p values of < 0.05 or < 0.01 were deemed a significant difference as indicated with one star (*) or two stars (**) respectively on the chart.

empty were conducted again using a variation of the single-flask transfection strategy optimised in Chapter 3. Briefly, a 30 mL 'transfection mix' (comprising 530 μg of the producer plasmid DNA mixture) was prepared and used to transfect 300 mL of HEK 293T cells at 1 x 10^6 cells/mL seeded into one 1000 mL flask. At this point, the culture was divided between 15 125 mL flasks, 19 mL per flask, and a series of second 'transfection mixes' was prepared sufficient, each, for three of these flasks. These second transfection mixes added 1 mL containing 10 μg of plasmid DNA (pcDNA3-TRBP1 and/or pcDNA3-empty) complexed with 5 μL of Lipofectamine 2000 to each flask for a total production volume of 20 mL. The experimental conditions were varied such that the ratio of pcDNA3-TRBP1:pcDNA3-empty was either: 0:10, 2.5:7.5, 5:5, or 10:0 μg respectively. Three of the fifteen flasks were not transfected a second time and acted as controls for the second transfections. Twenty-four hours after the transfection events, the media was changed by centrifugation and sodium butyrate was added. The virus was harvested after a further 48 hours' incubation. The virus titre for each flask was determined using flow cytometry (Section 2.5.1).

In Figure 4.3A, the results are presented normalised to the flasks transfected with 0:10μg of pcDNA3-TRBP1:pcDNA3-empty. When presented in this fashion, there was an apparent trend for an increase in vector production as the amount of pcDNA3-TRBP1 transfected increases. However, this trend did not reach significance. Crucially, when the results are presented normalised to the flasks that did not undergo a second transfection (Figure 4.3B), this apparent increase was not sufficient to compensate for an adverse impact of the second transfection on the observed titres.

Lipofectamine 2000 was selected as the second transfection agent in these studies in the hope that any confounding interaction between the first and second transfection

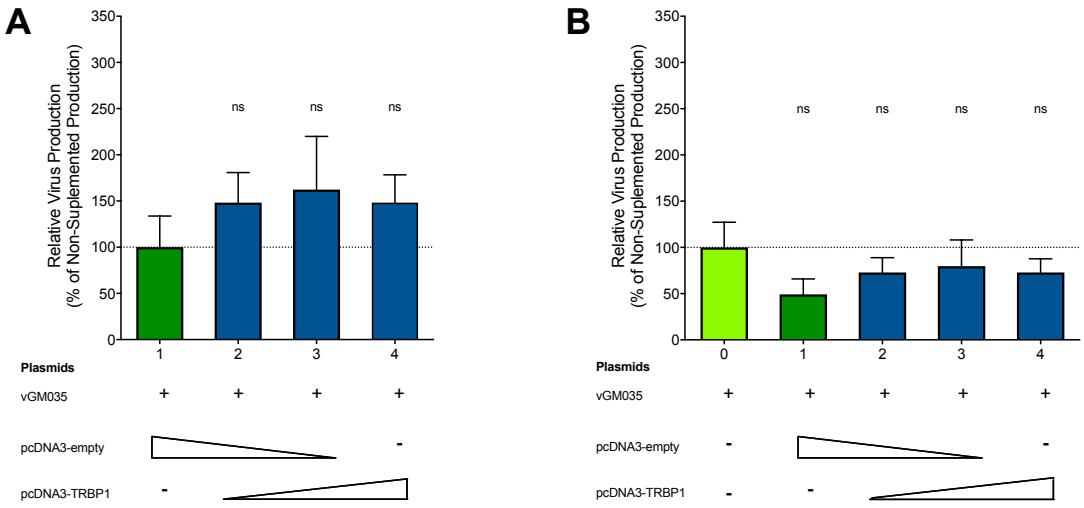


Figure 4.3 Effect of Lipofectamine 2000 transfection of a TRBP1 expression plasmid on lentiviral vector production. (A) HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV- EGFPLux) producer plasmids using 25 kDa branched PEI (Section 2.4). An additional transfection using Lipofectamine 2000 (Section 2.4.3) was performed with varying ratios of pcDNA3-TRBP1 and pcDNA3-empty such that total amount of additional transfected plasmid DNA remained constant at 10 µg. The ratio of pcDNA3-TRBP1:pcDNA3-empty was either: 0:10, 2.5:7.5, 5:5, or 10:0 – conditions 1 to 4 respectively. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to levels with empty plasmid alone ± SEM. (B) Same data normalised to an additional data set (0) where no additional plasmid was transfected. There were no statistically significant differences between group means as determined by one-way ANOVA: A) $F(3,8) = 0.4629$, $p = 0.7160$, B) $F(4,10) = 0.7429$, $p = 0.5946$. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on charts.

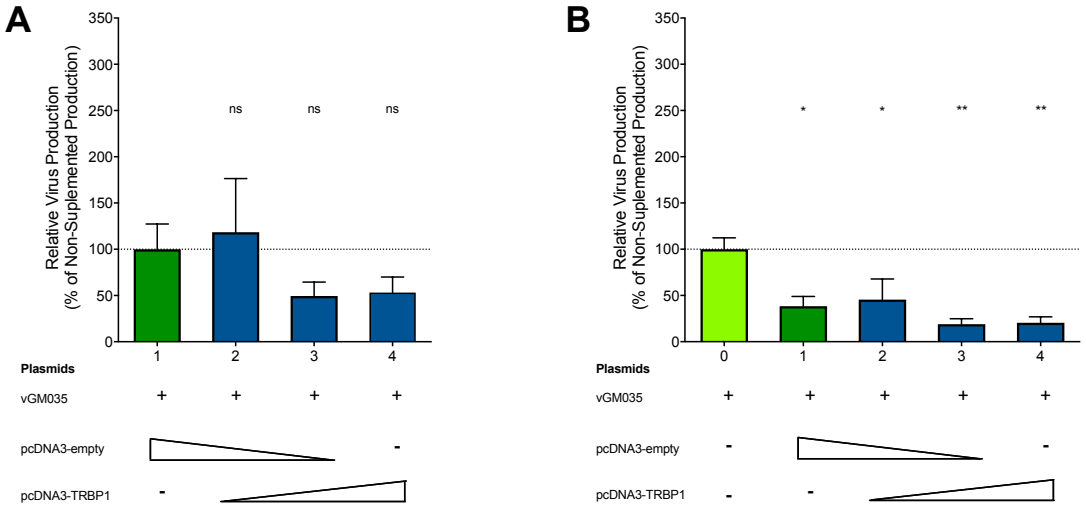


Figure 4.4 Effect of PEI transfection of a TRBP1 expression plasmid on lentiviral vector production. (A) HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV- EGFPLux) producer plasmids using 25 kDa branched PEI (Section 2.4). An additional transfection using PEI (Section 2.4.3) was performed with varying ratios of pcDNA3-TRBP1 and pcDNA3-empty such that total amount of additional transfected plasmid DNA remained constant at 10 µg. The ratio of pcDNA3-TRBP1:pcDNA3-empty was either: 0:10, 2.5:7.5, 5:5, or 10:0 – conditions 1 to 4 respectively. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to levels with empty plasmid alone ± SEM. (B) Same data normalised to an additional data set (0) where no additional plasmid was transfected. Statistically significant differences between group means were determined by one-way ANOVA: A) $F(3,8) = 1.018$, $p = 0.4341$, B) $F(4,10) = 6.511$, $p = 0.0076$, followed, if appropriate, by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on charts. Calculated p values of < 0.05 and < 0.01 were deemed a significant difference as indicated with one star (*) or two stars (**) respectively on charts

agent would be minimised. Given the apparent confounding interaction observed in Figure 4.3A and Figure 4.3B, a replicate study was performed where PEI, the transfection agent for the producer plasmids, was also used in the second transfection. Figure 4.4 shows that the use of the PEI as both first and second transfection agent resulted in significantly lower titres than the initial conditions.

4.2.3 Effect of ADAR1 on lentiviral vector production

To study the effects of ADAR1 overexpression on lentiviral vector production, the plasmids pcDNA3.1-ADAR1DCat-V5 and the parental plasmid pcDNA3.1-empty-V5 were used (Table 2.3). Here, a modified ADAR1-p150 protein with its catalytic domain removed was used to prevent any issues due to RNA editing and thus preserve the integrity of the vector's genome. This edited protein was tagged with the V5 epitope tag, a 14-amino acid oligopeptide derived from simian virus 5 (Hanke *et al.*, 1992), in the study where it was generated (Clerzius et al., 2009) and it was kept here. While this mutant has been shown to be less effective at reversing the effect of overexpressed PKR than the full-length molecule ADAR1-p150, this disadvantage was not apparent in experiments without overexpression of PKR (Clerzius et al., 2009) as it is the case here.

Lentiviral vector production studies incorporating pcDNA3.1-ADAR1DCat-V5 and/or pcDNA3.1-empty-V5 were conducted again using the modified single-flask transfection strategy. As described in Section 4.2.2, a single, large flask of transfected cells was prepared and immediately split into 15 flasks. A second transfection, in triplicates, was performed where the ratio of pcDNA3.1-ADAR1DCat-V5:pcDNA3.1-empty-V5 was either: 0:10, 2.5:7.5, 5:5, or 10:0 µg respectively. Three of the 15 flasks were not transfected a second time and acted as controls for the second transfections. Twenty-four hours after the transfection events, the media was changed by centrifugation and sodium butyrate was added. The virus was harvested after a further 48 hours' incubation.

The virus titre for each flask was determined using flow cytometry (Section 2.5.1). One flask each from the 5 and 10 µg triplicates had to be discarded due to bacterial contamination. In Figure 4.5A, the results are presented normalised to the flasks transfected with 0:10µg of pcDNA3.1-ADAR1DCat-V5:pcDNA3.1-empty-V5 and in Figure 4.5B to the flasks without a second transfection. There was no change in the production with any of the pcDNA3.1-ADAR1DCat-V5:pcDNA3.1-empty-V5 transfection ratios.

4.2.4 Effect of PACT on lentiviral vector production

To study the effects of PACT overexpression on lentiviral vector production, the plasmids pCMV-PACT-FLAG and the parental plasmid pCMV-empty-FLAG were used (Table 2.3). The PACT protein was tagged with the FLAG epitope tag, a synthetic 8-amino acid oligopeptide (Hopp *et al.*, 1988), in the study where it was generated (Clerzius et al., 2013) and it was kept here.

Lentiviral vector production studies incorporating pCMV-PACT-FLAG and/or pCMV-empty-FLAG were conducted again using the modified single-flask transfection strategy. As described in Section 4.2.2, a single, large flask of transfected cells was prepared and immediately split into 15 flasks. A second transfection, in triplicates, was performed where the ratio of pCMV-PACT-FLAG:pCMV-empty-FLAG was either: 0:10, 2.5:7.5, 5:5, or 10:0 µg respectively. Three of the 15 flasks were not transfected a second time and acted as controls for the second transfections. Twenty-four hours after the transfection events, the media was changed by centrifugation and sodium butyrate was added. The virus was harvested after a further 48 hours' incubation. The virus titre for each flask was determined using flow cytometry (Section 2.5.1).

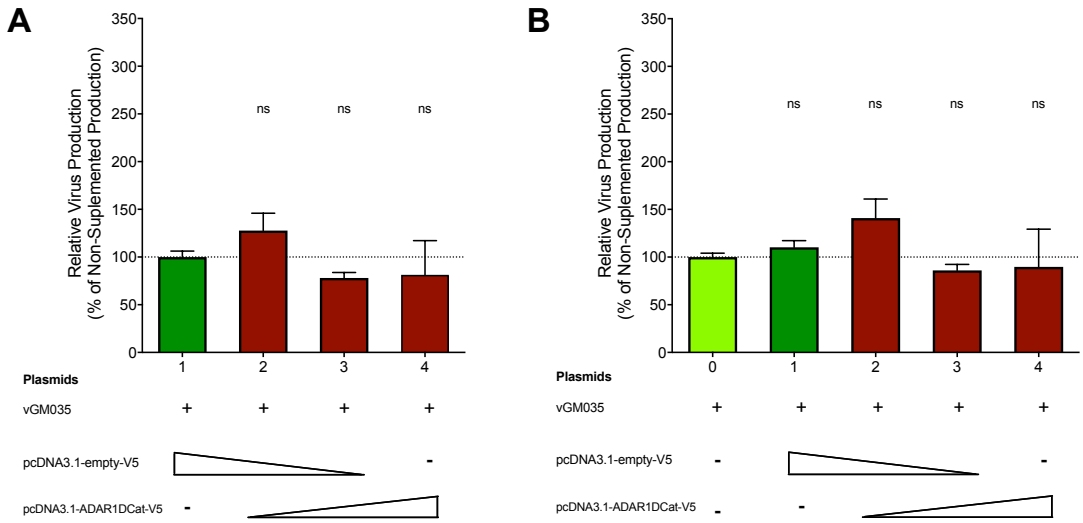


Figure 4.5 Effect of transfection of a catalytic-defective ADAR1 (ADAR1DCat) expression plasmid on lentiviral vector production. (A) HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV- EGFPLux) producer plasmids using 25 kDa branched PEI (Section 2.4). An additional transfection using Lipofectamine 2000 (Section 2.4.3) was performed with varying ratios of pcDNA3.1-ADAR1DCat-V5 and pcDNA3.1-empty-V5 such that total amount of additional transfected plasmid DNA remained constant at 10 µg. The ratio of pcDNA3.1-ADAR1DCat-V5:pcDNA3.1-empty-V5 was either: 0:10, 2.5:7.5, 5:5, or 10:0 – conditions 1 to 4 respectively. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to levels with empty plasmid alone ± SEM. (B) Same data normalised to an additional data set (0) where no additional plasmid was transfected. There were no statistically significant differences between group means as determined by one-way ANOVA: A) $F(3,6) = 1.641$, $p = 0.2770$, B) $F(4,8) = 1.699$, $p = 0.2425$. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on charts.

In Figure 4.6A, the results are presented normalised to the flask transfected with 0:10µg of pCMV-PACT-FLAG:pCMV-empty-FLAG, showing no change in production with any of the pCMV-PACT-FLAG:pCMV-empty-FLAG transfection ratios. However, as seen in Figure 4.6B, there was an apparent effect of the transfected empty plasmid, which seemed to affect virus production. The reason for the apparent effect of the empty plasmid is unclear, the only difference with previous experiments is that this plasmid pair was the only one to use the pCMV backbone. There is, nevertheless, a trend for higher titres with increasing amounts of transfected pCMV-PACT-FLAG compared to the flasks without a second transfection, including in the last condition where only pCMV-PACT-FLAG has been transfected, although this did not reach significance.

4.2.4 2-aminopurine supplementation

Other PKR inhibitors have been described in the literature for use in lentiviral vector production. In one study, 2-aminopurine supplementation and the plasmid pAdVAntage™ were assayed and it was found that both enhanced production of second-generation lentiviral vectors (Pernod et al., 2004). These two supplements were therefore assessed in preliminary studies using 6-well plates for production (Section 2.4).

The purine base 2-aminopurine can act as a DNA mutagen (Freese, 1959b; Freese, 1959a) and has been shown to inhibit PKR both *in vitro* (Sengupta *et al.*, 1990) and *in vivo* (Hu & Conway, 1993). As such, it has been reported to have a positive effect on lentiviral vector yields by incorporating 5 mM during the production phase (Pernod et al., 2004). The impact of 2-aminopurine on production yields was assessed using the same set of concentrations in single wells of a 6 well plate transfected for vector production. One well per condition was supplemented with 0.05 mM, 0.5mM or 5mM or only FreeStyle 293, as a control. After 24 hours, sodium butyrate was added to each well.

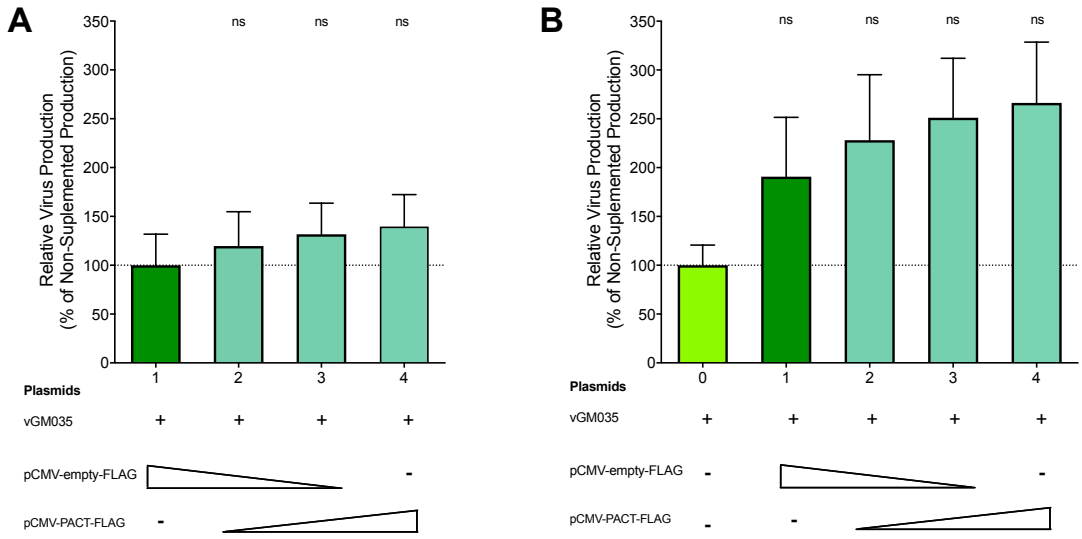


Figure 4.6 Effect of transfection of a PACT expression plasmid on lentiviral vector production. (A) HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV-EGFPLux) producer plasmids using 25 kDa branched PEI (Section 2.4). An additional transfection using Lipofectamine 2000 (Section 2.4.3) was performed with varying ratios of pCMV-PACT-FLAG and pCMV-empty-FLAG such that total amount of additional transfected plasmid DNA remained constant at 10 µg. The ratio of pCMV-PACT-FLAG: pCMV-empty-FLAG was either: 0:10, 2.5:7.5, 5:5, or 10:0 – conditions 1 to 4 respectively. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to levels with empty plasmid alone ± SEM. (B) Same data normalised to an additional data set (0) where no additional plasmid was transfected. There were no statistically significant differences between group means as determined by one-way ANOVA: A) $F(3,8) = 0.2734$, $p = 0.8430$, B) $F(4,10) = 1.357$, $p = 0.3156$. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on charts.

Due to the small volume, no media change was performed. Wells were harvested 48 hours later and titrated as described in Section 2.4. Carry-over of 2-aminopurine to the titration was possible in this setup, however the impact of such a transfer would have led to PKR inhibition in the cells used for titration and, therefore, benefited protein translation such as that of EGFPLux, the viral transgene. Figure 4.7A shows that the lower concentrations of 2-aminopurine had not effect while the higher concentrations had a very significant detrimental effect on production yields. This was hence not repeated at a larger scale.

4.2.5 pAdVAntage™ supplementation

pAdVAntage™ is a plasmid encoding the adenovirus virus-associated RNA I & II (VAI RNA & VAII RNA). VAI RNA has been described as a PKR activation inhibitor (Kitajewski et al., 1986; Akusjarvi et al., 1987). As such, it has been described to have a positive effect on lentiviral vector yields using 5 µg during production and doubling titres when using 10 µg (Pernod et al., 2004). The impact on yields of transfecting pAdVAntage™ during lentiviral vector production was assessed in corresponding amounts in single wells of a 6-well plate. One well per condition was transfected with 0.4 µg, 2 µg or 4 µg of pAdVAntage™ using PEI or only FreeStyle 293, as a control. After 24 hours, sodium butyrate was added to each well without media change. Wells were harvested 48 hours later and titrated as described in Section 2.4. Figure 4.7B shows that increasing amounts of pAdVAntage™ had a detrimental effect on production yields. This was consequently not repeated at a larger scale.

4.3 Discussion

The aim of the studies described in this chapter was to manipulate the intrinsic cell response to replicating viruses, in order to enhance lentiviral vector production. This was

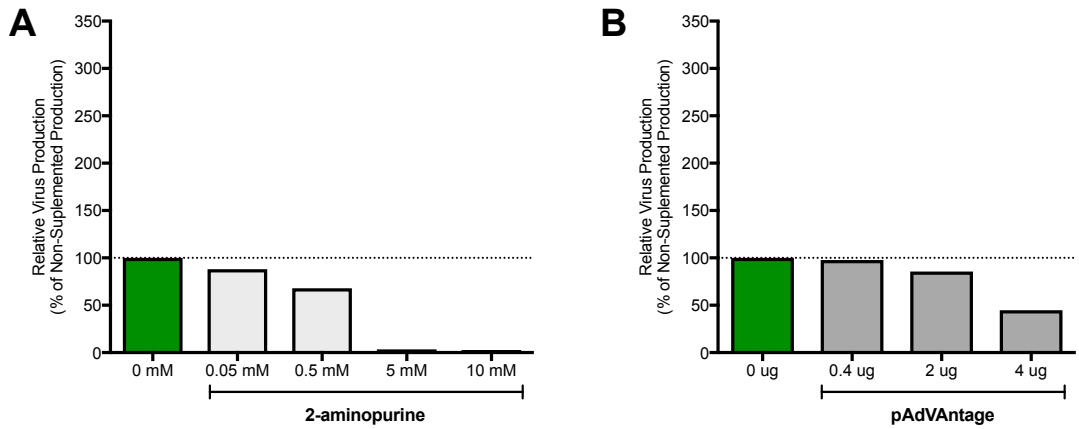


Figure 4.7 Effect of transfection of 2-aminopurine or pAdVAntage™ on lentiviral vector production. HEK 293T cells were transfected with the vGM035 (rHIV.F/HN CMV- EGFPLux) producer plasmids using 25 kDa branched PEI and with (A) 2-aminopurine or (B) Lipofectamine 2000 transfected pAdVAntage™. Titres were measured by flow cytometry (Section 2.5.1) and are shown relative to non-supplemented values. No statistical test was performed as this experiment was not done in replicates.

achieved by first assessing the effect of PKR overexpression on lentiviral vector production. As it is the case with HIV-1, PKR overexpression had a strong inhibitory effect on lentiviral vector production (Figure 4.2). From this result, it could be assumed that inhibiting PKR would enhance lentiviral vector production. Cellular inhibitors of PKR (TRBP1, ADAR1 and PACT), described as enhancers of HIV-1 production in the literature, were therefore assayed in overexpression studies to measure their impact on lentiviral vector production. While in some cases there was a slight tendency for overexpression of these proteins to increase titres, this never reached significance (Figures 4.3-7).

The discrepancy between higher production observed in other studies as compared to the lack of a significant change here can be explained, in part, by the different experimental setup. Experiments assessing PKR inhibitors used proviruses; these involve the transfection of only one plasmid and exhibit a different biology than vectors used here (Figures 4.3-6). The same is true for the discrepancy between the results obtained in published results (second-generation) (Pernod et al., 2004) and current results (third-generation) concerning 2-aminopurine and pAdVAntage™ (Figure 4.7).

The main difference between these systems is the number of plasmids being transfected, in addition to the plasmids for the PKR inhibitors, which might reduce the uniformity of production flasks. In the current experiments, a total of seven plasmids had to be transfected. The results obtained in the experiment where PEI was used for both lentiviral vector plasmids and TRBP1 shows how transfection interaction can have a serious effect on titres (Figure 4.4). While a single transfection of the lentiviral vector-producing plasmids and protein overexpression plasmids, rather than the two-step transfection setup used here, would have been ideal, it would have involved multiple ‘transfection mixes’ to generate the different concentrations. This would have increased assay

variability as observed in the in the multiple independent transfections strategy (Section 3.2.1). Skipping the transfection step using an inducible producer cell line might minimise experiential variability enhancing statistical opportunities to observe modest effects. Another possible source of discrepancy is that Tat is present in both the proviral system and the second-generation vectors while it is absent in the present experiments. Considering its role in the PKR pathway (Figure 4.1), its absence might lead to unknown consequences.

However, an alternative technique to using overexpression as a mean to mitigate intrinsic cellular response to viral replication, would be to directly inhibit host factors such as PKR that affect negatively lentiviral vector titres by either knock-down or knock-out. To investigate this possibility, a literature review, to identify HIV-1 host factors shown to inhibit HIV-1 in the portion of the life cycle applicable to vector production, is presented in Chapter 5. Chapter 7 also provides additional results regarding PKR and its inhibitors using RNA interference (RNAi), which confirmed previous results as well as trends observed here, but with the increased efficiency of using a knock-down strategy instead of overexpression.

Chapter 5 – Identification of inhibitory host factors affecting the late phase of the HIV-1 life cycle

5.1 Introduction

Chapter 3 investigated supplementation of the cell culture media as a way to enhance lentiviral vector production. This is a routine step in the optimisation and scale-up of transient transfection-based protocols to improve lentiviral vector production titres. This optimisation includes the selection of: the most efficient gene transfer reagents, the ratio of plasmid DNA components, the composition of cell culture media, supplementation with growth substrates and bioreactor growth conditions. Intriguingly, little attention has been paid to the optimisation of the mammalian host cell itself, with nearly all reported processes relying on commonly available cell lines that are amenable to transfection such as HEK 293 derivatives. Studies in Chapter 4 explored modulation of host factors involved in the intrinsic responses to viral infection; and showed that, in some cases, host factors can adversely affect lentiviral vector production. Thus, unmodified cell lines might not be optimal for lentiviral vector production because of intrinsically expressed factors.

In this chapter and subsequent ones, a rational design process to establish a cell line with enhanced lentiviral vector manufacturing properties is described. The current chapter aims at reviewing the HIV-1 literature to identify cellular factors, which might negatively affect virus production. Assuming that such factors are also active in the lentiviral vector production environment, it is hypothesised that this search will lead to the identification of gene targets, which could be ‘knocked-down’ or ‘knocked-out’ in order to increase production yields.

5.1.1 Cellular factors affecting HIV-1 replication

In order to replicate, viruses have evolved to exploit a large number of cellular factors, with an estimated 9.5% of human protein-coding genes proposed to affect HIV-1 replication (Bushman et al., 2009). These genes are classified here in four categories: essential factors, auxiliary factors, restriction factors and inhibitory factors (Figure 5.1).

5.1.1.1 Essential factors

The first category is composed of factors essential for virus replication, knock-down or knock-out of these genes will either be extremely detrimental to virus replication or completely abolish it. A number of genome-wide screens have studied essential factors and these are described in Section 6.1.1. Essential factors accomplish cellular functions that are required to produce viruses, or are exploited by the virus to complete its life cycle. A few examples include: CD4 which is required for cellular entry of wild-type HIV-1 (Dalgleish et al., 1984; Klatzmann et al., 1984); RANBP2 which is essential for nuclear import of HIV-1 pre-integration complex (Zhang et al., 2010); CCNT1 (cyclin T1) which is a tat cofactor mediating TAR RNA binding (Wei et al., 1998; Bieniasz et al., 1999); DDX3, a cofactor required for the Rev-RRE export function (Yedavalli et al., 2004); RAB9 which facilitates late endosome to trans-Golgi vesicular transport, a process implicated in HIV-1 particle assembly and export, (Murray et al., 2005) and TSG101, a factor involved in budding (Garrus et al., 2001; Martin-Serrano et al., 2001). The impact of these genes on lentiviral vector production might differ from their impact on HIV-1 viral replication as early steps in replication are bypassed either by transfection of the crucial virus factors, or by their activation in stable producer cell lines. Furthermore, lentiviral vectors have been modified and ‘pseudotyped’ with non-HIV-1 envelope protein(s) to enhance their cell-specific targeting.

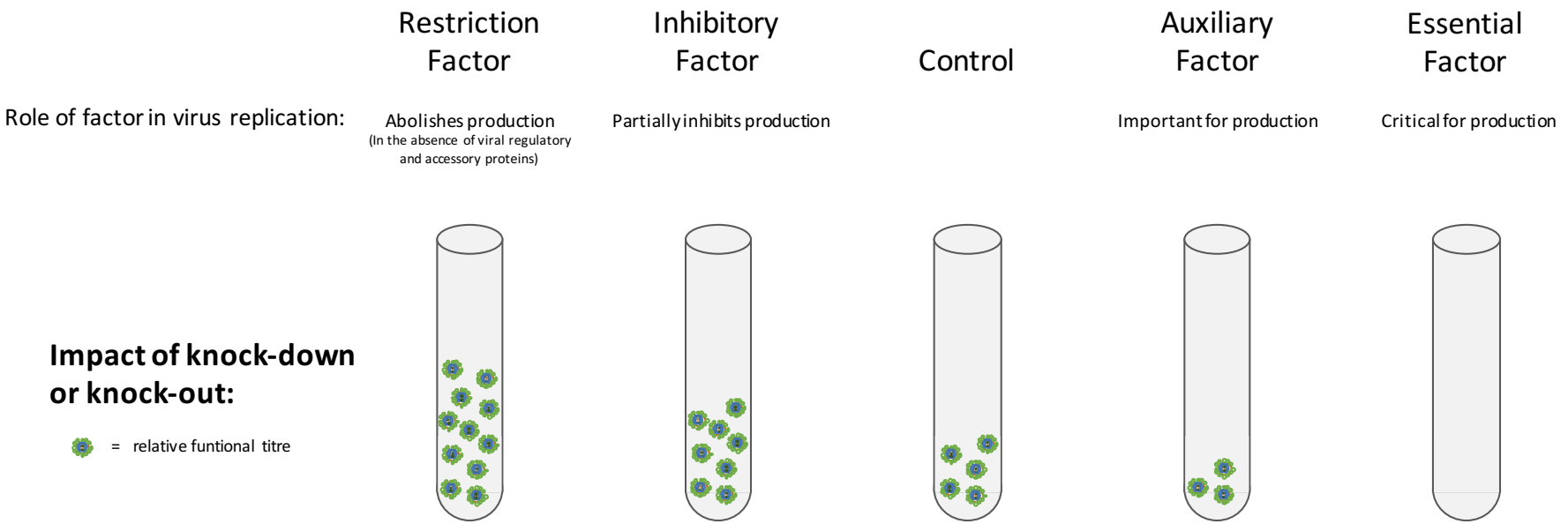


Figure 5.1 Categorisation of cellular factors involved in virus replication and the impact of their knock-down/knock-out on virus production. For the purposes of this study, cellular factors involved in virus replication were classified into four categories: restriction factors which abolish production in the absence of viral regulatory and accessory proteins, inhibitory factors which partially inhibit production, auxiliary factors which are important for virus production and essential factors which are required for virus production. The impact of the knock-down or knock-out of genes in each category of factor on subsequent virus production, compared to a control where no knock-down or knock-out has occurred, is indicated schematically.

5.1.1.3 Auxiliary factors

Host genes that have a positive impact on, but are not absolutely required for, virus production are identified here as auxiliary factors. In contrast to essential factors, decreasing the amount of these factors is expected to reduce, but not abolish, virus replication. Logically, increasing the levels of such factors might lead to increased replication, though little or no published evidence currently exists to support this hypothesis.

5.1.1.3 Restriction factors

Mammalian cells express specific factors to minimise virus replication. These restriction factors constitute a first line of defence even before the innate and adaptive immune systems have a chance to exert their antiviral action. Through mutual evolution, viruses have developed their own endogenous factors to counteract the activity of these host restriction factors in an ongoing 'arms race' (Figure 5.2). This is because virion production would be impossible if cellular restriction factors were not inhibited. In the case of lentiviruses, the regulatory and accessory proteins oppose restriction factors. HIV-1 is one of the most intensely studied viruses, generating a rich literature describing cellular factors that restrict HIV-1 replication including: APOBEC3G, BST2 (Tetherin), SAMHD1, SERINC3, SERINC5 and TRIM5α, are described in greater detail below.

The role of APOBEC3G in HIV-1 replication was discovered through the study of HIV-1 accessory proteins. Vif is an HIV-1 protein shown to be essential for replication in CD4+ T cells, but is dispensable in other cells such as the Jurkat T cell line (Gabuzda et al., 1992). APOBEC3G was identified as the principal factor mediating HIV-1 restriction in the absence of Vif (Sheehy *et al.*, 2002). Expression of APOBEC3G in permissive cells, which allows the replication of HIV-1 in the absence of Vif such as HEK 293T, inhibited

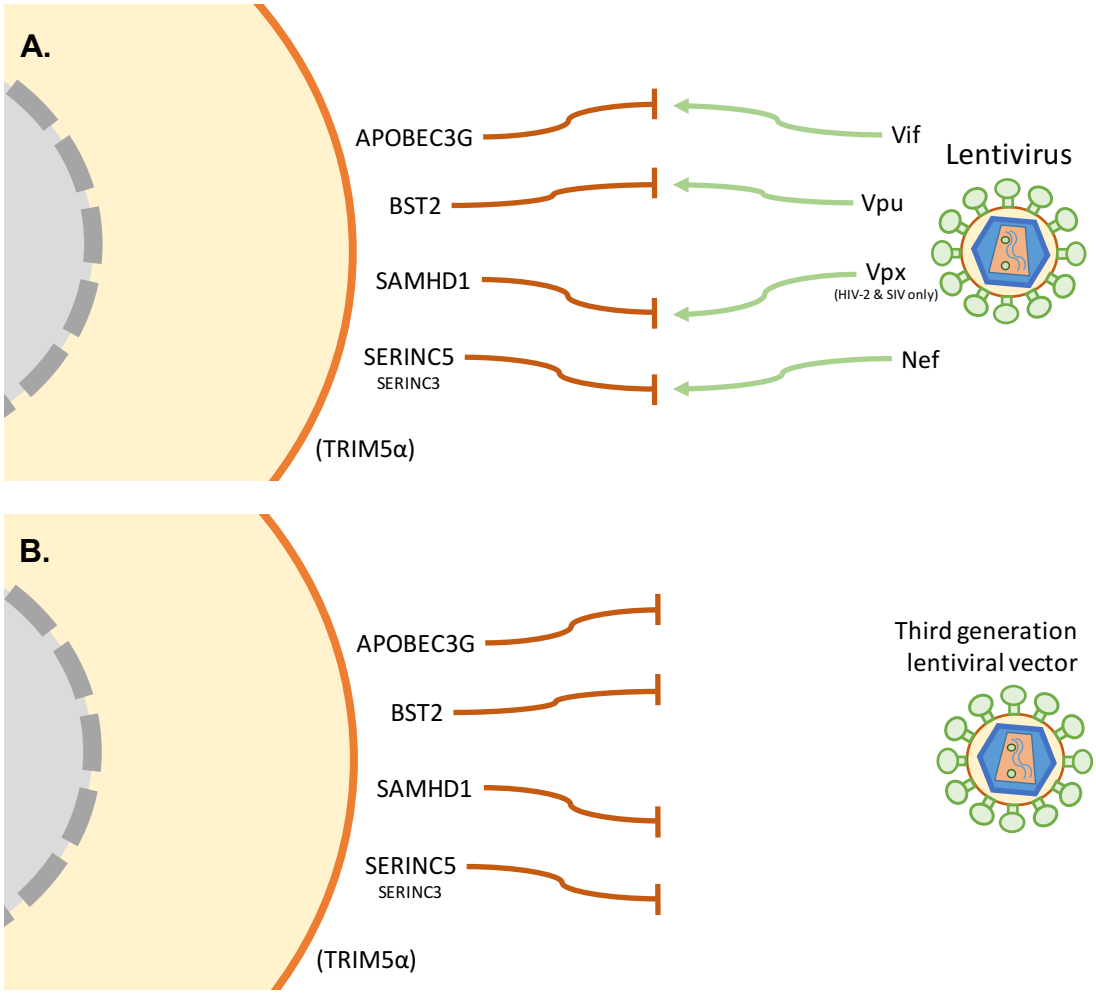


Figure 5.2. Lentiviral restriction factors along with their viral antagonists. ABOBEC3G, BST2 (Tetherin), SAMHD1, SERINC3, SERINC5 and TRIM5α are factors that restrict lentivirus replication. (A) In lentiviral infection of human cells, viral accessory proteins antagonise the effect of these restriction factors: APOBEC3G by Vif, BST2 by Vpu, SAMHD1 by Vpx (in HIV-2 & SIV only) and SERINC5 (and, to a lesser extent, SERINC3) by Nef. TRIM5α is a lentivirus restriction factor, but does not restrict HIV-1 infection of human cells, it is, however, a key restriction factor for HIV-1 in simian cells. (B) In third-generation lentiviral vector production and transduction none of the accessory proteins are present, rendering the vector susceptible to these host cell factors.

the infectivity of Vif-mutant HIV-1. ABOBEC3G is a homolog of APOBEC1 (Jarmuz et al., 2002) and both have been shown to act as DNA mutation inducers (Harris et al., 2002). Expression of APOBEC3G in HIV-1 producer cells renders the recipient cells non-permissive because, like a number of cellular proteins (Reviewed in Ott, 2008), APOBEC3G is packaged into virions (Sheehy *et al.*, 2002) and acts when the virus infects a subsequent cell, causing extensive deoxy-cytosine to deoxy-uracil mutations during reverse transcription of the minus strand DNA. This causes guanine to adenosine substitutions in the genomic strand and terminates the life cycle at reverse transcription (Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). Vif prevents APOBEC3G incorporation into virions by depleting its intracellular levels both by impairing the translation of APOBEC3G mRNA and accelerating the posttranslational degradation of the APOBEC3G protein (Conticello et al., 2003; Harris et al., 2003; Kao et al., 2003; Marin et al., 2003; Stopak et al., 2003; Yu et al., 2003). APOBEC3G can also restrict the replication of incoming viruses at the reverse transcription step (Guo et al., 2006). Other members of the ABOBEC3 family have also been linked with HIV-1 restriction, in particular APOBEC3F (Reviewed in Albin & Harris, 2010).

BST2, was found to be a Vpu-antagonised HIV-1 restriction factor (Neil *et al.*, 2008; Van Damme *et al.*, 2008). It localises to lipid rafts (Kupzig et al., 2003), where HIV-1 budding occurs, and prevents virion release through a tethering mechanism where virions at the cell surface are linked to the cellular membrane and to each other (Neil et al., 2007). It has also been shown to have the potential to block a number of other enveloped viruses through the same mechanism (Jouvenet et al., 2009). Additionally, BST2 can act as an innate sensor through activation of NF-κB (Galão et al., 2012). Vpu is required for efficient HIV-1 production in cell lines that express high levels of BST2 such as HeLa and primary T cells but not in cells lines such as HEK 293 that express very little or none (Neil *et al.*, 2008). Vpu acts by down-regulating BST2 from the cell surface, thus

counteracting this cellular antiviral defence mechanism (Van Damme *et al.*, 2008). The process through which BST2 down-regulation occurs is still unclear, Vpu could affect the intracellular trafficking of BST2 to the cell surface, sequester it to intracellular compartments by blocking its recycling after endocytosis or induce enhanced endolysosomal trafficking and degradation (Reviewed in Arias et al., 2012). In the case of HIV-2, which does not have a Vpu protein, its Env protein was found to fulfil a similar role (Bour & Strebel, 1996), and to be the antagonist of BST2 (Le Tortorec & Neil, 2009).

SAMHD1 is a restriction factor counteracted by Vpx (Hrecka *et al.*, 2011; Laguette *et al.*, 2011) and functions as a deoxynucleoside triphosphate (dNTP) triphosphohydrolase (Goldstone et al., 2011; Powell et al., 2011). It restricts HIV replication by depleting the intracellular pool of dNTPs (Lahouassa et al., 2012) necessary for reverse transcription. Exogenous Vpx strongly reduces SAMHD1 in dendritic cells and macrophages (Hrecka *et al.*, 2011; Laguette *et al.*, 2011). In SAMHD1-negative cells, exogenous expression reduces their sensitivity to HIV-1 while exogenous Vpx restores HIV-1's ability to infect these cells. The addition of exogenous deoxynucleoside alleviates the SAMHD1-mediated restriction (Lahouassa *et al.*, 2012). Furthermore, SAMHD1 silencing leads to an increased cell sensitivity to HIV-1 and vpx-null HIV-2 as well as an accumulation of viral DNA (Laguette *et al.*, 2011). However, only HIV-2 and SIV of macaques (SIV_{mac}) express Vpx and it is yet unclear how HIV-1 evades SAMHD1 restriction.

In 2015, a new family of restriction factors was identified through the study of HIV-1 accessory proteins. The protein Nef had long been known to promote HIV-1 infectivity (Chowers *et al.*, 1994), nevertheless, the host restriction factor targeted by Nef had not been identified. SERINC5 and, to a lesser extent, SERINC3, were shown to restrict HIV-1 infectivity and to be counteracted by Nef (Rosa *et al.*, 2015; Usami *et al.*, 2015). SERINC5 is localised in the plasma membrane and is incorporated into budding virions,

impairing their ability to translocate their content into the target cells' cytoplasm. Nef prevents virion incorporation of SERINC5 by redirecting it to an endosomal compartment (Rosa *et al.*, 2015). Double-knockout of SERINC3 and SERINC5 in CD4+ T cells leads to a 100-fold increase in the infectivity of Nef-deficient virions, while re-expressing SERINC3 and SERINC5 in these cells restores the restrictive phenotype (Usami *et al.*, 2015). Interestingly, SERINC3 and SERINC5 appear to have limited effect on the infectivity of VSV-g and Ebola virus glycoprotein pseudotyped viruses (Rosa *et al.*, 2015; Usami *et al.*, 2015). The VSV-g pseudotype targets HIV-1 entry to an endocytic pathway (Aiken, 1997), bypassing the SERINC3/SERINC5 block during the usual fusion with the cell membrane and also suppressing the requirement for Nef. It is also important to note that SERINC3 and SERINC5 expression varies considerably in cells; SERINC5 is expressed at high levels in Jurkat cells, but at low levels in HEK 293T (Rosa *et al.*, 2015; Usami *et al.*, 2015) whereas SERINC3 is expressed at higher levels in HEK 293T cells (Usami *et al.*, 2015). Consequently, in the context of VSV-g-pseudotyped lentiviral vectors produced in HEK 293T cells, SRINC3 and SERINC5 might only have a limited effect, even in the absence of Nef.

Viral infections are typically restricted to specific hosts and this has also been observed in the case of lentiviruses (Hofmann et al., 1999). In the case of primates, TRIM5α is a mediator of this restriction pattern by specifically recognising the capsid and promoting its rapid premature disassembly (Stremlau et al., 2006). It was identified in rhesus macaques (Stremlau et al., 2004) and African green monkeys (Hatziioannou et al., 2004; Keckesova et al., 2004; Yap et al., 2004) as a gene that, when introduced into permissive human cells, renders them resistant to HIV-1, but not to SIV_{mac} (Hofmann *et al.*, 1999). Human TRIM5α is not, however, a restriction factor for HIV-1 and SIV_{mac} in human cells; its antiviral activity against those viruses is very weak, but it potently restricts MLV (Stremlau *et al.*, 2004). The C-terminal domain of the protein determines the specificity

of retrovirus restriction (Ohkura et al., 2006) and a single amino acid substitution in the C-terminal domain of the human TRIM5α can confer it the ability to restrict HIV-1 (Yap et al., 2005).

The roles of these intrinsic restriction factors are well known in lentiviral infection, but they are also present in the producer cells during lentiviral vector production. Importantly, as described in Section 1.4, third-generation lentiviral vectors have been stripped of their accessory proteins for increased safety. This leaves the vector form of the lentivirus at the mercy of the host cell's restriction factors, which the viral accessory proteins are meant to counteract, and this could have a subsequent impact on production titre (Figure 5.2B). Helpfully, the HEK 293 cell line is known to express very low levels of the main restriction factors acting late in the HIV-1 life cycle (APOBEC3G, BST2 and SERINC5) (Tanner et al., 2007), these cells, therefore, are a better choice for lentiviral vector production than, for example, HeLa cells which express seven times more APOBEC3G and 252 times more BST2 (Tanner *et al.*, 2007; Havugimana et al., 2012).

5.1.1.4 Inhibitory factors

It would be surprising, of course, if a process as complex as virus inhibition could be achieved using only six cellular restriction factors. This, therefore, calls for another category of cellular factors, which have an adverse effect on virus production or infectivity, but which are not so crucial as to abolish virus production. Although such factors are usually referred to as *restriction* factors in the literature, here they are termed *inhibitory* factors here as they are not sufficiently critical to necessitate a direct countermeasure from the virus in the form of regulatory or accessory proteins. Such factors can, for example, accomplish cellular functions that indirectly inhibit virus production or affect cell growth. Most reviews of HIV-1-cell interaction, focus only on the

six canonical restrictions factors described in section 5.1.1.3 (Harris et al., 2012; Malim & Bieniasz, 2012; Santa-Marta et al., 2013; Strebel, 2013; Jia et al., 2015; Simon et al., 2015), disregarding inhibitory factors.

Inhibitory factors have mostly been studied in regards to viral entry, with several genes, such as CH25H, DDX58 (RIG-I) and MX2 (MxB), described as having an impact on the initial steps of HIV-1 replication. CH25H converts cholesterol into 25-hydroxycholesterol, a soluble antiviral factor, which broadly inhibits the growth of enveloped viruses including HIV-1 (Liu et al., 2013b). DDX58 is a cytoplasmic viral RNA sensor that is inhibited by the HIV-1 protease (Solis et al., 2011). MX2's orthologue in mouse, Mx1, is known to be an inhibitory factor for influenza replication (Lindenmann, 1964). Its paralogue in humans, MxA is a well-characterised inhibitory factor for a number of viruses (Pavlovic et al., 1990). Recently, MxB was shown to be an interferon-induced inhibitor of HIV-1 infection (Kane et al., 2013).

The inhibiting genes acting in the late phase of HIV-1 replication, and accordingly relevant to lentiviral vector production, are the target of the literature review conducted in this chapter.

5.1.2 Methods used to study inhibitory host factors

The study of restriction factors has largely focused on the interaction between viral proteins and virus infectivity in permissive and resistant cell lines, or through knock-down/knock-out studies. In order to find inhibitory factors, that are not necessarily linked to a viral protein in a direct antagonist way, a number of experimental strategies can be used. These include: monitoring the effects of gene silencing or gene overexpression, investigating host proteins found in virus particles or the effect of HIV-1 infection on host

gene expression levels, and the differential regulation of certain genes in different categories of HIV-infected patients. Each of these techniques has strengths and weaknesses which are explored below.

RNAi is a sequence-specific, post-transcriptional gene silencing process, initiated by a dsRNA with sequence homology to the silenced gene. It is well established that synthetic siRNA can mediate sequence-specific gene knock-down in both mammalian cell lines (Elbashir et al., 2001) and in mice (Mccaffrey et al., 2002). siRNAs targeting the mRNA of HIV-1 Gag or the cellular receptor CD4, for example, have been shown to inhibit virus production (Novina et al., 2002). siRNA can, therefore, be used to silence single genes of interest, or employed in a high-throughput, possibly genome-wide screen, to study the effects of many genetic factors. The silencing level depends both on transfection efficiency and the individual siRNA potency. Importantly, siRNAs have relatively short half-lives, which limits their use to short-term knock-down of gene expression. One alternative is to use short hairpin RNA (shRNA), an artificial RNA molecule constructed by connecting the sense and antisense strands of a siRNA duplex with a tight hairpin (Brummelkamp et al., 2002; Mcmanus et al., 2002; Paddison et al., 2002); after transcription, shRNAs are processed into siRNAs by the Dicer enzyme. The use of shRNA has the advantage of a lower rate of degradation and turnover compared with siRNAs (Reviewed in Rao et al., 2009). In addition, shRNA silencing can be accomplished through the use of plasmids, bacterial or viral vectors, to assess the impact of knock-down of gene expression in screens similar to siRNA screens.

A more recent technique uses clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) (CRISPR/Cas9)-based technology to knock-out genes from cell line allowing to study the effect of a permanent removal of the target gene on cells and processes such as viral replication. The

CRISPR/Cas9 technology is derived from an adaptable immune mechanism used by many bacteria and archaea to protect themselves from foreign nucleic acids, such as viruses or plasmids (Barrangou et al., 2007). The type II CRISPR system from *Streptococcus pyogenes* has been adapted to introduce sequence-specific double-stranded breaks which allow targeted genome editing (Jinek et al., 2012). This has proven to be an efficient tool to edit the genomes of human cells (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). To accomplish this, two components must be introduced into cells: Cas9 and a guide RNA. This guide RNA has 20 nucleotides at its 5′ end which direct Cas9 to a specific target DNA site creating a sequence-specific double-stranded break. This break can be repaired by the cells, but usually leads to insertions or deletions which can lead to the target gene's knock-out. Alternatively, this technique can also be used for gene knock-in using a similar strategy, but with the addition of a repair template (Reviewed in Sander & Joung, 2014).

In contrast to observing the effects of down-regulation of gene expression, the effects of gene over-expression can be investigated using expression plasmids, which allows the effect of artificially high concentrations of the desired gene product to be investigated.

Another indication that a gene product might be an important host factor in HIV-1 replication is when it is found packaged in virions. During virus assembly, a number of cellular proteins are packaged inside the virus or selectively included in the envelope (Reviewed in Ott, 2002; Cantin et al., 2005). This is not a random event; essential factors are packaged in the virion thereby facilitating replication in subsequent host cells and restriction factors are excluded. Some factors are also selectively packaged into virions in order to prevent the subsequent infection of the next host cells, as has been described for APOBEC3G (Sheehy *et al.*, 2002).

Finally, cellular factors involved in HIV-1 replication can be identified by studying the expression profile of genes *in vitro* following HIV-1 infection or in patients at different stages of infection (non-infected, acute infection, chronic infection, AIDS or under highly active antiretroviral therapy) as well as in patients with different levels of innate control of HIV-1 infection. If trends in gene expression can be established in these conditions, they could indicate that a specific gene might be an important factor in HIV-1 infection.

Inhibitory factors identified in the literature through these techniques, and that act late in the HIV-1 life cycle, could also inhibit lentiviral vector production or infectivity. In this context, it could be possible that the knock-down or knock-out of genes encoding such inhibitory factors would release the inhibition and consequently increase virus production titres. With this aim, this chapter describes an investigation of the scientific literature to identify inhibitory factors that could act in the late phase of the HIV-1 life cycle (which affect lentiviral vector production) for application in a siRNA screen described in Chapters 6 and 7.

5.2 Results

5.2.1 Systematic investigation of the literature

A search for publications was conducted on MEDLINE via the PubMed interface, using the following keywords in the Title/Abstract field: “HIV-1 restriction factor”, “inhibit HIV”, “repress HIV” or “restrict HIV”, with a publication date up to December 31, 2015. This search term combination led to the identification of 2,084 records. These were screened to select papers that identified genes that have an adverse impact on the late phase of the HIV-1 life cycle (Figure 5.3). Genes that were found to act early in the HIV-1 life cycle were not included in the results, but genes for which a step in the life cycle could not be identified were kept. Data from records that listed very large numbers of genes without

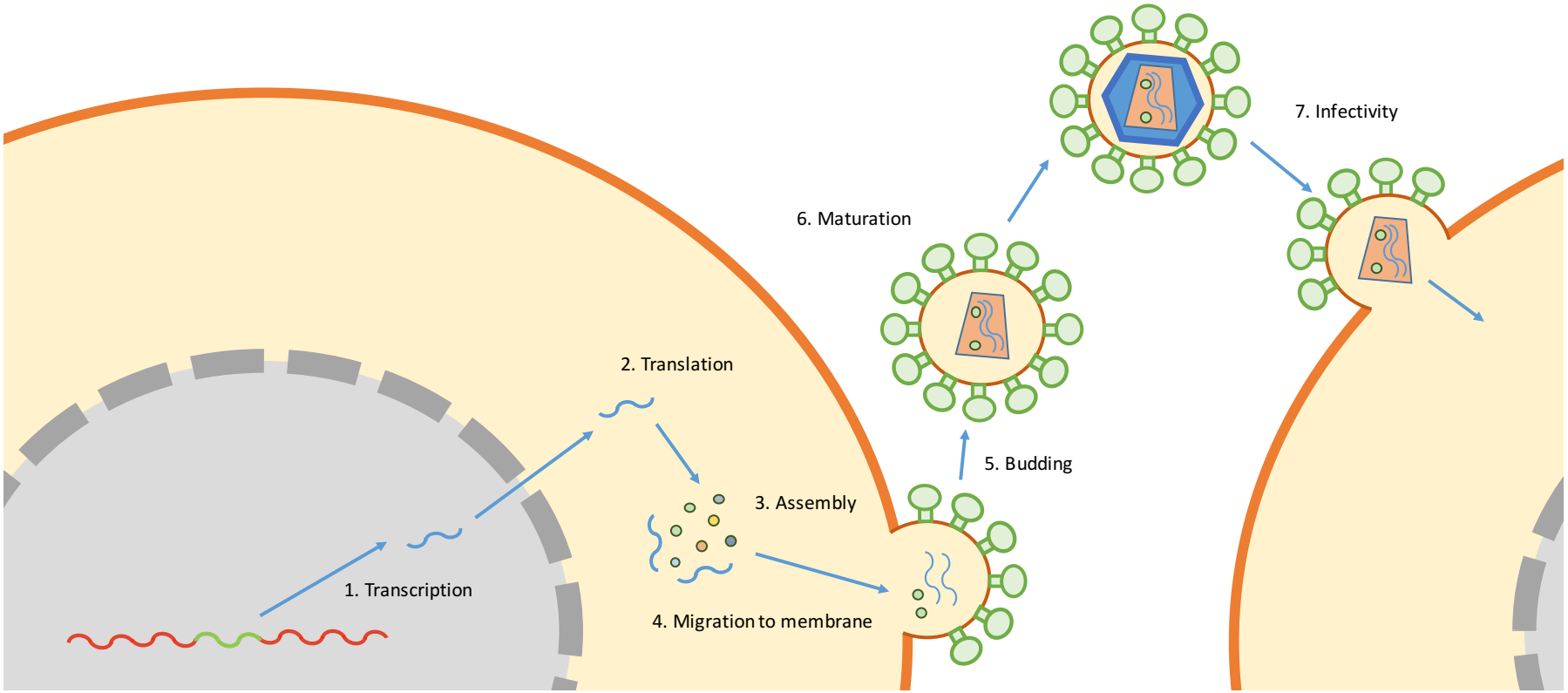


Figure 5.3. Schematic showing stages in the late phase HIV-1 life cycle. Production of lentiviral vectors via producer cell lines, or transient transfection, involves only the late steps of the HIV-1 life cycle (See also Figure 1.1). The genes identified in the literature search are identified by the step in which they are presumed to be active. Life cycle steps depicted: (1) transcription (2) translation, (3) assembly, (4) migration to the cell membrane, (5) budding, (6) maturation and (7) infectivity of the produced virions.

addressing the HIV-1 life cycle step in which these acted were retained, but only for information on genes also identified in other studies. The identified genes were termed 'candidate inhibitory factors', as they could potentially adversely affect lentiviral vector production. The reference lists of the papers identified in the search were also consulted to identify any published studies missed by the database search. Additional database enquiries were run using the name of candidate inhibitory factors and the keyword "HIV" to find further articles confirming findings. Studies providing conflicting results, informing the candidate inhibitory factor status, that were obtained from these additional searches were also included to obtain a more complete picture of the current state of knowledge on each of the identified genes. The Google Scholar and PubMed Gene database profile for each gene also provided supplementary studies that were investigated.

A total of 148 records that identified 152 candidate inhibitory factors were retained from this search (Table 5.1). A selection of auxiliary and essential factors is also presented in Table 5.2. These serve as controls in the experiments presented in Chapters 6 and 7. For each gene, in both tables, the protein absolute expression in particle per million (ppm) in HEK 293 cells (Tanner *et al.*, 2007) is listed as extracted from the MOPED database (Kolker et al., 2012). These data show the prevalence of the protein inside the cell and confirm that the restriction factors (Section 5.1.1.3) relevant to the late phase of the HIV-1 life cycle (APOBEC3G, BST2, SERINC3 and SERINC 5) are expressed at very low levels in HEK 293 cells (< 1.5 ppm), the lowest of all the cell lines and tissues listed in the database. Where possible, the HIV-1 life cycle step(s) where the gene is first described, or expected to be active, in the cited papers or in the HIV-1 interaction section of PubMed's Gene database, is indicated. The steps covered are transcription (1), translation (2), assembly (3), migration to the cell membrane (4), budding (5), maturation (6) and infectivity (7) as indicated in Figure 5.4. Two genes have an impact on cell apoptosis and are indicated as such in Table 5.1.

Table 5.1. Restriction and candidate inhibitory factors acting in the late phase of the HIV-1 life cycle identified in the literature review.

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
ABCA1	0.69	5 & 7	Mujawar-2006 Mujawar-2010 Cui-2012	4 4 1a, 4
ADAR (ADAR1)	87.60	2	(Clerzius-2009) Loke-2010 (Schoggins-2011) Biswas-2012 (Clerzius-2013) Weiden-2014	(1a, 2) 5b (2) 2 (1b, 2) 1a, 5a
AGO2	7.51	2-3	Nathans-2009	1a
AMT	Unknown		Dziuba-2012	1a
APOBEC3B	12.60	7	Bishop-2004 Yu-2004 Doehle-2005b	2 2, 3 2, 3
APOBEC3C	25.40	7	(Bishop-2004) (Wiegand-2004) Yu-2004 (Zheng-2004) (Doehle-2005b) Langlois-2005 Wang-2008a Abdel-Mohsen-2013 Raposo-2014	(2) (2) 2, 3 (2) (2) 2 3 5a 5b
APOBEC3D	Unknown	7	Dang-2008 Abdel-Mohsen-2013	2 5a
APOBEC3F	2.24	7	Bishop-2004 Liddament-2004 Wiegand-2004 Yu-2004 Zheng-2004 Doehle-2005b Langlois-2005 Dang-2008 Wang-2008a	2 2 2 2 2 2 2 2 3
APOBEC3G	0.54	7	Sheehy-2002 Kao-2003 Mangeat-2003 Mariani-2003 Marin-2003 Sheedy-2003 Zhang-2003 Bogerd-2004 Bishop-2004 Kobayashi-2004 Liddament-2004 Schröfelbauer-2004 Yu-2004 Doehle-2005a Doehle-2005b Langlois-2005 Bouazzaoui-2006 Dang-2008 Wang-2008b	2 2 3, 6 2 2 3 2 3 2 2 2 2 2, 3 2, 3 2 2 2 2 3
APOBEC3H	unknown	7	Dang-2008 Raposo-2014	2 5b
APOL1	0.57	2-3	Taylor-2014 McLaren-2015	2 2

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
APOL3	unknown	2	Loke-2010 McLaren-2015	5b 2
APOL6	0.66	2	McLaren-2015	2
AXIN1	3.42	2	Kameoka-2007 Kameoka-2009	1a 1a
BANP (SMAR1)	0.45	1	Sreenath-2010 Henderson-2012	1a, 2 1a
BST2 (tetherin, CD317)	1.27	5	Neil-2008 Van Damme-2008	1a, 2 1b, 2, 4a
CD151	33.80	5	Haller-2014	4
CD164	unknown		McLaren-2015	2
CD1A	3.39	after 2	McLaren-2015	2
CD37	0.79	5 & 7	Haller-2014	4
CD3E	unknown	after 2	McLaren-2015	2
CD3G	unknown		Voigt-2015	5b
CD53	unknown	5 & 7	Daneka-2007 Loke-2010 Tippett-2013 Haller-2014	3 5b 5a 4
CD63	21.60	5 & 7	Daneka-2007 (Chen-2008) (Ruiz-Mateos-2008) Sato-2008 Krementsov-2009 Haller-2014 (Li-2014) (Fu-2015)	3 (1a) (1a) 2, 3 2 4 (1a) (1a)
CD81	111.00	7	Daneka-2007 Sato-2008 Grigorov-2009 Krementsov-2009 Brégnard-2013 Tippett-2013 Haller-2014	3 2, 3 1b, 3 1b, 2 1a, 2, 3 4 4
CD82	unknown	5 & 7	Sato-2008 Krementsov-2009	2, 3 1b
CD9	unknown	5 & 7	Daneka-2007 Sato-2008 Krementsov-2009 Schoggins-2011 Tippett-2013	3 2, 3 2 2 4
CDH23	0.20		Dziuba-2012	1a
CDKN1A (p21)	4.17	1	Schoggins-2011 Abdel-Mohsen-2013 Farberov-2015	2 5a 4, 6
CHMP5	71.70	5	Ward-2005 (Schoggins-2011)	1a (2)
CNP	99.90	3	Wilson-2012 Linde-2013	2 3
COMMD1	1.19	1	Ganesh-2003	1a, 2
CTNNB1 (β-Catenin)	64.40	1	Henderson-2012 Narasipura-2012 Aljawai-2014	1a 1a 6
DDX5 (p68)	399.00	1, 5, 7	Naji-2012 Lorgeoux-2013 (Yasuda-Inoue-2013) (Zhou-2013) (Williams-2015)	1a 1a (2) (1a, 2) (1a)
DDX6 (RCK/p54)	217.00	2-3	Nathans-2009 (Reed-2012)	1a (1a)
DHX30	68.20	3	Zhou-2008b	2

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
DICER1	12.30	3	Bennasser-2005 Christensen-2007 Triboulet-2007 Nathans-2009 Qian-2009 Coley-2010 Casey Klockow-2013	6 1a 1a 1a 1a 4 1a, 4a
DLG1	21.80	7	Perugi-2009 Nzounza-2012	1a, 1b, 2 1b
DNAJA1 (HSP40A1)	218.00	1-7	(Kumar-2005) (Kumar-2011) (Dziuba-2012) Urano-2013	(1a, 2) (1a, 2) (1a) 2
DNAJB1 (HSP40B1)	unknown	1-7	(Kumar-2005) (Brass-2008) (Kumar-2011) Urano-2013	(1a, 2) (1a) (1a, 2) 2
DNAJB6 (HSP40B6)	14.60	1-7	(Kumar-2005) (Kumar-2011) Urano-2013	(1a, 2) (1a, 2) 2
DNAJC5 (HSP40C5)	96.60	1-7	(Kumar-2005) (Kumar-2011) Urano-2013	(1a, 2) (1a, 2) 2
DROSHA	4.42	2	Triboulet-2007 Nathans-2009	1a 1a
DUSP1	unknown	2	Kameoka-2007	1a
EIF2AK2 (PKR)	49.80	2	Benkirane-1997 Adelson-1999 Muto-1999 Dimitrova-2005 Ong-2005 Clerzius-2009 Sanghvi-2011 Clerzius-2013 Raposo-2014	2 2 2 4b 2 2 1a 2 5b
EIF3E	172.00	1	Jäger-2012	1a
EIF3F	186.00	1	Valente-2009 Jäger-2012	2 1a
EZR (VIL2, Ezrin)	558.00	5	Kameoka-2007 Haedicke-2008 Brégnard-2013 Linde-2013 (Wen-2014a)	1a 1a, 2 1a, 2 3 (1a, 1b)
FCGR3A	0.87		McLaren-2015	2
GBP5	unknown	7	(Schoggins-2011) McLaren-2015	(2) 2
GM2A	139.00		Zhou-2008a	1a
HAS2	unknown		Dziuba-2012	1a
HDAC1	202.00	1	Coull-2000 He-2002 Keedy-2009 Zhang-2011	6 6 6 1a
HDAC2	213.00	1	Keedy-2009	1a, 6
HDAC3	24.70	1	Keedy-2009	1a, 6
HERC5	17.00	3	Woods-2011 Raposo-2014	1b, 2, 5b 5b
HEXIM1	57.00	1	Barboric-2007	2
HSF1	3.52		Kameoka-2007	1a
HSP90AB1	8740.00		(Joshi-2013) Linde-2013	(2, 3) 3

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
HSPA12A	3.02	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA12B	5.65	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA13	13.10	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA14	55.60	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA1A	9820.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA1B	unknown	1	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA1L	711.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA2	55.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA4	611.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA4L	86.50	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA5	1580.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA6	685.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA7		1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA8	5520.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
HSPA9	828.00	1 & 7	Gurer-2002 Kumar-2011 Sugiyama-2011 Linde-2013	3 1a, 2 2 3
IFI16	14.30	1	(Schoggins-2011) McLaren-2015	(2) 2

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
IFI44	unknown		Loke-2010 Lu-2011 Schoggins-2011 Power-2015	5b 1b 2 1a, 2
IFITM1	13.50	2-3	Loke-2010 Lu-2011 Raposo-2014	5b 1b 5b
IFITM2	unknown	2-3	Loke-2010 Lu-2011 Schoggins-2011 Raposo-2014	5b 1b 2 5b
IFITM3	unknown	2-3	Lu-2011 Schoggins-2011 Raposo-2014	1b 2 5b
ISG15	54.90	5	Okumura-2006 Loke-2010 (Schoggins-2011) Woods-2011 Okumura-2013	1a, 2 5b (2) 2 6
KAT5 (Tip60)	3.87	apoptosis	(Kamine-1996) Creaven-1999 Col-2005	(2) 4a 4a
KCNK3 (TASK1)	0.58		Hsu-2004 Farberov-2015	2, 4 4, 6
LEF1		1	Narasipura-2012	1a
LIF	unknown	1	Patterson-2001 Tjernlund-2003 Tjernlund-2006 Tjernlund-2007	2, 5b 5b 5b 2
LPP	32.90		Dziuba-2012	1a
LSM1	119.00	2-3	Nathans-2009	1a
MAP3K5	0.33	apoptosis	Geleziunas-2001	6
MARCH8	unknown	7	Tada-2015	1b, 1c, 2
MB21D1 (cGAS)	unknown	7	Bridgeman-2015 Gentili-2015	6 6
MIR17	n/a	1	Triboulet-2007	2, 4a
MIR17HG	n/a	1	Triboulet-2007	4a
MIR18A	n/a	1	Triboulet-2007	4a
MIR19A	n/a	1	Triboulet-2007	4a
MIR19B1	n/a	1	Triboulet-2007	4a
MIR20A	n/a	1	Triboulet-2007	2, 4a
MIR29A	n/a	1-3	Nathans-2009 Sun-2011	1*, 2 1*, 2
MIR29B1	n/a	1-3	Nathans-2009 Sun-2011	1*, 2 1*, 2
MIR29B2	n/a	1-3	Nathans-2009 Sun-2011	1*, 2 1*, 2
MIR29C	n/a	1-3	Nathans-2009	1*, 2
MIR92A1	n/a	1	Triboulet-2007	4a
MOV10	73.70	2	Burdick-2010 Wang-2010 Abudu-2012 Raposo-2014	1a, 2, 3 1a, 2, 3 3 5b
NEAT1	n/a	3	Zhang-2013 Budhiraja-2015	1a, 4b 1a
NRON	n/a		Imam-2015	1a, 1b, 4a
NTRK3 (TRKC)	unknown		Dziuba-2012	1a
OAS1	unknown		(Schoggins-2011) McLaren-2015	(2) 2

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
PARP14	0.37		Lu-2011	1b
PINX1 (LPTL, LPTS)	17.00		Dziuba-2012	1a
PRKAA1 (AMPKa1)	25.20	1	(Zhou-2008a) Zhang-2009	(1a) 1a
PRKAA2 (AMPKa2)	4.44	1	Zhang-2009	1a, 6
PRKRA (PACT)	46.80	2	Patel-1998 Patel-2000 (Daher-2009) (Clerzius-2013)	6 6 (2) (1b, 2)
PRMT6	3.60	2-3	Boulanger-2005 Invernizzi-2006 Invernizzi-2007 Xie-2007 Singhroy-2013	1a, 2 1a, 2 6 1a, 2 2
RN7SL1	n/a	7	Wang-2007 Wang-2008a Wang-2008b	3, 6 6 6
RN7SL2	n/a		Wang-2007 Wang-2008a Wang-2008b	3, 6 6 6
RN7SL3	n/a		Wang-2007 Wang-2008a Wang-2008b	3, 6 6 6
RNASEL	2.41	2	Maitra-1998 Raposo-2014	2 5b
RNF115 (BCA2/Rabring7)	3.73	3 & 7	Miyakawa-2009 Nityanandam-2014	1a, 2 1b, 2
ROCK2	14.20	2	Kameoka-2007	1a
RSAD2 (Viperin)	unknown	3	Nasr-2012	1a, 2, 4b
RTP4	unknown		Lu-2011 (Schoggins-2011)	1b (2)
RUVBL2 (RVB2)	163.00		Mu-2015	1b, 2
SERINC3	1.44	7	Rosa-2015 Usami-2015	1c, 2 1a, 1c, 2, 3
SERINC5	unknown	7	Rosa-2015 Usami-2015	1c, 2, 3 1a, 1c, 2, 3
SETDB1	11.60	1	Van Duyne-2008	1a
SIRT1	16.00	1	(Pagans-2005) Zhang-2009	(1a, 2) 1a
SLC51A (OSTalpha)	unknown		Dziuba-2012	1a
SLFN11	41.10	2	Li-2012 Abdel-Mohsen-2013 Raposo-2014	1b, 2 5a 5b
SP3	8.91		Majello-1994	2
SPN (CD43)	unknown	7	McLaren-2015	2
SUMO1	242.00	7	Gurer-2005 de la Cruz-Herrera-2014	2, 3 6
SUMO2	426.00	7	de la Cruz-Herrera-2014	6
TCF4 (TCF-4)	0.35	1	Wortman-2002 Henderson-2012 Narasipura-2012	2 1a 1a
TDRD7	9.98		Lu-2011	1b

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
TFCP2			Romerio-1997 Coull-2000 He-2002	2 2, 6 6
TNFRSF10A	4.02		Schoggins-2011 McLaren-2015	2 2
TNFRSF10D	unknown		McLaren-2015	2
TRIM11	3.81	1	Uchil-2008 Raposo-2014	2 5a
TRIM15	unknown		Uchil-2008	2
TRIM21	3.72	1	Ohmine-2011 (Schoggins-2011) Raposo-2014	2, 3 (2) 5b
TRIM22 (STAF50)	unknown	1, 3-5	Bouazzaoui-2006 Barr-2008 Uchil-2008 Loke-2010 Kajaste-Rudnitski-2011 Ohmine-2011 Raposo-2014 Turrini-2015	2, 4b 1b, 2 2 5b 1b, 2 2, 3 5b 1b, 2
TRIM26	32.00	1	Uchil-2008 Abdel-Mohsen-2013	2 5a
TRIM32 (HT2A)	7.69	1	Uchil-2008 Abdel-Mohsen-2013	2 5a
TSPAN7 (CD231)	unknown	5 & 7	Sato-2008 Haller-2014	2, 3 4
TSPO	20.00	2	Zhou-2014	1b, 1c, 6
UBA7 (UBE1L)	0.23	5	Okumura-2006	2
UBASH3A (TULA)	0.34	3	Smirnova-2008	2
UBASH3B (TULA-2)	0.69	3	Smirnova-2008	1a, 2
UBE2L6 (UBCH8)	unknown	5	(Schoggins-2011) Woods-2011	(2) 2
UBP1 (LBP-1)	11.60		Parada-1995	2
YY1	40.00	1	Margolis-1994 Romerio-1997 Coull-2000 He-2002 Sundaravaradan-2010	2 2 2, 6 2, 6 5a
ZC3H12A (MCPIP1)	1.15	1-3	Liu-2013a	1a, 2
ZNF10	unknown	1	Nishitsuji-2015	1a, 1b, 2
ZNF350 (ZBRK1)	unknown	1	Nishitsuji-2012	1b, 2

Table 5.2. Selection of essential and candidate auxiliary factors acting in the late phase of the HIV-1 life cycle.

Gene	[ppm] in HEK 293	HIV-1 Life Cycle Step	Source	Screen Type
ATP1A1	181.00	1	(Laird-2014)	(6)
DDX24	50.40		(Ma-2008)	(1a)
LIMK1	0.35	5	(Wen-2014a) (Wen-2014b)	(1a) (1a, 1b)
PLK1	26.90		N/A	N/A
PRKACA	236.00	7	(Cartier-2003)	(3, 6)
TARBP2 (TRBP1)	25.80	2	(Benkirane-1997) (Ong-2005) (Christensen-2007) (Clerzius-2009) (Sanghvi-2011) (Clerzius-2013)	(6) (2) (1a) (2) (1a) (2)
TSG101	30.90	5	(Garrus-2001) (Martin-Serrano-2001) (Demirov-2002) (Ward-2005) (Zhou-2008b) (Wen-2014a) (Fu-2015)	(1a) (6) (6) (1a) (1a) (1a) (1a)
UBE2I (UBC9)	224.00	4 & 7	(Jaber-2009) (Bohl-2013)	(1a) (1a, 6)

Legend for Tables 5.1 and 5.2 These tables list restriction and candidate inhibitory factors (Table 5.1) or candidate auxiliary and essential factors (Table 5.2) acting in the late phase of the HIV-1 life cycle that were identified in the literature review. Each **Gene** is identified with its HUGO Gene Nomenclature Committee official symbol (Gray *et al.*, 2015), with alternative names used in cited papers in parentheses. The **[ppm] in HEK 293** refers to the protein concentration in particle per million (ppm) in Human Embryonic Kidney 293 (HEK 293) cells as described in (Tanner *et al.*, 2007). The **HIV-1 Life Cycle Step** refers to the step in the Human Immunodeficiency Virus type 1 (HIV-1) life cycle where the gene is affecting production (Figure 5.3), specifically: (1) transcription, (2) translation, (3) assembly, (4) migration to the cell membrane, (5) budding, (6) maturation, and (7) infectivity. Two genes regulate cellular apoptosis and are labelled as such. The **Source** refers to the studies identifying the gene as a candidate HIV-1 inhibitory factor in the late phase of the HIV-1 life cycle or a candidate essential/auxiliary factor if listed in parentheses. The **Screen Type** indicates the technique or study used to identify the candidate host factor, ranked by the perceived value of the evidence: (1a) siRNA knock-down, (1b) shRNA knock-down, (1c) CRISPR/Cas9 knock-out, (1*) miRNA inhibition, (2) protein over-expression, (3) virion incorporation, (4) active down-regulation during *in vitro* studies, (5a) down-regulated in HIV-infected patients, (5b) over-expression in HIV-infected patients and (6) any other evidence (Section 5.2.1), once again parentheses are used if the study identified the gene as a candidate essential/auxiliary factor.

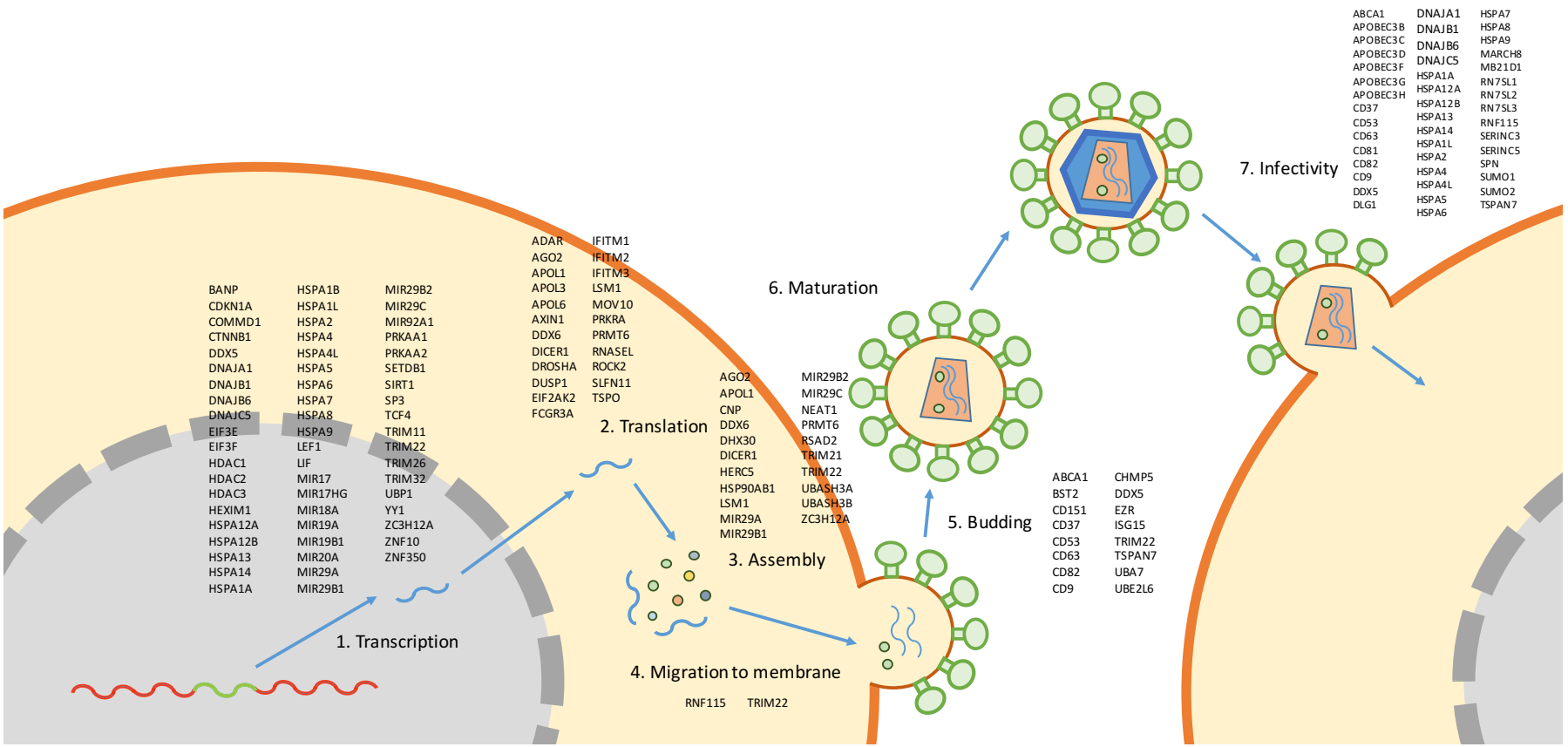


Figure 5.4 Candidate inhibitory factors and the step in the late phase HIV-1 life cycle where they are inferred to act. The genes identified in the literature search are indicated in the diagram near the step where they are presumed to be active in the late stages of the HIV-1 life cycle, as derived from the literature review. Steps depicted are (1) transcription, (2) translation, (3) assembly, (4) migration to the cell membrane, (5) budding, (6) maturation and (7) infectivity of the produced virions. Genes without precisely identified steps are not shown.

Each paper was scrutinised to determine the techniques that lead to the identification of the candidate inhibitory factor. A ranking was established to evaluate the relative 'value' of the data presented in the papers. The most reliable data were obtained from silencing or knock-out studies (ranked 1), followed by overexpression studies (ranked 2), detection of virion incorporation of a gene product (ranked 3), active down-regulation by the virus during cell culture studies (ranked 4), change in expression in HIV-infected patients (ranked 5) and any other evidence (ranked 6), the lowest in value. The reliability of the identification of a specific gene as a candidate inhibitory factor involved in HIV-1 inhibition was further evaluated based on the number of publications citing the gene, with more confidence given to genes identified in multiple studies.

5.3 Discussion

In this chapter, the literature was investigated to identify candidate inhibitory factors involved in the late phase of HIV-1 replication, manipulation of which could potentially increase titres during lentiviral vector production. A total of 152 genes expressing potential inhibitory factors were identified and are shown in Figure 5.4, alongside the respective step where they act in the viral life cycle. Approximately 40.1% of these candidate inhibitory factors (61/152) were identified in papers published since 2010, indicating that this is a very dynamic field of research. The literature investigation performed here was done thoroughly, using broad research terms, followed by a repeated search using the gene names in two additional databases to extend the findings. It is possible that some studies, and hence some candidate factors, may have been overlooked. Nevertheless, with more than a hundred genes implicated, this suggests that this systematic search is likely to be more representative of a complex phenomenon such as viral infection inhibition, compared with the small number of restriction factors often cited in published reviews for the entire HIV-1 life cycle. This

literature review constitutes, therefore, a first step to explore inhibitory factors exclusively in the late phase of HIV-1 replication.

Not all the evidence identifying candidate inhibitory factors was deemed of equal value. Nearly half of these candidate genes (74/152: 47.4%) were identified in a single article and did not appear to have been validated in further studies. Furthermore, the technique used to identify the genes varied vastly. Gene knock-down and knock-out studies give results that are very representative of the effect of a gene. These techniques were used to identify more than half (67/152, 57.2%) of the genes found here. Only three studies induced a complete knock-out of a gene, using CRISPR/Cas9, but it is expected that many more studies using this technology will be published in future. On the other hand, overexpression studies create an 'artificial' expression profile which might never exist in a normal cell. This might explain why several studies used a combination of both knock-down and overexpression strategies to assess the effect of a gene on HIV-1 production. The other types of evidence for identifying candidate inhibitory factors were the fact that a protein is packaged into virions, that its level is increased or decreased during infection of that expression levels vary between different infected patients or uninfected individual. While these techniques might hint at a specific role for that particular gene in HIV-1 replication, it is not sufficient to confirm this effect. Fortunately, most genes identified using these techniques were also identified in studies using RNAi-mediated knock-down. Studies described in the next chapter partially address the issue of validation of these candidate genes, by testing them in a high-throughput siRNA screen.

Chapter 6 – Development of a high-throughput screen to validate candidate inhibitory factors

6.1 Introduction

This chapter continues the focus on virus-cell interactions, in order to ultimately enhance upstream lentiviral vector production. In Chapter 5, an investigation into the HIV-1 literature was performed which generated a list of candidate inhibitory factors acting in the late phase of the HIV-1 life cycle. To determine if these candidate factors are relevant to lentiviral vector production, a high-throughput screening protocol was designed here.

6.1.1 High-throughput genome-wide screens

High-throughput screening is a drug-discovery process that uses automation to assay the biological or biochemical activity of numerous drug-like compounds. High-throughput screening is also a powerful strategy that allows the identification of factors involved in virus replication not previously linked to virus infection, as well as confirmation of previously established relationships. Most genome-wide screens for genes affecting human viral infections, published to date, have tended to focus on essential/auxiliary factors with only three specifically focussing on inhibitory factors (Table 6.1). However, screens that did look for inhibitory factors usually focussed on the early steps of the viral life cycle (viral entry to integration) as these are most relevant as targets for drugs blocking early infection. This is also true in the case of HIV-1 with six genome-wide screens performed, four of which looked only for essential/auxiliary factors, one for both essential/auxiliary and inhibitory factors and one looking at inhibitory factors acting early in the HIV-1 life cycle (Table 6.1). The characteristics of the different screens are presented in Table 6.2 and helped inform the design of a new screen, described in this chapter.

Table 6.1 High-throughput genome-wide screens for genes impacting human viral infections

Virus	Study	Organism	Cellular factors	Screen type
AAV	(Mano et al., 2015)	Human	Inhibitory factors (early phase)	Genome-wide
coronavirus	(Wong et al., 2015)	Human	Essential/auxiliary factors	Genome-wide
coxsackievirus B & poliovirus	(Coyne et al., 2011)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
dengue virus	(Sessions et al., 2009)	Drosophila / Human	Essential/auxiliary factors	Genome-wide / Targeted
hepatitis C virus	(Li et al., 2009)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
hepatitis C virus	(Tai et al., 2009)	Human	Essential/auxiliary factors	Genome-wide
HIV	(Nguyen et al., 2007)	Human	Essential/auxiliary factors	Genome-wide
HIV	(Brass *et al.*, 2008)	Human	Essential/auxiliary factors	Genome-wide
HIV	(Konig et al., 2008)	Human	Essential/auxiliary factors	Genome-wide
HIV	(Zhou *et al.*, 2008a)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
HIV	(Yeung et al., 2009)	Human	Essential/auxiliary factors	Genome-wide
HIV	(Liu et al., 2011)	Human	Inhibitory factors (early phase)	Genome-wide
influenza	(Brass et al., 2009)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
influenza	(Hao et al., 2008)	Drosophila / Human	Essential/auxiliary and inhibitory factors	Genome-wide / Targeted
influenza	(Karlas et al., 2010)	Human	Essential/auxiliary factors	Genome-wide
influenza	(Konig et al., 2010)	Human	Essential/auxiliary factors	Genome-wide
influenza	(Prusty et al., 2011)	Human	Essential/auxiliary factors	Genome-wide
influenza	(Su et al., 2013)	Human	Essential/auxiliary factors	Genome-wide
Rift Valley fever virus	(Hopkins et al., 2013)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
Sindbis virus	(Ooi et al., 2013)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
Sindbis virus	(Panda et al., 2013)	Drosophila	Essential/auxiliary and inhibitory factors	Genome-wide
VSV	(Panda et al., 2011)	Human	Essential/auxiliary factors	Genome-wide
VSV	(Lee et al., 2014:)	Human	Essential/auxiliary factors	Genome-wide
West Nile virus	(Krishnan et al., 2008)	Human	Essential/auxiliary and inhibitory factors	Genome-wide
West Nile virus	(Yasunaga et al., 2014)	Drosophila	Inhibitory factors (early phase)	Genome-wide
yellow fever virus	(Le Sommer et al., 2012)	Human	Essential/auxiliary factors	Genome-wide

Table 6.2 Comparison of high-throughput screen for essential, auxiliary and inhibitory factors implicated in HIV-1 replication

Study	Screen type Number of genes (Looking for)	Steps covered	Cell Line	cDNA/siRNA/ shRNA transfection (Number/gene)	Time from transfection to challenge	Virus / Vector	Harvest; Readout in new cells	Readout	Cut-off	Hits found
(Nguyen *et al.*, 2007)	Sub-genomic 15,000 genes (Essential/Auxiliary Factors)	Entry to release	HeLa ($CD4^+$, β-gal reporter) with HIV-LTR-Luciferase reporter plasmid	Gene Juice (1 cDNA/gene)	24 hours	Live HIV-1 (III B) infection	72 hours	Luc reporter in producer cells	2-fold	315
(Brass *et al.*, 2008)	Genome-wide 21,121 genes (Essential/Auxiliary Factors)	Entry to infectivity	HeLa ($CD4^+$, β-gal reporter)	Oligofectamine (4 siRNA/gene)	72 hours	Live HIV-1 (III B) infection	48 hours; 48 hours	p24 (CA) in screened cells & reporter activation in infected new cells	2-SD	273
(Konig *et al.*, 2008)	Genome-wide 19,628 genes (Essential/Auxiliary Factors)	Entry to translation	HEK 293T	Lipofectamine 2000 (6 siRNA/gene)	48 hours	VSV-g-pseudotyped HIV-1 Luc vector transduction	24 hours	Luc reporter in screened cells	1.45-fold	295
(Zhou *et al.*, 2008a)	Genome-wide 19,709 genes (Essential/Auxiliary and Inhibitory Factors)	Entry to infectivity	HeLa ($CD4^+$, β-gal reporter)	Oligofectamine (3 siRNA/gene)	24 hours	Live HIV-1 (HXB2) infection	48 & 96 hours; 48 hours	reporter activation in screened cells & reporter activation in infected new cells	2-SD or 2-fold	232 (81)
(Yeung *et al.*, 2009)	Genome-wide 59,509 transcripts (Essential Factors)	Entry to release	Jurkat	VSV-g pseudotyped FIV (3-5 shRNA/gene)	3 weeks	HIV-1 NL4-3	4 weeks	cell survival after puromycin and HIV-1 selection	2-fold	252
(Liu *et al.*, 2011)	Genome-wide 19,121 genes (Inhibitory Factors)	Entry to translation	HeLa ($CD4^+$)	HIPErfect (4 siRNA/gene)	72 hours	$HIV^{89.6R}$ (EGFP reporter)	48 hours	reporter activation in screened cells	3-SD	114
Current screen	Targeted 89 genes (Inhibitory Factors)	Transcription to Infectivity	293SF-LVP	Lipofectamine 2000 (4 siRNA/gene)	72 hours	VSV-g pseudotyped HIV-1 EGFP vector production	72 hours; 72 hours	reporter activation in transduced new cells	2-SD	9

Legend Steps covered: steps of the HIV-1 life cycle covered by the screen (Figure 1.3), **Harvest; Readout in new cells**: single values indicate a readout at harvest while two values indicate both the harvest time and the time before results readout in a different set of cell transduced or infected with the virus produced in the original cells, **Cut-off**: criteria for selection of hits, **Hits**: genes recognised to be essential, auxiliary or inhibitory factors to HIV-1 replication in the screen.

This leaves an opportunity in the unexplored field of inhibitory factors acting late in the HIV-1 life cycle, which is the focus of the current study. In Chapter 7, a screen is presented that significantly differs from these published screens, as a targeted approach was adopted in order to focus on the siRNA-mediated knock-down of selected inhibitory factors identified in Chapter 5.

The strategy used in previously published high-throughput screens (infection or transfection with a provirus or a lentiviral vector) involves going through early steps of the HIV-1 life cycle and hence early inhibitory factors. To focus on the late phase of the life cycle, an inducible producer cell line and associated titration cell line were used in the current screen and are described below.

6.1.2 Cell lines used in the assay

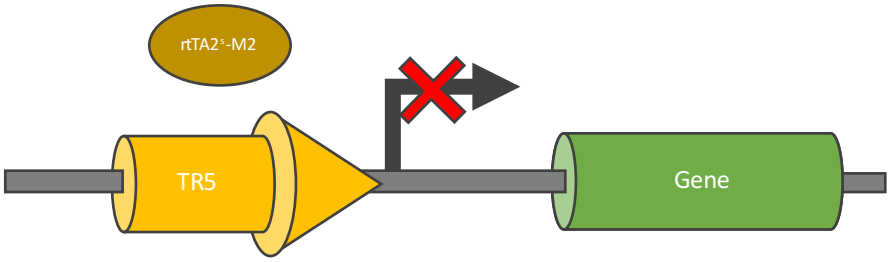
To assess the impact of a gene's knock-down on lentiviral vector production, a siRNA molecule targeting that gene needs to be introduced into lentiviral vector producing cells. In the experiments described in Chapters 3 and 4, a transient transfection process was used to generate lentiviral vector producing cells. Using a similar transient transfection for the plasmids necessary to initiate lentiviral vector production in a siRNA knock-down screen, however, would necessitate two concurrent transfection events for the vector-producing cells: one transient transfection to introduce the virus production plasmids, and one transient transfection to introduce the knock-down siRNAs. As observed with experiments involving two transfections in Chapter 4, it is possible that the two transfections events would introduce additional variability into the experiment. Furthermore, the two concurrent transfection steps could constitute a confounding factor leading to difficulties in results interpretation. Hence, to circumvent the need for two transfection steps, and to increase reproducibility, an alternative

process of initiating lentivirus production using an established inducible producer cell line was employed.

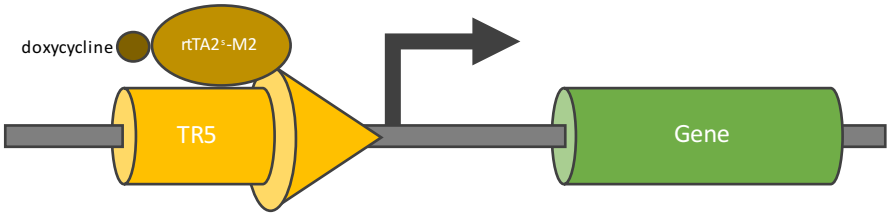
The HEK 293-based cell line 293SF-PacLV #29-6-14 (293SF-LVP; Table 2.1) (Broussau *et al.*, 2008), obtained from R. Gilbert (National Research Council of Canada, Montréal, Canada), was selected. This is an inducible lentiviral vector producer cell line controlled by two independent switches, which generates LVR2-GFP (rHIV.VSV-g CMV GFP), a third-generation, self-inactivating, HIV-based lentiviral vector pseudotyped with VSV-g, with a cytomegalovirus promoter that directs the expression of GFP. The choice of a VSV-g pseudotype-based cell line was motivated by the need to reduce the number of steps involved since the produced vectors don't need to be activated like it was the case for Sendai virus F/HN pseudotyped-vectors in Chapters 3 and 4. Here again, a fluorescent reporter gene, GFP, was deemed ideal for quick detection of transduced cells at the titration step.

This cell line is based on the HEK 293SF cells (Table 2.1) (Cote *et al.*, 1998), a cell line adapted to grow in a serum-free, low-calcium medium which was itself derived from HEK 293S (Table 2.1) adapted for suspension culture (Stillman & Gluzman, 1985). In the 293SF-LVP cells, lentiviral vector production is repressed by an inducible tetracycline-regulated gene expression system (Tet-On) (Gossen et al., 1995) and a cumate repressor system (Mullick et al., 2006) described below. Lentiviral vector production from 293SF-LVP cells is, therefore, only initiated when doxycycline (a tetracycline analogue) and cumate are added to the media. To create the 293SF-LVP cell line, HEK 293SF cells were first modified to express the repressor of the cumate switch (CymR) (Mullick *et al.*, 2006) and the reverse transactivator of the tetracycline switch (rtTA2^{s}-M2) (Urlinger et al., 2000) (Figure 6.1 & 6.2A). This allows the presence of doxycycline and cumate in the media to activate expression of the genes regulated

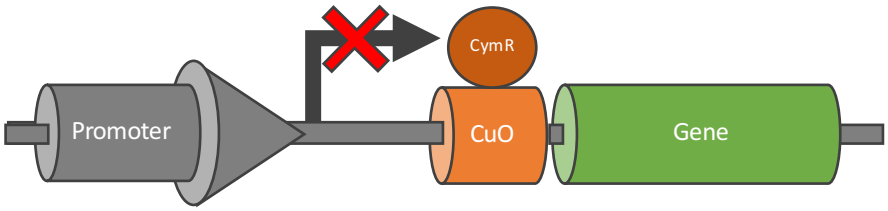
A **Off (no doxycycline)**



On (with doxycycline)



B **Off (no cumate)**



On (with cumate)

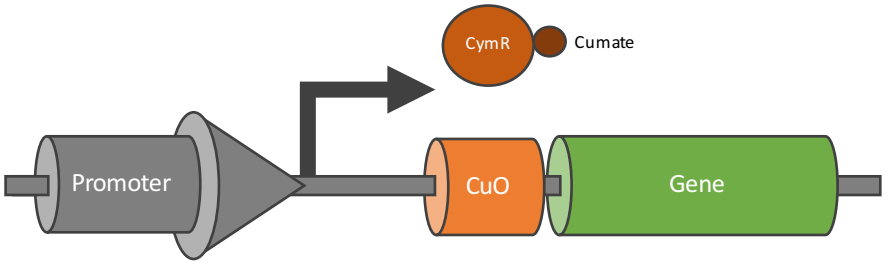
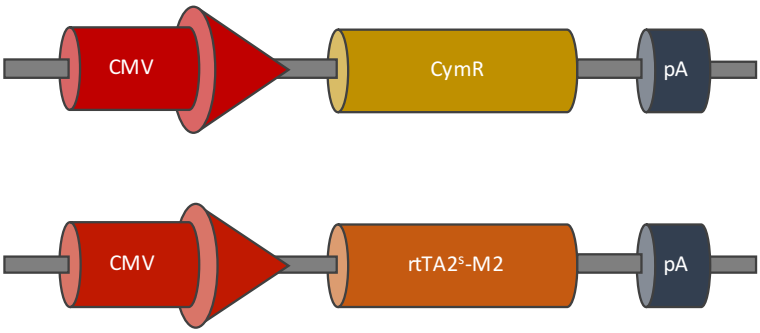
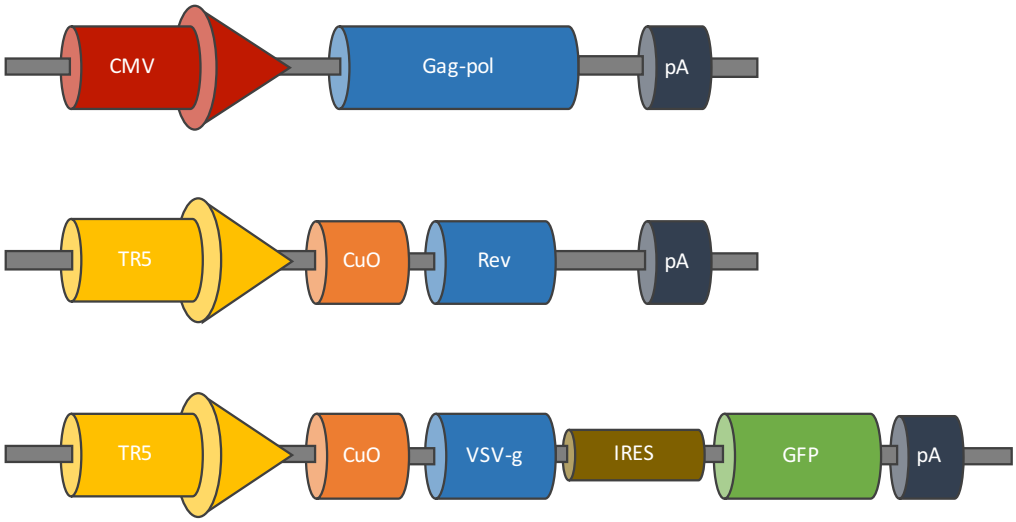


Figure 6.1. The doxycycline and cumate inducible gene expression systems. A) Doxycycline induces binding of the reverse transactivator of the tetracycline switch (rtTA2^{S}-M2) to the tetracycline-regulated promoter (TR5) inducing transcription. No binding of rtTA2^{S}-M2 occurs in the absence of doxycycline. B) In the absence of cumate, the repressor of the cumate switch (CymR) binds to the cumate operator (CuO) and prevents transcription. Cumate inhibits binding of CymR to CuO which allows transcription. Adapted from (Broussau et al., 2008).

A **Switches**



B **Packaging plasmids**



C **Transfer vector**

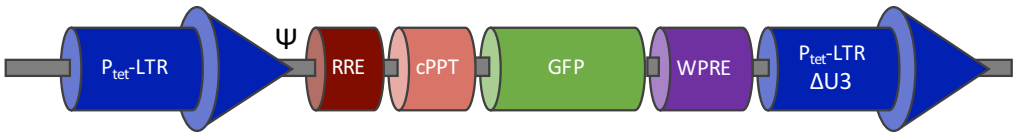


Figure 6.2. Plasmids used in the construction of the stable 293SF-LVP producer cell line (Broussau et al., 2008). (A) Schematic of plasmid components used to introduce the repressor of the cumate switch (CymR) and the reverse transactivator of the tetracycline switch (rtTA2^{S}-M2) generating the 293SF-CymR-rtTA2^{S}-M2 (293SF-Titr) titration cell line. (B) Schematic of components of additional plasmids, that were used to generate the 293SF-PacLV (Table 2.1) packaging cell line. (C) Key components of the transfer vector used to generate the stable producer cell line 293SF-LVP (Table 2.1). CMV, immediate early enhancer and promoter of cytomegalovirus; TR5, tetracycline-regulated promoter and binding site for the doxycycline-bound rtTA2^{S}-M2; CuO, cumate operator, binding site for CymR; IRES, internal ribosome entry site; Ptet-LTR, tetracycline-regulated long terminal repeat and binding site for rtTA2^{S}-M2; Ψ, packaging signal; ΔU3, deletion of the U3 region that removes its promoter activity; RRE, rev-responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; GFP, green fluorescent protein; cPPT, central polypurine tract; VSV-g, glycoprotein of vesicular stomatitis virus. Adapted from (Vigna et al., 2002) and (Broussau et al., 2008).

by these switches. Doxycycline promotes the binding of rtTA2^{S}-M2 to the TR5 promoter allowing transcription (Figure 6.1A). The rtTA2^{S}-M2 functions at low doxycycline concentrations, is stable in eukaryotic cells and causes minimal background expression in the absence of doxycycline (Urlinger et al., 2000). Cumate works in the opposite way, by promoting the release of CymR, expressed in these cells from the cumate operator (CuO), thus allowing transcription to occur (Mullick et al., 2006) (Figure 6.1B). This cell line, 293SF-CymR-rtTA2^{S}-M2 (293SF-Titr) was also obtained from R. Gilbert (Table 2.1) and was used in this study for titration of lentiviruses generated by the producer cells. The addition of doxycycline to these cells during titration allows binding of rtTA2^{S}-M2 to, and transactivation of, the TR5 promoter in transduced viruses previously produced using 293SF-LVP cells. This, in turn, activates GFP production, which allows detection of virus-infected cells by fluorescence microscopy.

The 293SF-Titr cell line was further modified to create a packaging cell line by co-transfection of the plasmids pMPG-CMV-Gag/polRRE, pTR5-CuO-Rev, pTR5-CuO-VSVG-IRES-GFPq and pPuro (described in (Broussau et al., 2008)), generating the 293SF-PacLV cell line (Figure 6.2B & Table 2.1). These plasmids encode the viral protein Gag and Pol, the accessory protein Rev, the VSV-g pseudotype, and the resistance for puromycin, respectively. Rev is required for the efficient expression of the protease from the *gag* gene and the protease has been reported to be cytotoxic (Kaplan & Swanstrom, 1991). Thus, due to the constitutive expression of Gag in these cells even a relatively small amount of Rev could be detrimental to cell survival. The same is true for VSV-g, which is cytotoxic, and its expression is, therefore, usually regulated in packaging cell lines (Ory *et al.*, 1996). As a consequence, in 293SF-PacLV cells, Rev and VSV-g are regulated by both the TR5 tetracycline-regulated promoter and the CuO switch (Figure 6.2B). This was a key characteristic in the selection of this

cell line for this screen as viability will already be negatively affected by the siRNA transfection. The best clone of the 293SF-PacLV cells, in terms of GFP expression following doxycycline and cumate addition, was selected. It was then transfected with the transfer vector pLVR2-GFP ((Vigna et al., 2002) & (Figure 6.2C)), a tetracycline-regulated SIN HIV-based lentiviral genome which allows the production of LVR2-GFP (rHIV.VSV-g CMV GFP) (Table 2.2). This generated the 293SF-LVP producer cell line (Table 2.1), which was used for lentiviral vector production in the studies described below.

6.2 Results

6.2.1 Assay Development

6.2.1.1 Preliminary conditions for virus production in 96-well format using HEK 293T cells and transient transfection

The transient transfection protocol used in Chapter 3 and 4 for virus production is routinely performed in 20 mL, or greater, volume cultures grown in shake flasks (Figure 2.1 & Section 2.4). Hence, to establish a high-throughput screen, a number of parameters required adjustment for use in a 96-well format. For preliminary studies, transient transfection of adherent HEK 293T cells was used to scale down the current process before transitioning to the 293SF-LVP producer cells. At such a small scale, suspension culture was not possible and would have also been problematic for reading the plates where the titration was conducted as it could have required imaging multiple focal planes. It was, nevertheless, decided to keep the cells in suspension until the cells were seeded into the 96-well plates, as the inducible producer cell line has been adapted to growth in suspension in serum-free media. To assess ideal cell seeding densities, 12 wells of a 96-well plate were seeded with four different cell concentrations in triplicate, at 2×10^4, 1×10^4, 5×10^3, and 2.5×10^3 cells per well in 100 µL of FreeStyle

293. As previously done for vector production in flasks (Section 2.4), the wells were individually transfected with the five plasmids (Section 2.3.1) required to produce vGM035 (rHIV.F/HN CMV- EGFPLux) (Table 2.2) constituting a total of 1.5 µg DNA/1 x 10^6 cells. Three days after seeding, the cells were observed using light microscopy and the cell confluence evaluated (Figure 6.3). A cell seeding density of 2 x 10^4 per well was deemed ideal for virus production based on confluence, while seeding at a lower cell density (1 x 10^4 cells per well) was more appropriate for titration so that individual cells could be more easily distinguished and counted. However, under fluorescence microscopy, none of the cells appeared positive for EGFP expression (Figure 6.3). This was thought to be due to inefficient transfection – a potential issue when using such small volumes. It was therefore decided that, in subsequent studies, the cells would be first transiently transfected as a pool in a larger volume (30 mL) and then seeded into the 96-well plate. This should improve reproducibility as previously observed in Chapter 3 as well as more closely resembling the state of an inducible producer cell line after activation.

6.2.1.2 Preliminary conditions for titration in 96-well format using HEK 293T cells

To establish the titration conditions, a single 250 mL flask containing 1 x 10^6 cells/mL in 30 mL media was transfected with the plasmid mixture required to produce vGM035 (rHIV.F/HN CMV- EGFPLux) (Section 2.4) at 1.5 µg DNA/1 x 10^6 cells and the cells were then directly seeded at 2 x 10^4 per well in a 96-well plate. After three days, a 40 µL aliquot of the supernatant of each well was transferred to the corresponding well of a separate 96-well plate. This virus was activated with 6 µL trypsin 0.05% (w/v) for 1 hour, followed by trypsin inactivation with 4 µL Defined Trypsin Inhibitor and dilution in 200 µL of FreeStyle 293 medium for a final volume of 250 µL. Volumes ranging from 5 to 50 µL were then added to a new 96-well plate containing 1 x 10^4 HEK 293T cells

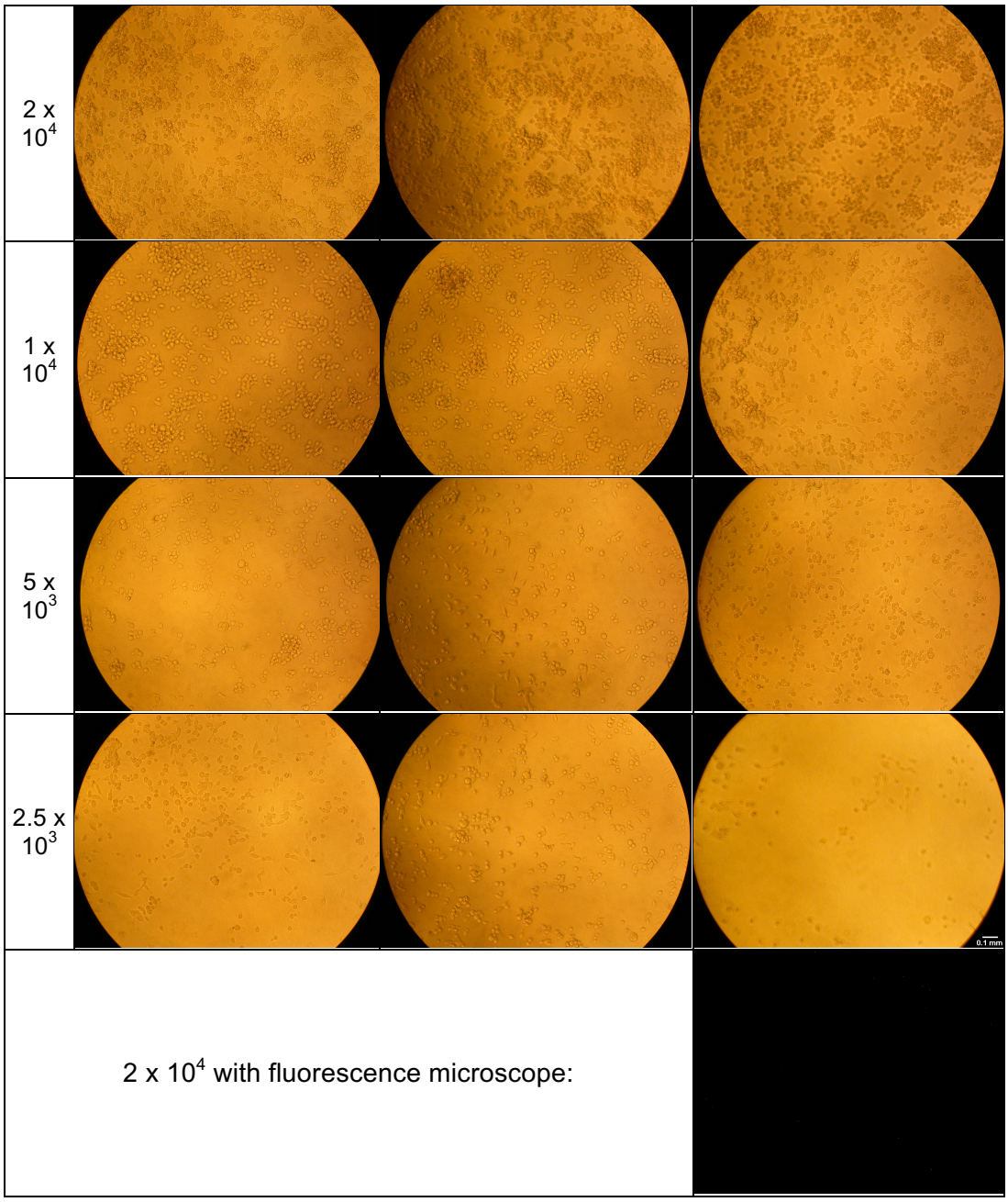


Figure 6.3 Light and fluorescence microscopy images of the 96-well plate used to determine cell seeding densities for the siRNA production and titration. Top four rows: Light microscopy images showing representative fields of wells of a 96-well plate. Each row of wells was seeded with the indicated number of cells and transiently transfected with the plasmids required for vGM035 (rHIV.F/HN CMV- EGFPLux) production three days prior to imaging. Bottom row: Representative fluorescence microscopy field of the first 2 x 10^4 well, showing no visible green cells.

in FreeStyle 293 for a final volume of 200 µL per well. This created final dilutions of activated virus ranging from 1:20 to 1:200 (the flask production protocol with titration in 12-well plates uses a dilution of 1:190). As a positive control, a trypsin-activated sample from a previous vGM035 (rHIV.F/HN CMV- EGFPLux) flask production was used. Negative controls included a non-activated supernatant from the 96-well production process, along with a well without the addition of supernatant.

After 72 hours' incubation, a gradient was observed in the numbers of EGFP positive cells per well corresponding to the dilutions, indicating the success of the dilution series. Transduction of 2-20% of the cells was deemed ideal as it would allow quantification of the virus production with a low risk of having more than one virus transducing each EGFP positive cell. The number of green cells appeared to be relatively low throughout, though, suggesting that even the 1:20 dilution might be too dilute for the experiment. A possible cause for this is the absence of the sodium butyrate addition or the media change steps. These steps were omitted because of the small volumes involved here. While this might negatively affect titres, it would not be an issue with the inducible producer cell line as its use circumvents the need for a transfection step.

There were also some very bright green cells observed in the non-activated, negative control wells. This would suggest that despite the cells being grown adherent at this stage, there was some 'carry-over' of transfected cells from the production process, as experience with this system suggests that transduced cells are rarely as 'bright' as the producer cells. To address this issue, a filtration step using a 96-well filter plate was introduced at the harvest stage, to avoid cell carry-over. This is similar to the flask production process (Section 2.4.1) where a 0.45 µm filter was used at virus harvest, following centrifugation to prevent cell carry-over. Only 0.2 µm filter plates are available

in the 96-well format, but the impact of this change on titres, if any, should be consistent throughout the plate and therefore should not affect comparisons within the same plate.

6.2.1.3 Establishment of cell conditions for the 293SF-LVP inducible producer cell line screen

Having established the production and titration conditions with transiently transfected HEK 293T cells in a 96-well format (Sections 6.2.1.1 and 6.2.1.2), the 293SF-LVP inducible producer cell line was then introduced. For the initial assessment, a flask of 293SF-LVP cells was activated with 1 µg/mL doxycycline and 10 µg/mL cumate. When activated, green 293SF-LVP cells were observed within 24 hours under the fluorescence microscope due to the GFP transgene being present in both the integrated genome and pseudotyping constructs. Samples were harvested and frozen daily following activation until day 4. Cell viability, measured using a haemocytometer, diminished on day 3 which might indicate cytotoxicity from high virus production at that point. A titration of the daily samples was performed using a 12-well plate following the titration protocol used in studies described in Chapters 3 and 4 (Section 2.5.1), with the following modifications: 293SF-Titr cells, 1 µg/mL doxycycline-supplemented media and both 1:10 and 1:100 dilutions of production supernatant. Qualitative data from the fluorescence microscopy observation of this plate 24 and 72 hours after transduction is presented in Table 6.3. Together, these observations suggested that harvesting three days after virus production activation, followed by a titration protocol lasting three days, would be appropriate.

For production in the 96-well format, the seeding density for 293SF-LVP was evaluated, as previously done for HEK 293T, using 2×10^4, 1×10^4, 5×10^3, and 2.5×10^3 cells per well (Section 6.2.2.1). Three days after seeding, the cells were observed

Table 6.3 Summary of vector titration of virus harvested on different days and titrated at different dilutions

Observation done on	Day 1 of titration					Day 3 of titration				
Cells from production day	0	1	2	3	4	0	1	2	3	4
1:10 virus dilution used	-	-	-	+	+	-	+	+	++	++
1:100 virus dilution used	-	-	-	-	-	-	-	-	+	+

Legend Qualitative data from fluorescence microscopy of a titration of viral vectors produced in 293SF-LVP cells in a 96-well plate, harvested and frozen on different days, and then titrated at different dilutions. 293SF-Titr cells were used in a 96-well plate and observed both one and three days after transduction. Legend: -: no green cells, +: few green cells, ++: 2-20% green cells.

using light microscopy and the cell confluence evaluated. As previously (Figure 6.3), cell seeding densities of 2 x 104 and 1 x 104 were deemed ideal in terms of confluence for production and titration respectively.

6.2.1.4 Establishment of conditions for the 96-well 293SF-Titr titration

To establish the titration conditions, a 96-well plate was seeded for vector production with 2 x 10^4 activated 293SF-LVP cells per well and harvested on day 3. A new 96-well plate was seeded for titration with doxycycline-treated 293SF-Titr cells, 1 x 10^4 cells per well in 100 µL. These cells were transduced with different volumes of filtered virus from the production stage ranging from 80 µL to 5 µL (in 5 µL increments) and 2.5µL to 0.02 µL in successive two-fold dilutions. The volume of each well was kept constant at 200 µL by the addition of appropriate volumes of FreeStyle 293. After 72 hours' incubation, a gradient of dilution in terms of the number of GFP positive cells was observed; and the appropriate amount of virus supernatant to reach 20% green cells was established to be between 10 and 30 µL.

6.2.1.5 Establishment of experimental controls

Dharmacon ON-TARGET Plus siRNAs were selected as the preferred source of siRNAs on the basis that they are available as pools of four individual siRNAs and are guaranteed by the manufacturer to silence target gene expression by at least 75% at the mRNA level when used under optimal delivery conditions. This feature is described as having been determined using a validated positive control and measured at the mRNA level 24 to 48 hours after transfection using 100 nM siRNA. The manufacturer further claims that the siRNAs are highly functional at a working concentration of 5 to 25 nM. They also contain a 'dual-strand modification pattern' to reduce off-targets. This is where the sense strand bases and base linkages are modified to reduce interaction with the RNA-induced silencing complex (RISC), in turn, favouring antisense strand

usage. Additionally, the antisense strand seed region, the region responsible for recognition of the target mRNA, is also similarly modified to destabilise off-target activity and enhance target specificity (Jackson et al., 2006).

To establish siRNA transfection conditions, four control siRNAs were used: (1) a *non-target control*, which consists of a pool of four siRNAs designed and tested by the manufacturer for minimal targeting of human, mouse or rat genes and recommended for establishing baseline cellular responses in RNAi experiments. This constitutes the negative control in each experiment to which all results are normalised. (2) A *transfection efficiency control* consisting of a siRNA targeting GFP. This siRNA, the GFP Duplex I, (target sequence: 5'-GCA AGC TGA CCC TGA AGT TC–3') has shown high-efficiency silencing of GFP and EGFP at both the mRNA and protein level (Caplen et al., 2001). Its effect on viral titres was evaluated to determine if it could also act as a viral production knock-down control, possibly by targeting the GFP-containing viral RNA before its packaging in virions. (3) A *cell proliferation control*, consisting of a pool of siRNAs targeting the polo-like kinase 1 (PLK1), a critical regulator of mitotic cell division (Reviewed in Van De Weerdt & Medema, 2006), was used. Using antisense oligonucleotides, it has been shown that downregulation of cellular PLK1 levels in cancer cells altered cell cycle progression with potent anti-proliferative effects in cell culture (Spankuch-Schmitt et al., 2002b). Using siRNAs, cell proliferation was reduced by 66-99%, 48 hours after transfection. Cell apoptosis was also increased from 1–5% to 13–50% in transfected cells (Spankuch-Schmitt et al., 2002a). (4) A *production knock-down control*, which consists of a pool of siRNAs targeting a known essential factor, the tumour susceptibility 101 (TSG101) gene. It has been demonstrated that depletion of cellular TSG101 expression by siRNA arrests HIV-1 budding at a late stage and that budding can be rescued by reintroduction of TSG101 (Garrus *et al.*, 2001). Importantly, knock-down of TSG101 has been used as a positive control for

knock-down of HIV-1 production in several of the screens described in Table 6.2 (Nguyen et al., 2006; Zhou *et al.*, 2008a; Wen *et al.*, 2014a).

6.2.1.6 Establishment of siRNA conditions for the production phase of the screen

Having established baseline production and titration conditions suitable for a 96-well format, subsequent studies assessed: (1) cell concentration of 1 x 10^4 or 2 x 10^4 cells per well, (2) the day of siRNA transfection (GFP or Non-Target), from 2 days before, to 2 days after, activation of virus production and (3) the transfection reagent concentration from 0.05 to 0.5 µL per well. The transfection reagent used was DharmaFECT 1, the proprietary transfection reagent recommended by GE Dharmacon, the manufacturer of the siRNAs which were used at a concentration 25 nM. Previous titration studies (Section 6.2.1.2), suggested that a 10-30 µL volume of virus was appropriate for titration studies; thus 25 µL was used here. To increase the likelihood of achieving the desired ~2-20% transduction in the titration duplicate titration studies were performed using 1 x 10^4 and 5 x 10^3 cells per well. All other parameters were as established previously.

Upon inspection of the plates under the fluorescence microscope, it was apparent that the virus produced successfully transduced the titration cells, although a very strong 'edge effect' was observed where the external rows and columns of the plates had extreme results (few, or no, green cells in top and bottom rows, very low titres in first and last columns). An inspection of the plates used for virus production revealed that the external columns and rows of plates were subject to pronounced evaporation, despite being maintained in a humidified cell culture environment. From the original 100 µL production volume, 90 µL was left in the central wells, 85 µL in the external wells and 65 µL in the corner wells. This may have significantly altered the virus

production dynamics leading to the edge effect observed in the titration results. It is of note that the production phase plates showed more pronounced evaporation than the titration phase plates which most likely reflects their extended incubation (five as opposed to three days). Data from the inner wells, where minimal or no evaporation was observed, were insufficient to compare cells transfected with GFP or Non-Target siRNAs. Nevertheless, a production cell density of 2×10^4 cells per well was deemed to be sufficient to obtain relevant virus production levels. Another observation was that there was apparent toxicity due to the siRNA transfection with DharmaFECT 1. Positive production values in the central wells indicated that a cell seeding density of 5×10^3 cells per well using 25 µL of supernatant was appropriate and these conditions were used in subsequent experiments. Finally, particularly poor virus production was noted in all conditions where siRNAs were transfected if doxycycline and cumate (to induce virus production) were added on the same day, possibly due to an interaction between the reagents.

In subsequent experiments, only the central wells were used for virus production and the outer two rows and columns of each plate were filled with 200 µL DPBS to compensate for the observed evaporation. This noticeably reduced experimental variability. Additionally, substitution of DharmaFECT 1 with either Lipofectamine 2000 or 3000 was evaluated in an attempt to reduce transfection related toxicity. In contrast to DharmaFECT 1, there was no evident toxic effect from increasing Lipofectamine 2000 concentrations and no apparent impact of using Lipofectamine 2000 to transfect siRNAs on the day of virus production activation. Lipofectamine 3000 proved to be a poor gene transfer agent under the experimental conditions evaluated and was thus not taken further.

Additional experiments assessed the *cell proliferation control* (PLK1) and the *production knock-down control* (TSG101), two siRNAs for genes expected to have a negative impact on titres. As per the manufacturer's protocol, concentrations of Lipofectamine 2000 between 0.2-0.5 µL per well (0.2, 0.35 and 0.5 µL) were used. Concentrations of siRNAs of 25, 50 and 100 nM were used as well as a 'no siRNA' control. The transfection window was extended to include siRNA transfection between three days before, and 2 days after, virus production activation. The large number of conditions evaluated precluded replicates, not allowing statistical analysis of the results. Nonetheless, the greatest reduction in virus titre (PLK1: 13% and TSG101: 14% of FreeStyle only control) was obtained with 0.5 µL Lipofectamine 2000 and 100 nM siRNA per well three days before virus production activation.

6.2.1.7 Validation of the siRNA conditions for the production phase of the screen

A follow-up experiment evaluated the effect of the four siRNA controls transfected at three different concentrations (25, 50 and 100 nM) in triplicates, three days before virus activation, with 0.5 µL Lipofectamine 2000 per well onto 2 x 10^4 293SF-LVP cells in 100 µL FreeStyle 293. Twenty-four hours before harvest, the appearance under the fluorescence microscope of the cells transfected with 100 nM siRNA confirmed that these conditions showed high transfection efficiency (Figure 6.4) while there was little difference between the wells containing the 25 and 50 nM siRNA concentrations (data not shown). At 100 nM, the GFP siRNA reduced the number of green cells (its target) without reducing the total number of cells, confirming that it was an appropriate control for transfection. From observation of the cells in this well, it was evident that a number of cells were expressing high levels of GFP, similar to non-transfected cells or cells transfected with the Non-Target siRNA, and therefore that the knock-down efficiency was not 100%. This needs to be taken into account in the interpretation of the effect of

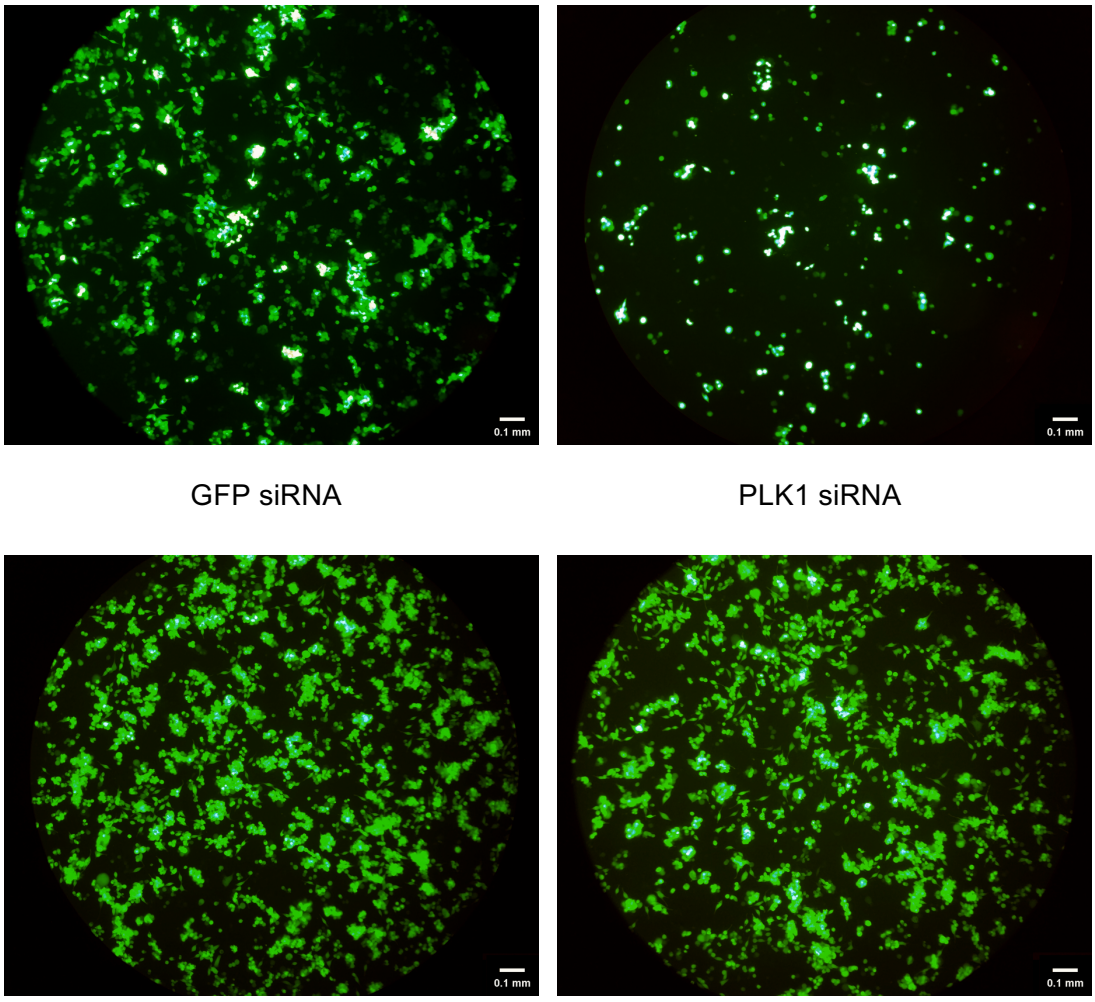


Figure 6.4 Fluorescence microscopy images showing the effect of control siRNAs transfection on producer cells. The indicated siRNAs (GFP, PLK1, Non-Target and TSG101) were used to transfect 293SF-LVP cells (2 x 10^4 cells in 100 µL FreeStyle 293, 100 nM siRNAs and 0.5 µL Lipofectamine 2000 per well) three days before activation with doxycycline (1 µg/mL) and cumate (10 µg/mL). Photographs were taken 72 hours post-activation.

all siRNAs as some cells will not be affected by the knock-down and will express normal levels of the target gene product. The PLK1 siRNA did not affect the relative green fluorescence of the cells, but there was a reduced number of cells present, and therefore overall fluorescence of the well (Figure 6.5) confirming its effectiveness as a control for cell proliferation. Even at 100 nM, there was no visible difference between the appearance and number of cells in the Non-Target and TSG101 siRNA transfected wells. Figure 6.5 shows that there was no statistically significant difference in terms of fluorescence intensity of cells in the entire well, as measured using the Envision, between Non-Target and TSG101 siRNA transfected cells, while there was a 2-fold decrease in fluorescence when GFP or PLK1 siRNAs were transfected.

At the end of the titration phase, it was evident that both the GFP and PLK1 siRNAs inhibited virus production very significantly, with the number of green cells per well being notably lower than with the Non-Target siRNA (Figure 6.6 and 6.7). This indicates that the GFP siRNA inhibited the virus production, possibly by targeting viral RNA for degradation and that the PLK1 siRNA also inhibited virus production, in addition to blocking cells from replicating as observed during production (Figure 6.4). The TSG101 siRNAs reduced the number of green cells compared with the Non-Target siRNAs to a lesser extent, not reaching significance in this experiment (Figure 6.6 and 6.7).

Several published studies identified in Chapter 5 (Table 5.1) investigated transfections of siRNAs at two separate time-points. The objective of this strategy was to ensure that the knock-down of the protein was efficient throughout virus production, which requires at least three days. To evaluate the potential of this approach, three plates were seeded for virus production. To minimise issues with the evaporation of medium from the outer wells, only the internal 32 wells of each plate were utilised and the two

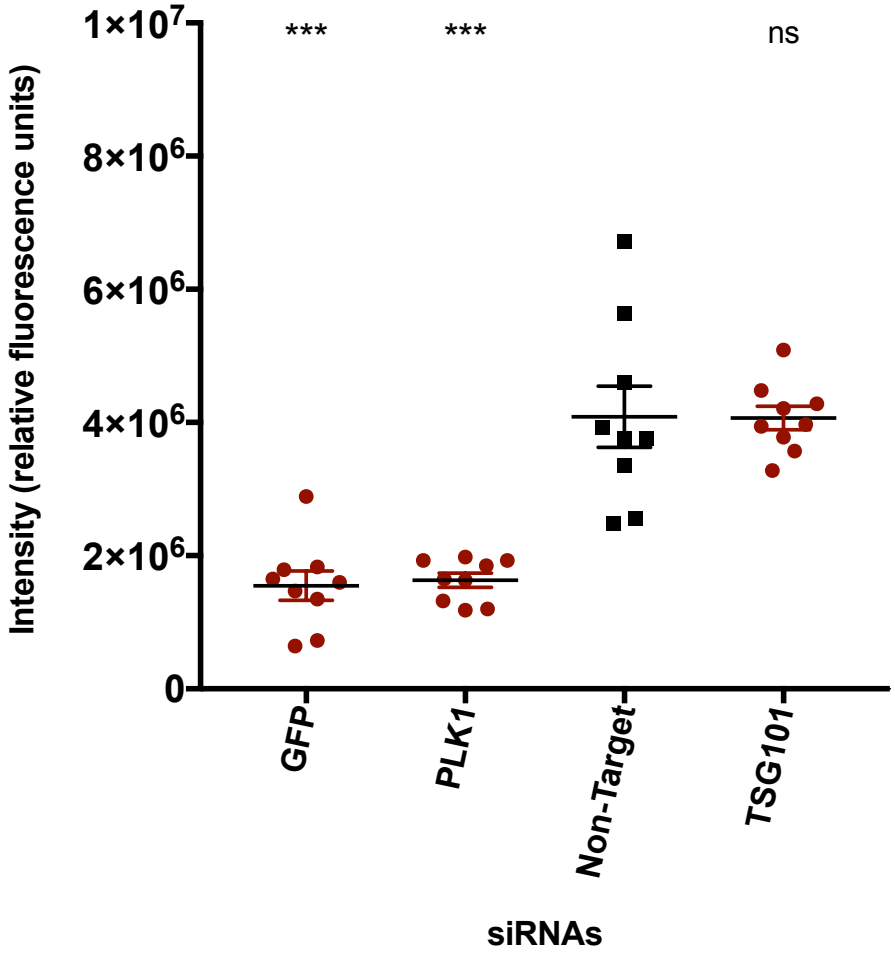


Figure 6.5 Fluorescence intensity readings showing the effect of control siRNA transfections on producer cells. The indicated siRNAs (GFP, PLK1, Non-Target and TSG101) were used to transfect 293SF-LVP cells (2 x 10^4 cells in 100 µL FreeStyle 293, 25-100 nM siRNAs and 0.5 µL Lipofectamine 2000 per well in triplicates) three days before activation with doxycycline (1 µg/mL) and cumate (10 µg/mL). Individual readings as well as mean intensity (relative fluorescence units) ± SEM are shown for all wells of the indicated siRNAs, regardless of the concentration used, and were measured using the Envision (Section 2.4.4). Statistically significant differences between group means were determined by one-way ANOVA: $F(3,32) = 27.24$, $p < 0.001$, followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on figures. Calculated p values < 0.001 were deemed a significant difference as indicated with three stars (***) on the chart.

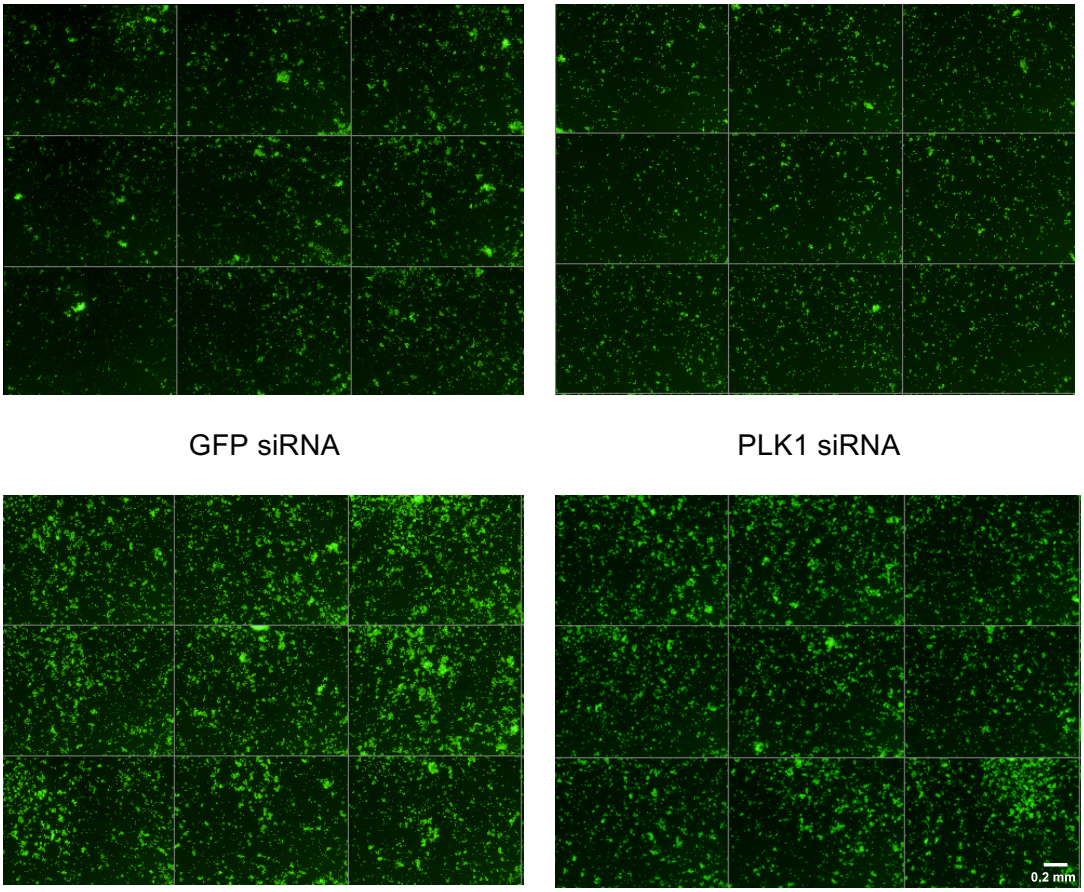


Figure 6.6 Fluorescence microscopy images of the titration of lentiviral vector productions showing the effect of control siRNA transfections on titres. Fluorescence microscope images of the central fields of nine wells in the 96-well plate where virus produced in the presence of the indicated siRNAs was titrated (taken with the Operetta High-Content Imaging System (Section 2.6.2)). The relative number of green cells shows the effect on titres of the transfection of different siRNA controls during lentiviral vector production: GFP (*transfection efficiency control*), PLK1 (*cell proliferation control*), Non-Target control and TSG101 (*production knock-down control*).

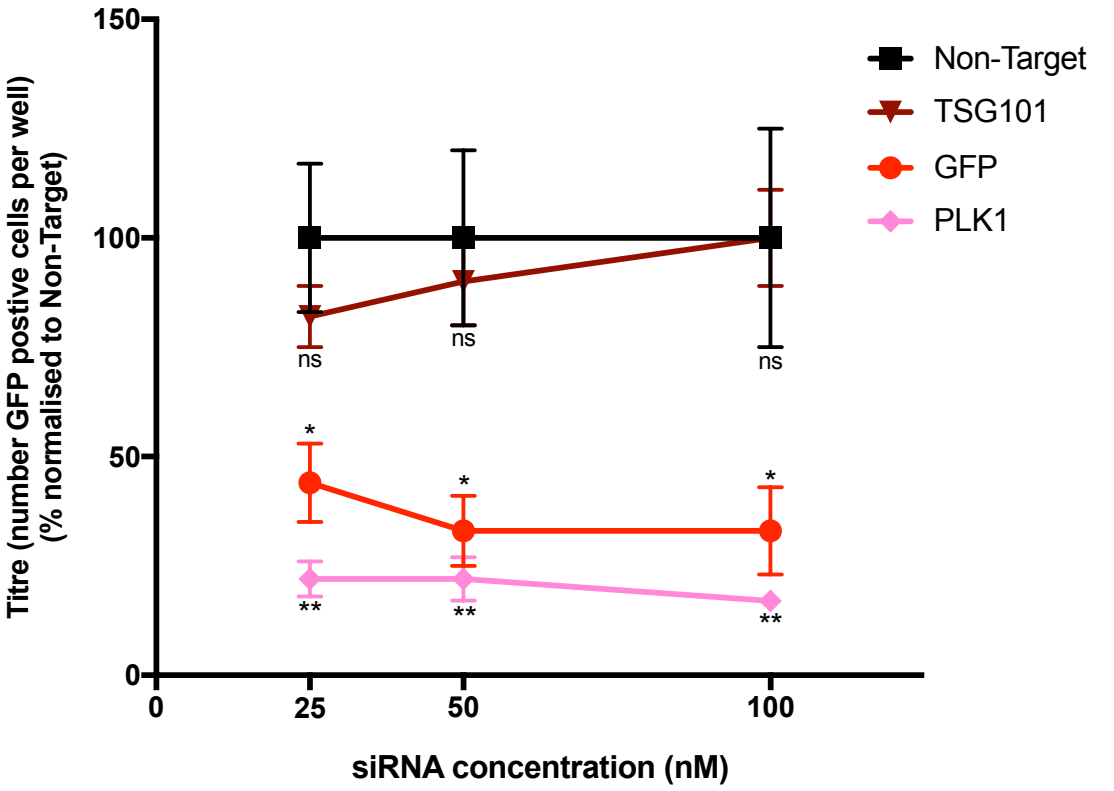


Figure 6.7 Comparison of titres obtained with different concentrations of the four siRNA controls. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced and assessed as described in Section 6.2.1.7 and reported normalised to the average Non-Target control siRNA pool results for each concentration set at 100% (black squares) ± SEM. Statistical significance was determined by one-way ANOVA on the data points for each siRNA concentration individually, followed by Dunnett's post-test: 25 nM) $F(3,8) = 11.55$, $p < 0.01$, 50 nM) $F(3,8) = 10.56$, $p < 0.01$, 100 nM) $F(3,8) = 9.024$, $p < 0.01$. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05 and < 0.01 were deemed a significant difference and indicated with one (*) or two stars (**) on the chart, respectively.

external wells rows and columns were filled with 200 µL DPBS. One plate was transfected with requires at least three days. To evaluate the potential of this approach, three plates were seeded for virus production. To minimise issues with the evaporation of medium from the outer wells, only the internal 32 wells of each plate were utilised and the two external wells rows and columns were filled with 200 µL DPBS. One plate was transfected with siRNAs three days before virus activation only, one was transfected three days and 1 day before virus activation and one was transfected three days before and 1 day after virus activation (Figure 6.8A). This time, the knock-down in virus production using TSG101 siRNA was significantly different from the result obtained with the Non-Target control siRNA for the single transfection. While no significant difference could be observed in double transfections because of the small number of samples, there was no apparent difference between the magnitude of the knock-down in single or double transfections and the double transfection protocol was, therefore, abandoned (Figure 6.8B).

6.2.1.8 Statistical analysis and power calculation for the confirmation of the effect of candidate inhibitory factors

Using the preliminary data sets outlined above, the final set of conditions for the screen were established and are described fully in Sections 2.4.4 and 2.5.2 and schematically represented in Figure 2.2. Using these conditions, a final control experiment was performed in which 32 replicate wells (all the usable wells in one 96-well plate when filling the two external rows and columns with 200µL DPBS) were transfected with Non-Target control siRNA during virus production (Figure 6.9). One well (number 24) failed to produce any virus, and as an outlier, was excluded from further analysis. Statistical analysis of the titration results of the remaining 31 wells demonstrated that a siRNA was, therefore, established as the ‘cut-off’ point for confirming the inhibitory effect of candidate

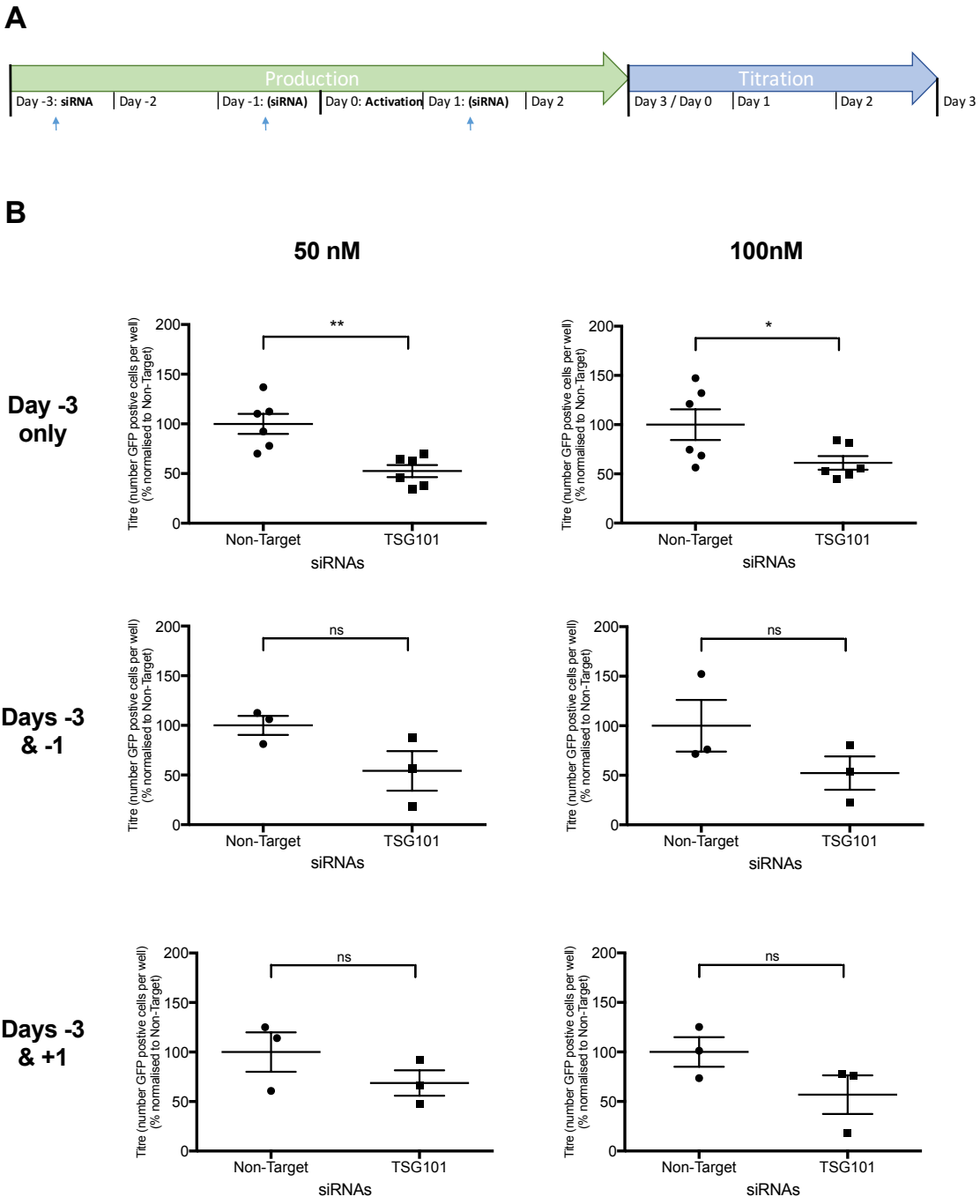

A
Production
Titration
Day -3: siRNA
Day -2
Day -1: (siRNA)
Day 0: Activation
Day 1: (siRNA)
Day 2
Day 3 / Day 0
Day 1
Day 2
Day 3
B
50 nM
100nM
Day -3 only
Days -3 & -1
Days -3 & +1
Titre (number GFP postive cells per well) (% normalised to Non-Target)
Non-Target
TSG101
siRNAs
**
*
ns

Figure 6.8 Evaluation of single and double transfections, their timing and dosing. A) Timeline of the experiment with the single siRNA transfection (arrow) indicated three days (Day -3: siRNA) before activation of production (Day 0: Activation) and time points for the second transfections (arrows) in double transfection indicated in parentheses (Day -1: (siRNA) & Day 1: (siRNA)). B) Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in the presence of the indicated siRNAs transfected once (n=6) or on two separate occasions (n=3) and normalised to their respective Non-Target control siRNA pool results set at 100% (solid line) ± SEM. Statistical significance for each graph was determined by a Student t test. A calculated p value of > 0.05 was deemed non-significant and indicated with ns on graphs. Calculated p values of < 0.05 or < 0.01 were deemed a significant difference and indicated with one (*) or two stars (**) respectively on the charts.

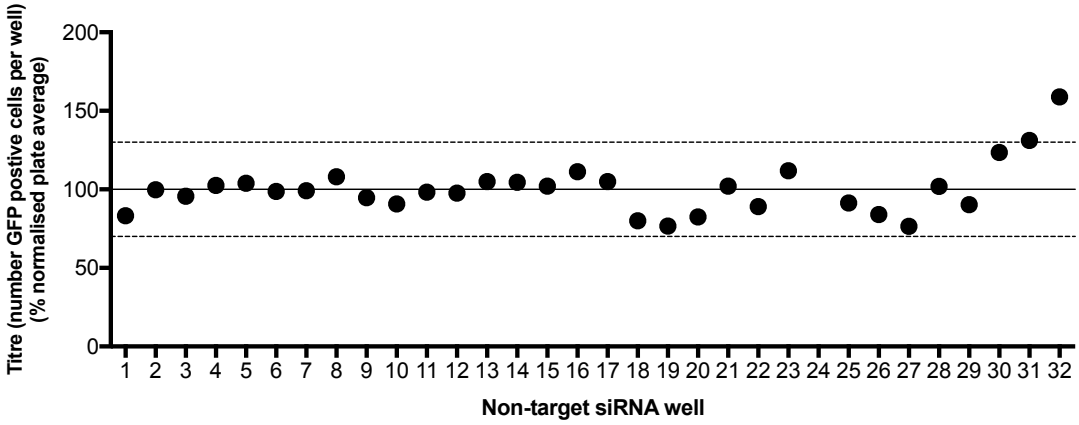


Figure 6.9 Effect of Non-Target siRNA transfection on lentiviral vector production. Lentiviral vector production from 293SF-LVP cells was assessed in 32-replicate wells of a 96-well plate and each well was transfected with a pool of Non-Target control siRNA under the conditions described in Section 2.6. No GFP expression was observed in well number 24 which, as an outlier, was excluded from further analysis. The titre from each of the remaining 31 replicates was determined as the mean of two independent titration studies. Results were normalised to the overall mean of the samples, and plotted for each well against the nominal well number. The overall mean of the 31 wells is represented by the solid horizontal line. The horizontal dotted lines represent the mean ± two standard deviations of the mean.

inhibitory factors ('hits') using this screen. Furthermore, this is consistent with several of the published genome-wide screens described previously (Table 6.2), which experiment, performed in quadruplicate, would be powered at 80% to detect a 1.4-fold increase in titre. This also approximates to twice the SD of the experiment (19.77%) and also used two SD, 40% difference compared to the control or numerically similar values (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008a). It was also calculated that quadruplicate samples would be powered at 80% to detect a 40% reduction in titre (two SD below Non-Target control), which would indicate that knocking-down the gene decreases virus production and that the target is either an auxiliary or an essential factor.

6.3 Discussion

The aim of this chapter was to establish a screening protocol to assess the candidate inhibitory factors identified in Chapter 5. This was accomplished by adapting both the production protocol in flasks and the titration protocol in 12-well plates, described in Chapters 3 and 4, for use in the high-throughput 96-well format. From the adapted conditions using HEK 293T cells, 293SF-LVP and 293SF-Titr cells were used for production and titration, respectively, to determine the experimental controls. These were established as follows: GFP, a *transfection efficiency control*, PLK1, a *cell proliferation control*, TSG101, a *production knock-down control*, and a *non-target control*. The plate setup, siRNA transfection reagent, siRNA concentration and timing for seeding, transfection and harvest were established and are detailed in Section 2.4.4. Finally, the statistical threshold for detecting 'hits' with 80% power was identified as a change of 40% of the production value of the Non-Target control, which is also twice the SD of titres of an entire Non-Target siRNA plate.

Table 6.2 shows the similarities and differences between the screen developed here and previously published high-throughput screens. The main difference in the approach described in this chapter is the focus on the late phase of the replication cycle. This approach was facilitated by the use of an inducible vector producer cell line during the production phase of the assay, thereby avoiding a transient transfection step. The use of this cell line, however, means that the results of this screen might be less relevant to processes using transient transfection. The size of the wells used in this high-throughput screen might also limit applicability to factors associated with virus production in cells grown in suspension, although this was minimised by keeping the cells in suspension until seeding and in the same media throughout.

Figure 6.4 showed that following transfection of 100 nM of a GFP siRNA, a number of cells were still expressing high levels of GFP when activated. This indicated that the knock-down efficiency of this screening protocol is not 100%. This could be due to a less than optimal transfection efficiency, however, a high concentration of siRNA was used here and the use of pools usually compensate for any under-performing siRNA sequence. Alternatively, this could be due to the siRNA molecules being 'diluted out' by cell division over the course of the 6 days of the assay (Reviewed in Sharma & Rao, 2009). This needs to be taken into account in the interpretation of the effect of all siRNAs as some cells will not be affected by the knock-down and will express normal levels of the target gene product and, therefore, produce normal levels of viruses, which will drive the mean value towards non-significance.

There is also a concern regarding the possible adverse effect of the non-targeting siRNA on virus production (Reed *et al.*, 2012). In that study, some non-targeting siRNAs reduced intracellular Gag levels and virus particle production by 2-100-fold compared with mock-transfected cells. This is however not an issue with the siRNA pool used here,

as no significant difference was observed in triplicate Non-Target siRNA transfected wells and corresponding wells where only Lipofectamine 2000, Non-Target siRNA or FreeStyle was added at the time of transfection (Figure 6.10).

The studies outlined in this chapter demonstrate how careful selection of conditions for reproducible transfection, and the control of issues such as evaporation of small sample volumes, can produce an assay proven to be robust and allow a low statistical threshold to identify hits to be carried forward. This assay is now ready to be used for the evaluation of the candidate inhibitory factors identified in Chapter 5.

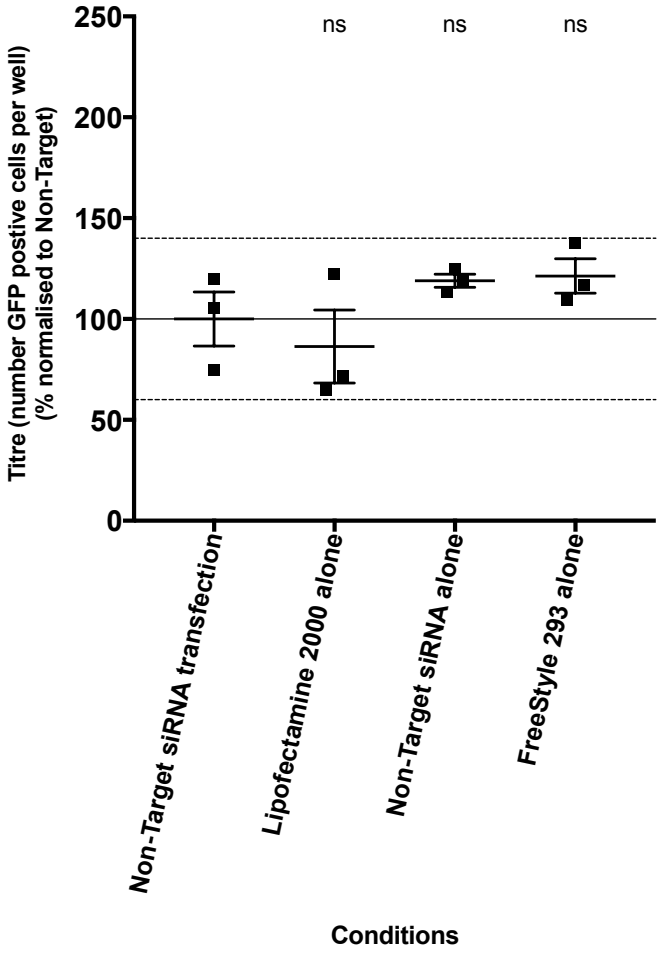


Figure 6.10 Comparison of titres from Non-Target siRNA transfected cells and cells treated only with Lipofectamine 2000, Non-Target siRNA or FreeStyle 293. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) assessed as described in Section 2.5.2 and reported normalised to the average Non-Target control siRNA pools results set at 100% (solid line) ± SEM. The dotted lines indicate the 'hit' threshold (± 40% value of Non-Target siRNA transfection). Samples titrated were produced as described in Section 2.4.4, in the presence of either Lipofectamine 2000-transfected Non-Target siRNA (0.5 µL & 100 nM each well), Lipofectamine 2000 only (0.5 µL/well), Non-Target siRNA 100 nM only or FreeStyle 293 alone. There were no statistically significant differences between group means as determined by one-way ANOVA: A) $F(3,8) = 1.854$, $p = 0.2156$. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart.

Chapter 7 – Evaluation of candidate inhibitory factors with the high-throughput siRNA screen

7.1 Introduction

Following initial studies in Chapters 3 and 4 that looked at supplementation and regulation of intrinsic immunity to boost lentiviral vector production, the focus shifted to inhibitory factors in the host cell. A list of candidate inhibitory factors acting late in the HIV-1 life cycle was established in Chapter 5 and a high-throughput screening protocol was designed and optimised in Chapter 6 to evaluate the effect of knocking-down genes encoding these inhibitory factors using siRNAs. Studies in this chapter describe the use of this screening protocol to evaluate a selection of these candidate inhibitory factors. The study was performed in four independent parts, termed sub-screens below, with the targets of each screen listed in Table 7.1.

7.2 Results

7.2.1 Establishment of further controls for the screen

7.2.1.1 Sub-screen 1

An initial set of 10 targets was selected from the lists established in Chapter 5 (Table 5.1 and 5.2) to validate the assay. This selection of genes (Table 7.1) included BST2 (tetherin) as a representative of the restriction factors (see Section 5.1.1.1), the knock-down of which was expected to have no effect due to its low expression in HEK 293 cells (Tanner et al., 2007). Included as well were five candidate inhibitory factors (CHMP5, DNAJA1, EIF3F, HERC5 and ISG15) and four candidate essential or auxiliary factors (DDX24, TSG101, UBE2I and VTA1) that had previously been assayed in independent siRNA/shRNA studies (Table 7.2). Of note, while DNAJA1 was identified as a candidate

Table 7.1 Host factors evaluated per sub-screen

Restriction and Candidate Inhibitory Factors						Non-Target Control	Candidate Essential and Auxiliary Factors		
Original 4 controls (Chapter 6)									
						Non-Target Control	**GFP** **PLK1**	**TSG101**	
Sub-screen 1 (10 genes and 4 controls)									
BST2	DNAJA1	HERC5				**Non-Target Control**	DDX24	**TSG101**	VTA1
CHMP5	EIF3F	ISG15					**GFP**	TSG101	
							PLK1	UBE2I	
Complete set of 8 screen controls									
BST2	**CHMP5**					**Non-Target Control**	**GFP** **PLK1**	**TSG101** **UBE2I**	**VTA1**
Sub-screen 2 (24 genes and 8 controls)									
ABCA1	CD81	DUSP1	MOV10	SLFN11		**Non-Target Control**	**GFP**	**PLK1**	**UBE2I**
APOBEC3G	**CHMP5**	EIF2AK2	PRMT6	SUMO1			LIMK1	**TSG101**	**VTA1**
APOL1	CNP	EIF3E	RNF115	TRIM22					
AXIN1	DDX5	EZR	RSAD2	UBA7					
BST2	DLG1	HSPA1A	SIRT1	UBASH3A					
Sub-screen 3 (48 genes and 8 controls)									
ADAR	BANP	DICER1	HSPA8	PRKRA	*TRIM28*	**Non-Target Control**	ATP1A1	PRKACA	**UBE2I**
AGO2	**BST2**	DNAJAB1	IFI16	RNASEL	TRIM32		**GFP**	TARBP2	USP18
APOBEC3A	CD3G	DNAJB6	IFITM1	ROCK2	TSPO		**PLK1**	**TSG101**	**VTA1**
APOBEC3B	CD63	DNAJC5	IFITM2	SUMO2	UBE2L6				
APOBEC3C	**CHMP5**	DROSHA	IFITM3	TCF4	UBP1				
APOBEC3D	COMMD1	HEXIM1	LSM1	TRIM11	ZC3H12A				
APOBEC3F	DDX6	HSPA1B	MARCH8	TRIM21					
APOBEC3H	DHX30	HSPA4	PRKAA1	TRIM26					
Sub-screen 4 (24 genes and 8 controls)									
BST2	(CD81)	**CHMP5**	HDAC2	MB21D1	SPN	**Non-Target Control**	*DDX58*	*MX2*	**TSG101**
(CD63)	CD82	CTNNB1	HSP90AB1	RUVBL2	TSPAN7		**GFP**	*SAMHD1*	**UBE2I**
CD151	CD9	GBP5	KAT5	SETDB1			**PLK1**	*TRIM5*	**VTA1**
CD53	CDKN1A	HDAC1	MAP3K5	SP3					

Gene names in *italics* are factors outside the scope of the screen (early stages of the HIV-1 life cycle).

Table 7.2 Characteristics of published studies using siRNA/shRNA knockdown of the 10 targets selected for sub-screen 1

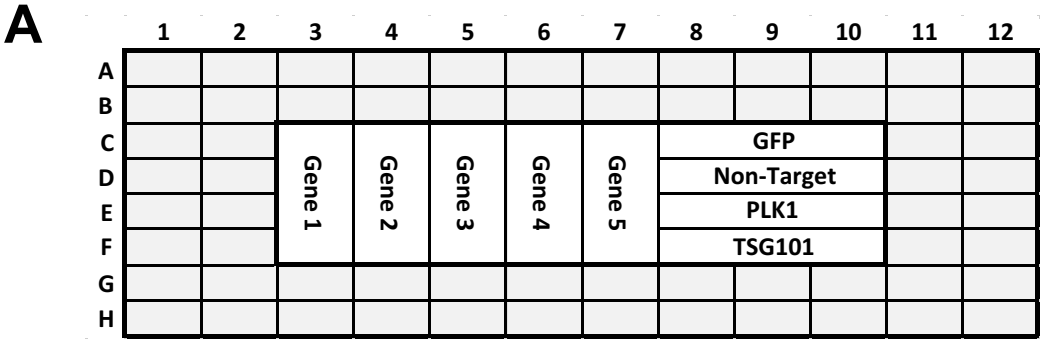
siRNA target	Study (Year)	Cell Line	siRNA transfection reagent	Time from siRNA transfection to challenge	Virus / Vector	Harvest	Result (~ % of control)
CHMP5, VTA1, TSG101	(Ward *et al.*, 2005)	HEK 293T or HeLa	Lipofectamine 2000	24 hours & 0 hours	rHIV.VSV-g EF-1α LacZ (transfection)	48 hours	232%, 31%, 12%
DDX24	(Ma *et al.*, 2008)	HEK 293T	Lipofectamine 2000	Not disclosed	BH10 HIV-1	Not disclosed	50-33%
DNAJA1	(Kumar & Mitra, 2005)	HEK 293T	Lipofectamine 2000	24 hours	pNL4-3	48 hours	40%
DNAJA1	(Dziuba *et al.*, 2012)	TZM-bl HeLa	Lipofectamine 2000	48 hours	HIV-1 LAV	48 hours	25%
DNAJA1	(Kumar *et al.*, 2011)	HEK 293T	Amine FX	24 hours	pNL4-3	72 hours	60%
EIF3F	(Jager *et al.*, 2011)	HeLa P4/R5	RNAiMAX	48 hours	pNL4-3	48 hours	33-20%
HERC5 (shRNA)	(Woods *et al.*, 2011)	HEK 293T and HeLa	Lipofectamine 2000	0 hours	HIV-1 pR9	24 hours	190%
ISG15	(Okumura *et al.*, 2006)	HEK 293T	Lipofectamine 2000	0 hours	pNL4-3 (+ IFN)	72 hours	160%
TSG101	(Garrus *et al.*, 2001)	HEK 293T	Lipofectamine 2000	24 hours & 0 hours	HIV-1 R9 DNA	48 hours	10-2%
TSG101	(Zhou *et al.*, 2008a)	HeLa P4/R5	Oligofectamine	24 hours	HIV-1 HBX2	48 or 96 hours	30%
TSG101	(Wen *et al.*, 2014b)	HeLa	Lipofectamine 2000	24 hours & 0 hours	pNL4-3	48 hours	20%
TSG101	(Fu *et al.*, 2015)	U1/HIV-1	Oligofectamine	48 hours	HIV-1 (induction)	72 hours	50%
UBE2I	(Jaber *et al.*, 2009)	HEK 293T	Lipofectamine 2000	24 hours & 0 hours	pNL4-3	72 hours	10%
UBE2I	(Bohl *et al.*, 2013)	HEK 293T	Lipofectamine 2000	48 hours & 24 hours	pNL4-3	24 hours	50-40%
Multiple	Current screen	293SF-LVP	Lipofectamine 2000	72 hours	LVR2-GFP (rHIV.VSV-g CMV GFP) (induction)	72 hours	207%-16%

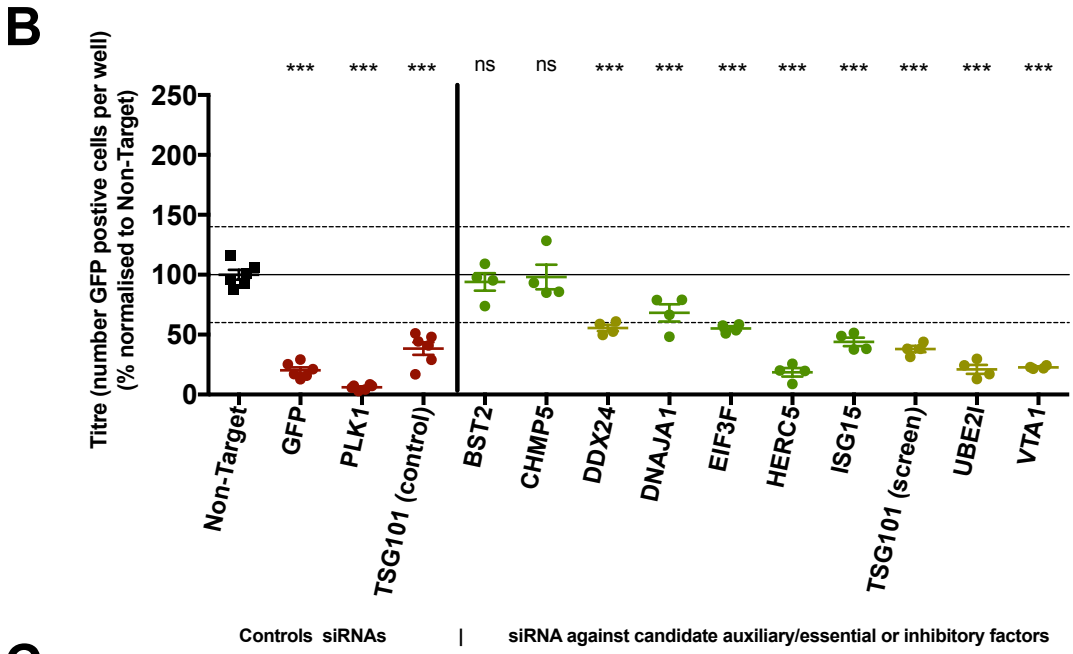
Legend for Table 7.2 Time from siRNA to challenge: in the timeline of the experimental setup, time between when the siRNA/shRNA is transfected and the cells are challenged with a transfection, infection or activation of virus production, **Cell Line**: Cell line in which the experiment was performed (Legend: TZM-bl HeLa (HeLa cell line with integrated copies of the luciferase and β-galactosidase genes under control of the HIV-1 promoter (Wei et al., 2002)), HeLa P4/R5 (HeLa cells that express CD4 and CCR5 and have Tat-inducible β-galactosidase activity (Charneau et al., 1994)), U1/HIV-1 (subclone of U937 cells chronically infected with HIV-1 in which the addition of phorbol myristate acetate induces HIV-1 expression (Folks et al., 1987)), **Virus / Vector**: virus or vector used to challenge the cells (Legend: rHIV.VSV-g EF-1a LacZ (3rd generation VSV-g pseudotyped HIV-1 expressing LacZ under a human elongation factor-1 alpha (EF-1α) promoter), LacZ (β-galactosidase reporter gene), BH10 HIV-1 (BH10 isolate of HIV1), pNL4-3 (proviral plasmid), HIV-1 LAV (LAV isolate of HIV-1), HIV-1 pR9 & HIV-1 R9 DNA (HIV-1 molecular clone), **Harvest**: time from challenge to harvest of the vector or assay readout, **Result (% of control)**: effect of the siRNA/shRNA treatment on virus production when compared to the control of the screen.

inhibitory factor in an over-expression study, while siRNA studies pointed in the direction of it being an essential/auxiliary factor. The siRNA/shRNA studies described in Table 7.2 all used a broadly similar assay to the one developed here. Most used HEK 293T and/or HeLa cells and used Lipofectamine 2000, even when using GE Dharmacon's siRNAs, supporting the choice of transfection reagent. The main difference between the studies in Table 7.2 and the current screen is the timeline of the experimental setup. As indicated in the 'Time from siRNA to challenge' column, all studies transfected siRNAs 0-48 hours before challenge (transfection, infection or activation of virus production) compared to the 72 hours here. This can be explained by the fact that an inducible cell line is used here and it is assumed that activation of production in these cells leads quicker to virus production than would a transient transfection or an infection and, therefore, the siRNAs need to be fully active earlier to be effective when viral transcription is initiated.

This first set of 10 siRNAs was assayed in quadruplicate on two 96-well plates, five genes per plate. Each plate also had triplicate of the control siRNAs: GFP, Non-Target, PLK1 and TSG101 (Figure 7.1A). In this particular plate format, the titration values for the triplicate Non-Target controls on each plate were averaged and all data for that plate were normalised to this average.

Figures 7.1B and C show the results of sub-screen 1. The control siRNAs (Section 6.2.1.5) performed as previously with knock-down of TSG101, *production knock-down control*, GFP, *transfection efficiency control*, and PLK1, *cell proliferation control*, all leading to significant reductions in titre. Very similar results were obtained for controls between the two plates (Figure 7.2A-D) and from the two independent preparations of the commercial siRNA pool targeting TSG101 (Figure 7.2E), with no significant difference between any of these pairs. As expected, the inhibition of BST2 had no impact


A
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Gene 1
Gene 2
Gene 3
Gene 4
Gene 5
GFP
Non-Target
PLK1
TSG101


B
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
*** *** *** ns ns *** *** *** *** *** *** *** ***
Non-Target
GFP
PLK1
TSG101 (control)
BST2
CHMP5
DDX24
DNAJA1
EIF3F
HERC5
ISG15
TSG101 (screen)
UBE2I
VTA1
Controls siRNAs
siRNA against candidate auxiliary/essential or inhibitory factors

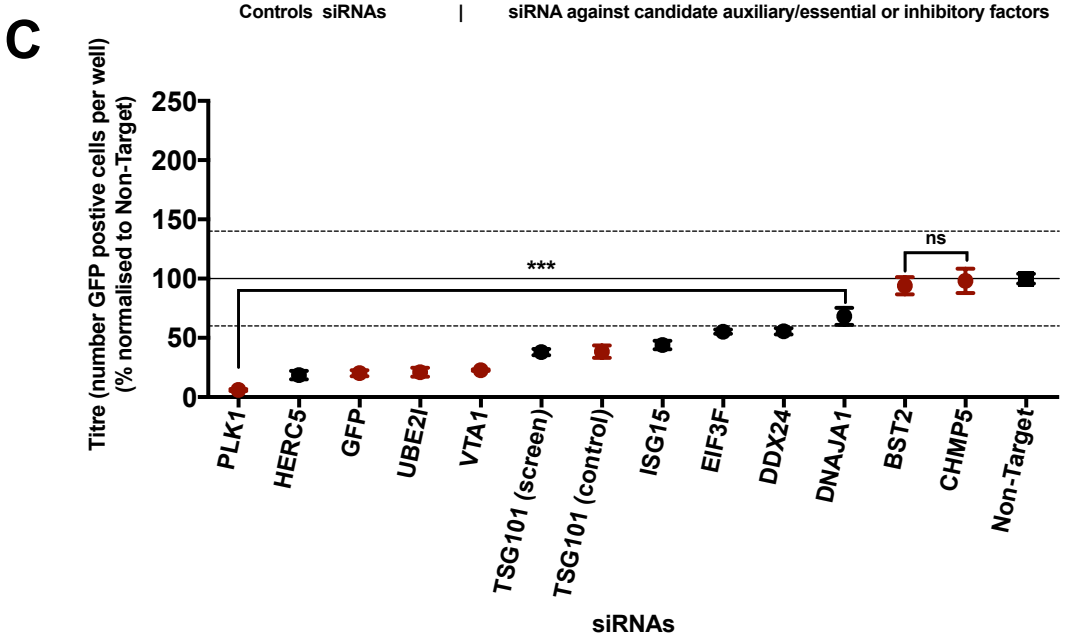

C
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
***
ns
PLK1
HERC5
GFP
UBE2I
VTA1
TSG101 (screen)
TSG101 (control)
ISG15
EIF3F
DDX24
DNAJA1
BST2
CHMP5
Non-Target
siRNAs

Figure 7.1 Plate format and results of sub-screen 1 evaluating the effect of 10 siRNA pools and four controls on lentiviral vector production in activated 293SF-LVP cells to identify standard controls. A first set of 10 genes, including the restriction factor BST2, five candidate inhibitory factors (CHMP5, DNAJA1, EIF3F, HERC5 and ISG15), and four candidate essential or auxiliary factors (DDX24, TSG101, UBE2I and VTA1), together with four controls (Non-Target, GFP, PLK1 and TSG101), were screened to evaluate the impact of their knock-down on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. A) Plate format of each of the two plates. Two rows and columns of outer wells (indicated in grey) were unused (filled with 200 µl DBPS). Screened siRNAs were tested in quadruplicates on one plate while control siRNAs were tested in triplicates on each plate. B & C) Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in the presence of the indicated siRNAs (n=4 or 6) and normalised to the Non-Target control siRNA pools results of their respective plate set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target), established in Section 6.2.1.8. Statistically significant differences between group means were determined by one-way ANOVA ($F(13,50) = 49.83$, $p = < 0.001$) followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.001 were deemed a significant difference as indicated with three stars (***) on the chart. B) Comparative results of control siRNAs with selected siRNAs targeting auxiliary, essential or inhibitory factors listed in alphabetical order. siRNAs pools colour coding: Non-Target: black squares, screen controls: dark red circles, restriction and candidate inhibitory factors: green circles, essential and candidate auxiliary factors: dark yellow circles. C) Mean titration results ± SEM, ordered by ascending normalised value. siRNAs pools colour coding: Non-Target: black squares, screen controls: dark red circles.

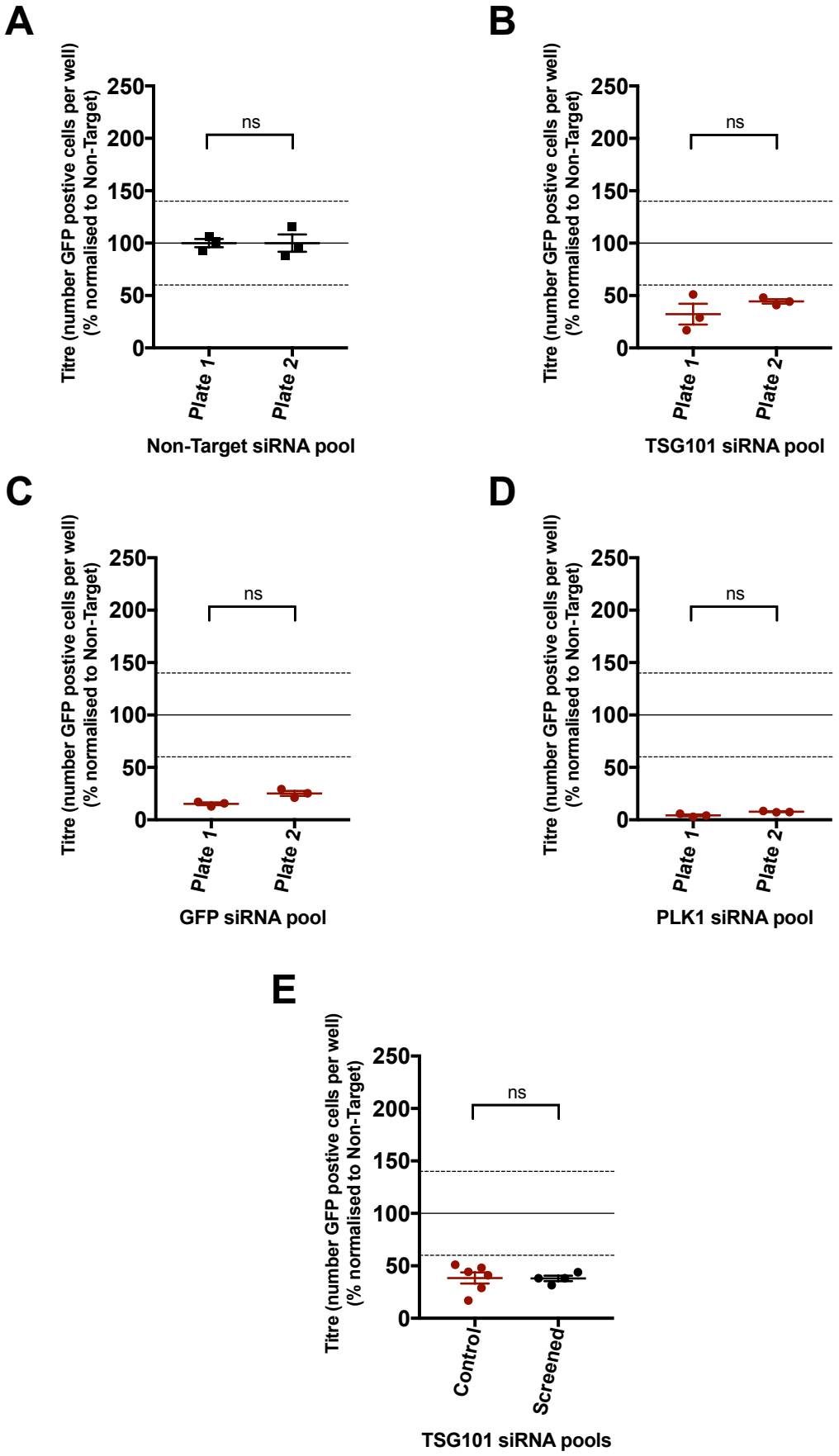

A
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
ns
Plate 1
Plate 2
Non-Target siRNA pool
B
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
ns
Plate 1
Plate 2
TSG101 siRNA pool
C
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
ns
Plate 1
Plate 2
GFP siRNA pool
D
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
ns
Plate 1
Plate 2
PLK1 siRNA pool
E
Titre (number GFP postive cells per well)
(% normalised to Non-Target)
250
200
150
100
50
0
ns
Control
Screened
TSG101 siRNA pools

Figure 7.2 Selected results of sub-screen 1 evaluating the effect on lentiviral vector production in activated 293SF-LVP cells of control siRNA pools on separate plates and of the two independent preparations of the commercial siRNA pool targeting TSG101. A-D) Compared mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in the presence of the indicated siRNAs on separate plates (n=3 on each plate) E) Compared mean titre of virus (number of GFP positive cells per well) produced in the presence of two independent preparations of the commercial siRNA pool: the control TSG101 siRNA pool (dark red circles) or the screened TSG01 siRNA pool (black circles). A-E) All results were normalised to the average of the Non-Target control siRNA pools results (black squares in (A)) of their respective plate set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target), established in Section 6.2.1.8. Statistically significant differences between group means were evaluated by a Mann-Whitney test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on charts.

on virus production, as, even though it is a restriction factor, it is only expressed at very low levels in HEK 293 (Tanner *et al.*, 2007). All siRNAs inhibiting candidate essential or auxiliary factors led to significant decreases in viral production titres compared to the Non-Target control. In the case of candidate inhibitory factors, CHMP5 led to no change in viral titres, while the others, surprisingly, led to decreased titres.

From the combined results of this preliminary study (sub-screen 1), BST2, as well as the best performing candidate inhibitory and essential or auxiliary factors, were selected: CHMP5, UBE2I and VTA1 (Figure 7.1B) and new independent preparations of the commercial siRNA pools were used as comparative controls for all the subsequent sub-screens, combined with the four original controls: GFP, Non-Target, PLK1 and TSG101.

7.2.3 Screen of candidate genes

7.2.3.1 Sub-screen 2

The next set of 24 targets selected for sub-screen 2 (Table 7.1) included other candidate inhibitory factors identified in siRNA/shRNA studies: ABCA1, AXIN1, CD81, DDX5, DLG1, DUSP1, EIF2AK2, EIF3E, EZR, HSP1A1, MOV10, PRMT6, RNF115, RSAD2, SIRT1, SLFN11 and TRIM22 (Table 5.1). Additional candidate inhibitory factors included: APOL1, CNP, SUMO1, UBA7 and UBASH3A, which were identified in overexpression studies. The restriction factor, APOBEC3G, was also included as it affects the late part of the HIV-1 life cycle. Again, its knock-down was expected to have no effect due to its low expression in HEK 293 cells (Tanner et al., 2007). The candidate essential/auxiliary factor LIMK1 was selected as a control as it was shown that a siRNA targeting LIMK1 reduces virus particle output to 22% of the output of scrambled siRNA control-transfected HeLa cells (Wen *et al.*, 2014b). From this sub-screen onwards, the late format (Figure 7.3A) included a single well for each of the 24 evaluated genes and

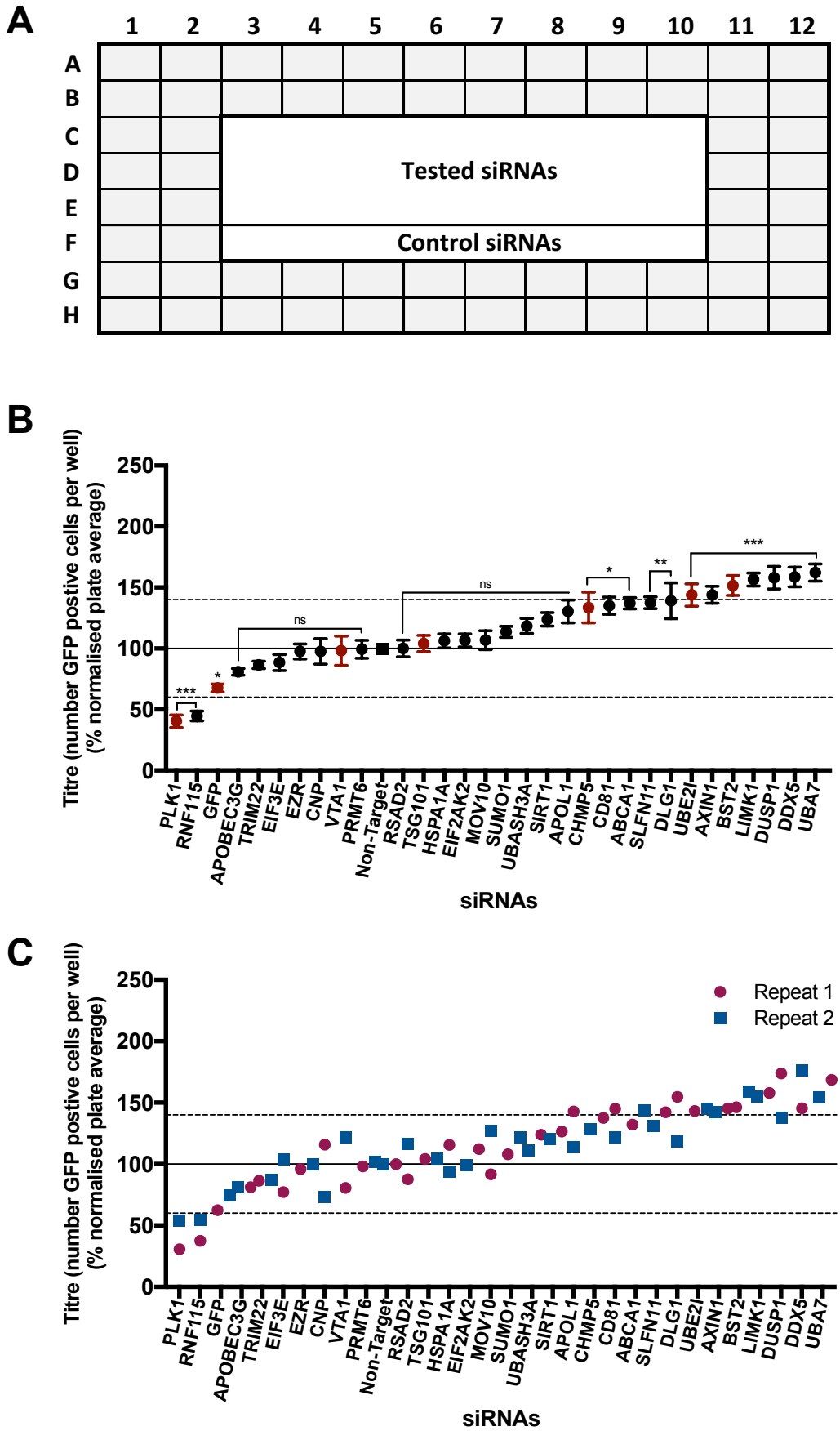

A
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Tested siRNAs
Control siRNAs
B
Titre (number GFP postive cells per well)
(% normalised plate average)
250
200
150
100
50
0
***
*
ns
ns
*
**
***
PLK1 RNF115 GFP APOBEC3G TRIM22 EIF3E EZR CNP VTA1 PRMT6 Non-Target RSAD2 TSG101 HSPA1A EIF2AK2 MOV10 SUMO1 UBASH3A SIRT1 APOL1 CHMP5 CD81 ABCA1 SLFN11 DLG1 UBE2I AXIN1 BST2 LIMK1 DUSP1 DDX5 UBA7
siRNAs
C
Titre (number GFP postive cells per well)
(% normalised plate average)
250
200
150
100
50
0
Repeat 1
Repeat 2
PLK1 RNF115 GFP APOBEC3G TRIM22 EIF3E EZR CNP VTA1 PRMT6 Non-Target RSAD2 TSG101 HSPA1A EIF2AK2 MOV10 SUMO1 UBASH3A SIRT1 APOL1 CHMP5 CD81 ABCA1 SLFN11 DLG1 UBE2I AXIN1 BST2 LIMK1 DUSP1 DDX5 UBA7
siRNAs

Figure 7.3 Plate format and results of sub-screen 2 repeats evaluating the effect of 24 siRNA pools and eight controls on lentiviral vector production in activated 293SF-LVP cells. A second set of genes, including APOBEC3G, 22 candidate inhibitory factors and one candidate essential/auxiliary factor (LIMK1) as well as the eight screen controls (Non-Target (black square in (B)) and BST2, CHMP5, GFP, PLK1, TSG101, UBE2I and VTA1 (dark red circles in (B))), was screened to evaluate the impact of their knock-down on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. A) Plate format for sub-screens 2-4. Two rows and columns of outer wells (indicated in grey) were unused (filled with 200 µl DBPS). Screened siRNAs were tested in quadruplicate on four plates (sub-screens 2 (repeat 1) and 4) or two sets of four plates (sub-screen 3), or in triplicate on three plates sub-screen 2 (repeat 2)). B & C) Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced as described in Section 2.4.4 in the presence of the indicated siRNAs. Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target), established in Section 6.2.1.8. Results are sorted (left to right) in ascending order of the average value obtained for two repeats. B) Averaged titres of two repeats of virus production in the presence of the indicated siRNAs (n=7). Statistically significant differences between group means were determined by one-way ANOVA ($F(31,192) = 18.3$, $p = < 0.001$) followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart. C) Means of two of the sub-screen 2 repeats (Repeat 1 (maroon circles): n=4, Repeat 2 (blue squares): n=3).

eight control genes on each plate. Multiple independent plate productions were performed for each sub-screen and each plate titrated on two independent plates. For each factor, the single titration plate value was normalised to the associated Non-Target control value found on its plate. The average of the two titration plates was then calculated, before the average of all productions was collected. To assess the reproducibility of this approach, this sub-screen was performed with the same 24 siRNA pools on two independent occasions. On the first occasion, four independent replicate plates were used, on the second, three independent plates were used. Figure 7.3B shows the average of the combined data from the two repeats while Figure 7.3C shows the variation between the two repeats. The results were broadly similar between the two independent repeats of sub-screen 2 – the Pearson correlation coefficient, with a 95% confidence interval, for these two repeats, was $r=0.80$ $p< 0.001$.

Sub-screen 2 identified four candidate inhibitory factors (AXIN1, DUSP1, DDX5, UBA7) as 'hits', their knock-down increasing vector production by more than 40% (Figure 7.3). Also having a similar effect was the siRNA pool against LIMK1, a candidate essential/auxiliary factor. This was surprising, although, the study identifying LIMK1 as a candidate essential/auxiliary factor (Wen *et al.*, 2014b) was undertaken in a different cell line (HeLa), using a proviral transfection (NL4-3) and measuring particle output (p24 enzyme-linked immunosorbent assay (ELISA)), rather than the inducible HEK 293-based producer line and functional titre used here. These many differences could account for differences in observed effects of LIMK1 knock-down. UBE2I had an opposite effect in sub-screen 2 compared to the result in sub-screen 1. The reason for this is unknown, the only difference being that it came from a different independent preparation of the commercial siRNA pool. Knock-down of four genes (DLG1, SLFN11, ABCA1, CD81) significantly increased titres, but the experiment was not sufficiently powered to confirm this increase. Such genes can be qualified as 'near-hits'. Knock-down of most other

genes (15) had no significant effect on vector titres except for RNF115 which significantly decreased titre.

7.2.3.2 Sub-screen 3

A further round of targets was selected with the next 43 most promising candidate inhibitory factors in sub-screen 3 (Table 7.1). These included the remaining members of the APOBEC3 family. Of these, all except APOBEC3A have been linked with HIV-1 infectivity inhibition, although with contradictory evidence for APOBEC3C (Table 5.1). There is some evidence for APOBEC3A's involvement in the early phases of HIV-1 infection (Peng et al., 2007; Berger et al., 2011). Also included were a number of TRIM family members shown to act late in the HIV-1 life cycle, as well as TRIM28, which restricts HIV-1 early in the life cycle, at the integration step (Allouch et al., 2011). Additional heat shock proteins were also tested here. The PKR inhibitors described in Chapter 4 were included in the selection. This is explained by the fact that, despite having been shown to enhance HIV-1 replication, ADAR1 (gene ADAR) was also recognised as an inhibitory factor in two studies (Biswas *et al.*, 2012; Weiden *et al.*, 2014). PACT (gene PRKRA) (Patel & Sen, 1998; Patel *et al.*, 2000) was included for having been reported as a PKR activator and therefore had been identified in the literature review (Table 5.1). For completeness, TRBP1 (gene TARBP2) was also included, together with three other candidate auxiliary/essential factors: ATP1A1, PRKACA and UBP18 (Table 5.2). In a high-throughput screen of small molecular inhibitors of HIV-1, cardiac glycosides were identified to act on the late phase of the viral life cycle through inhibition of ATP1A1 (Laird *et al.*, 2014), it could, therefore, be an essential/auxiliary factor. USP18 is an ISG15 de-conjugating enzyme, which was shown to partially rescue HIV-1 release in ISG15-expressing cells (Okumura *et al.*, 2006). PRKACA was found to be required for viral infectivity and to interact with the viral capsid protein (Cartier *et al.*, 2003).

Sub-screen 3 was performed using the same plate format as in sub-screen 2 (Figure 7.3A) on two plates which each included a single well for each of the 24 evaluated siRNAs per plate and the eight control siRNA on each plate. Four independent productions were performed simultaneously and titrated on two independent plates each. For each factor, the single titration plate value was normalised to the associated Non-Target control value found on its plate. The average of the two titration plates was then calculated, before the average of all the plates for each siRNA target was collected.

The results of this sub-screen are shown in Figure 7.4. As these were predicted to be less promising candidates from sub-screens 1 and 2 (21/43 (48.8%) candidate inhibitory genes identified by siRNA studies in sub-screen 3 compared to 22/29 (75.9%) for sub-screens 1 and 2 combined), it is perhaps not surprising that proportionally fewer candidate inhibitory factors were identified as 'hits' in this sub-screen, their knock-down increasing vector production by more than 40%, CD63 and the candidate essential/auxiliary factor ATP1A1 being the only two. As observed in sub-screen 2, UBE2I knock-down increased virus production, the opposite to the result in sub-screen 1. In contrast to sub-screen 2 where the knock-down of candidate inhibitory factors had no effect on titres, except identified 'hits', and RNF115 which significantly reduced titres, here for 21 genes it led to decreased vector titres. Amongst these, the knock-down of the PKR inhibitors ADAR (ADAR1) and PRKRA (PACT) led to strong decreases in vector titres, confirming results from some HIV-1 studies, as did the knock-down of the candidate essential/auxiliary factor USP18.

7.2.3.3 Sub-screen 4

During sub-screen 3 described above (Section 7.2.3.2), the top hit, CD63, when knocked-out, increased titres by 236.8% over the Non-Target control; this warranted

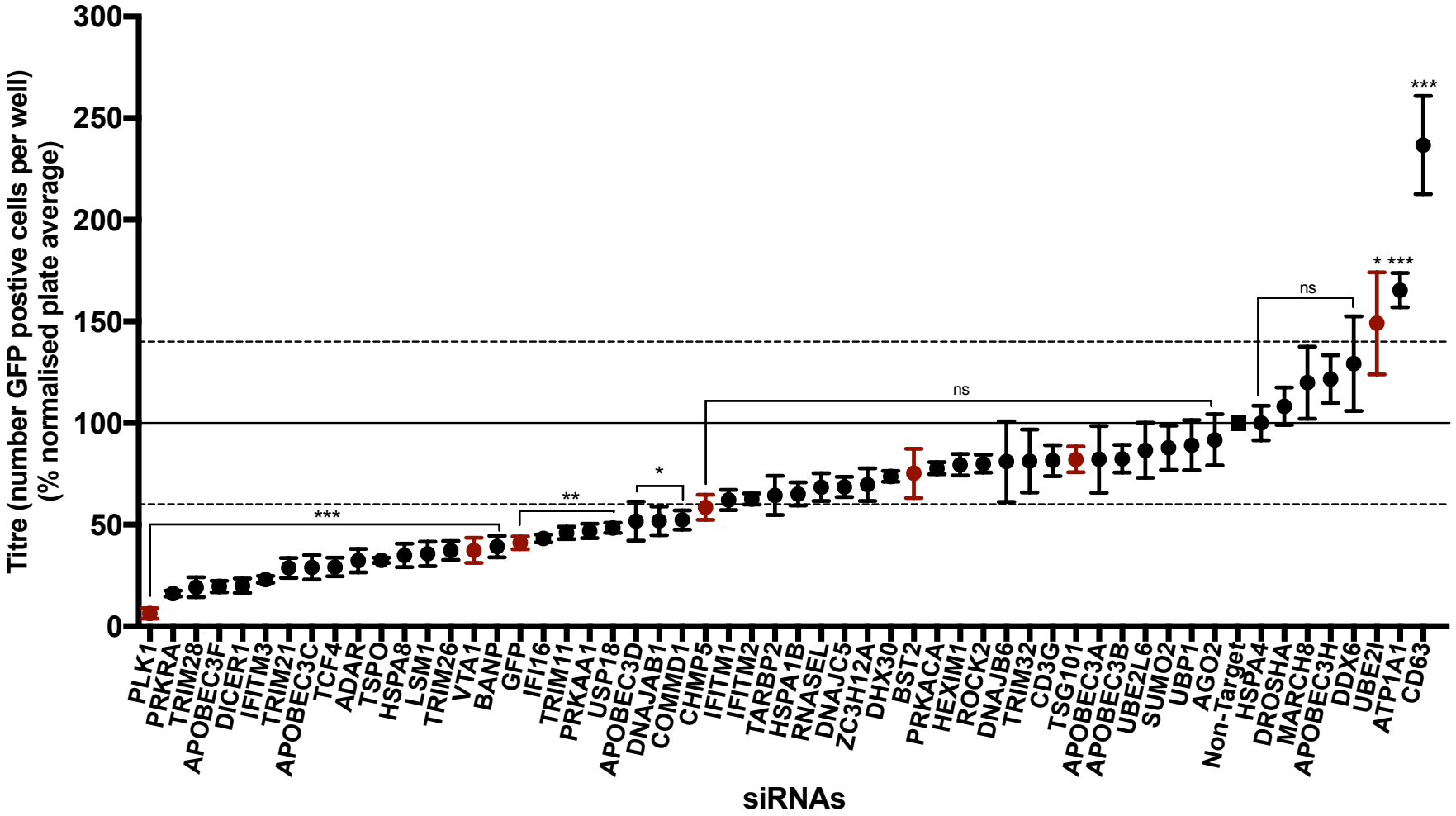

Titre (number GFP postive cells per well)
(% normalised plate average)
300
250
200
150
100
50
0
***
**
*
ns
ns
* ***
***
PLK1
PRKRA
TRIM28
APOBEC3F
DICER1
IFITM3
TRIM21
APOBEC3C
TCF4
ADAR
TSPO
HSPA8
LSM1
TRIM26
VTA1
BANP
GFP
IFI16
TRIM11
PRKAA1
USP18
APOBEC3D
DNAJAB1
COMMD1
CHMP5
IFITM1
IFITM2
TARBP2
HSPA1B
RNASEL
DNAJC5
ZC3H12A
DHX30
BST2
PRKACA
HEXIM1
ROCK2
DNAJB6
TRIM32
CD3G
TSG101
APOBEC3A
APOBEC3B
UBE2L6
SUMO2
UBP1
AGO2
Non-Target
HSPA4
DROSHA
MARCH8
APOBEC3H
DDX6
UBE2I
ATP1A1
CD63
siRNAs

Figure 7.4 Results of sub-screen 3 evaluating the effect of 48 siRNA pools and eight controls on lentiviral vector production in activated 293SF-LVP cells. A third set of genes, 45 candidate inhibitory factors (2 of them, APOBEC3A and TRIM28, being outside of the scope of the screen and used as controls), and four candidate essential/auxiliary factors (ATP1A1, PRKACA, TARBP2, USP18) and eight controls (Non-Target (black square) and BST2, CHMP5, GFP, PLK1, TSG101, UBE2I and VTA1 (dark red circles)), was screened to evaluate the impact of their knock-down on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistically significant differences between group means were determined by one-way ANOVA ($F(55,168) = 18.1$ $p = < 0.001$) followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart.

further attention. CD63 is a member of the tetraspanin family which are known regulators of the HIV-1 replication cycle (Reviewed in Thali, 2009) and CD81, another member of the tetraspanin family, was a 'near-hit' in sub-screen 2. Lentiviral accessory protein Nef has been shown to significantly downregulate CD37, CD53, CD63, CD81, CD151 and TSPAN7 expression at the cell surface (Haller *et al.*, 2014) and CD63 over-expression led to > 60% decrease in infectivity (Sato *et al.*, 2008). A similar effect was noted with overexpression of CD9, CD81 and CD82, while overexpression of CD231 (TSPAN7) decreased infectivity by 90% (Sato *et al.*, 2008). Furthermore, overexpression of selected tetraspanins reduced the infectivity of cell-free virus as well and cell-to-cell transmission of virions (with best results obtained with CD63) (Krementsov *et al.*, 2009) and the tetraspanin CD53 was shown to be present in the plasma membrane domain where HIV-1 assembles in macrophages along with CD63, CD81 and CD9 (Deneka *et al.*, 2007). In summary, these findings suggest an interesting role for tetraspanins, and especially CD63, in virus production. As several tetraspanins identified in Chapter 5 remained untested, it was decided to test these (CD9, CD53, CD82, CD151 and TSPAN7) in parallel with repeats of CD63 and CD81 knock-downs, as well other selected candidate inhibitory factors (Table 7.1).

Also included in sub-screen 4 (Table 7.1) were two restriction factors not yet tested (SAMHD1 and TRIM5) although their mechanism of action predicted they were unlikely to affect the virus production steps evaluated in this screen (Section 5.1.1.3). Finally, DDX58 (RIG-I) and MX2 (MxB) were also included as controls as they are well recognised inhibitory factors, but that act early in the life cycle (Section 5.1.1.4).

This sub-screen was performed using the same plate format as in sub-screen 2 (Figure 7.3A) with a single well for each of the 24 evaluated siRNAs and the eight control siRNA on a single plate. Four independent productions were performed simultaneously and

titrated on two independent plates each. For each factor, the single titration plate value was normalised to the associated Non-Target control value found on its plate. The average of the two titration plates was then calculated, before the average of all the plates for each siRNA target was collected.

Figure 7.5 shows the results of sub-screen 4, with Figure 7.6 focussing on the selected tetraspanins (average of two independent sub-screens for CD63 and CD81). The previously assessed CD63 and CD81 were the only tetraspanins which inhibition resulted in > 40% increase in titres in this sub-screen. CD53 led to significantly higher viral titres, but fell below the cut-off. The knock-down of the other tetraspanins appeared to have either a slightly adverse effect on virus titres, or none at all. A new target from this sub-screen, SPN, was identified as a 'hit' although a high variability between replicates was noted.

Finally, Figure 7.7 and Table 7.3 show the results for the whole siRNA screen identifying nine 'hits' which led to a 40% increase in vector production compared to the Non-Target siRNA pool in the averaged results of the whole screen. In Figure 7.8, the spread of all the data from all the sub-screens is shown. There is limited variation in the variance for most tested siRNA except for the eight controls (dark red open circles) which are affected by inter-plate variation. This is mostly due to the fact that the data is normalised to the Non-Target siRNA and therefore dependent on the variation of this value. The plate setup in sub-screens 2-4 included only one replicate of the Non-Target control which made results from these sub-screens more dependent to the variability of this single Non-Target control result. Future studies would benefit from having more replicates of the Non-Target siRNA on each plate.

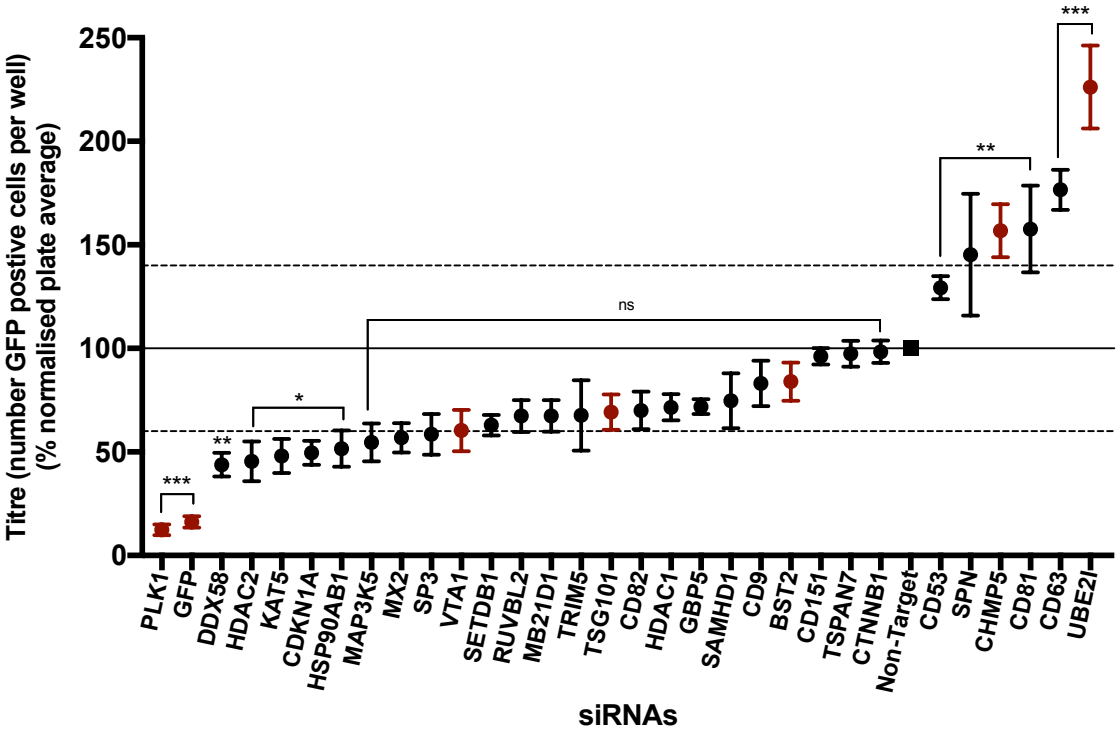

Titre (number GFP postive cells per well)
(% normalised plate average)
250
200
150
100
50
0
***
**
*
ns
**
***
PLK1
GFP
DDX58
HDAC2
KAT5
CDKN1A
HSP90AB1
MAP3K5
MX2
SP3
VTA1
SETDB1
RUVBL2
MB21D1
TRIM5
TSG101
CD82
HDAC1
GBP5
SAMHD1
CD9
BST2
CD151
TSPAN7
CTNNB1
Non-Target
CD53
SPN
CHMP5
CD81
CD63
UBE2I
siRNAs

Figure 7.5 Results of sub-screen 4 evaluating the effect of 24 siRNA pools and eight controls on lentiviral vector production in activated 293SF-LVP cells. A fourth set of genes, 20 candidate inhibitory factors (including two repeats from previous screens, CD63 and CD81), four control early inhibitory factors (DDX58, MX2, SAMHD1, TRIM5) and the eight standard controls (Non-Target (black square) and BST2, CHMP5, GFP, PLK1, TSG101, UBE2I and VTA1 (dark red circles)), was screened to evaluate the impact of their knock-down on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistically significant differences between group means were determined by one-way ANOVA ($F(31,96) = 18.59$ $p = < 0.001$) followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart.

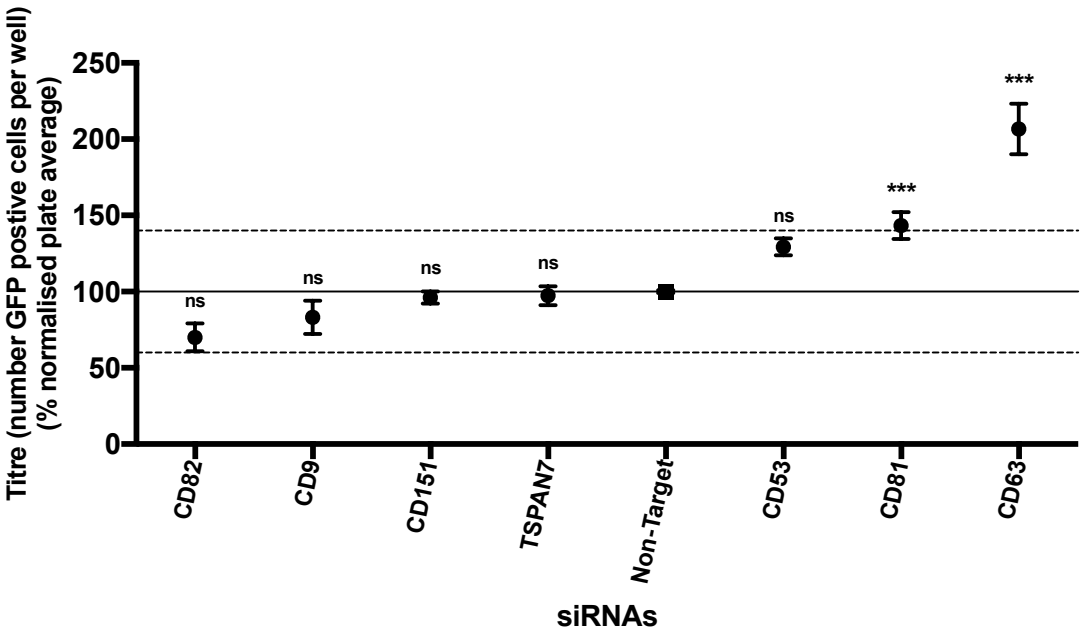


Figure 7.6 Selected averaged results of sub-screens 2-4 evaluating the effect of siRNA pools targeting members of the tetraspanin protein family on lentiviral vector production in activated 293SF-LVP cells. A selection of members of the tetraspanin family, were screened for their impact on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in sub-screens 2-4 as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-7). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set (black square) at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.001 were deemed a significant difference as indicated with three stars (***) on the chart.

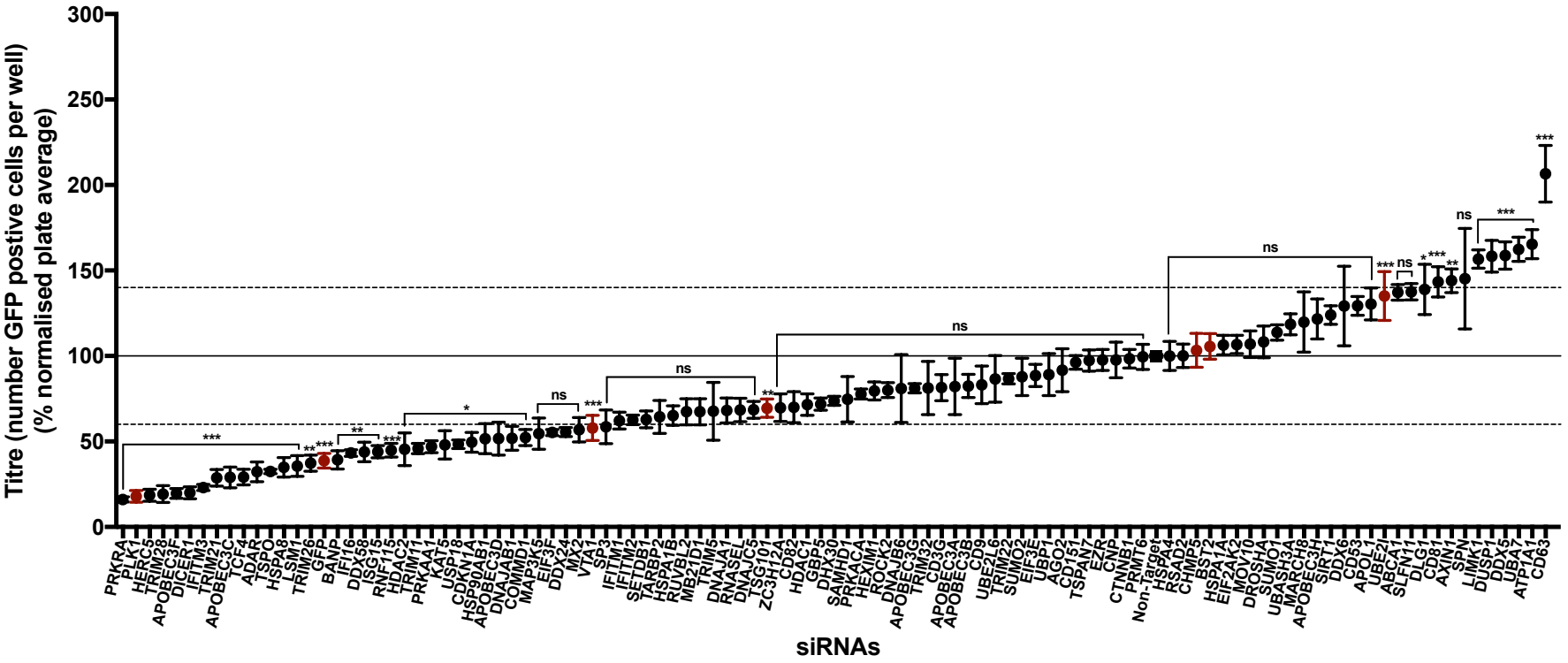

Titre (number GFP postive cells per well)
(% normalised plate average)
300
250
200
150
100
50
0
siRNAs
PRKRA
PLK1
HERC5
TRIM25
APOBEC3F
DICER1
IFITM3
TRIM21
APOBEC3C
TCF4
ADAR
TSPO
HSPA8
LSM1
TRIM56
GFP
BANF1
IFI16
DDX58
ISG15
RNF115
HDAC2
TRIM11
PRKAA1
KAT5
USP18
CDKN1A
HSP90AB1
APOBEC3D
DNAJB11
COMMD1
MAP3K5
EIF3F
DDX24
MX2
VTA1
SP3
IFITM1
IFITM2
SETDB1
TARBP2
HSPA1B
RUVBL2
MB21D1
TRIM5
DNAJA1
RNASEL
DNAJC5
TSG101
ZC3H12A
CD82
HDAC1
GBP5
DHX30
SAMHD1
PRKACA
HEXIM1
ROCK2
DNAJB6
APOBEC3G
TRIM32
CD3G
APOBEC3A
APOBEC3B
CD9
UBE2L6
TRIM22
SUMO2
EIF3E
UBP1
AGO2
CD151
TSPAN7
EZR
CNP
CTNNB1
PRMT6
Non-Target
HSPA4
RSAD2
CHMP5
BST2
HSPA1A
EIF2AK2
MOV10
DROSHA
SUMO1
UBASH3A
MARCH8
APOBEC3H
SIRT1
DDX6
CD53
APOL1
UBE2J1
ABCA1
SLFN11
DLG1
CD81
AXIN1
SIPA1L1
LIMK1
DUSP11
DDX5
UBA7
ATP1A1
CD63
ns
***
**
*

Figure 7.7. Mean results of the whole screen evaluating the effect of siRNA pools-mediated knockdown of host factors and GFP on lentiviral vector production in activated in 293SF-LVP cells. The effect of siRNA-mediated knockdown of 105 host cell factors and GFP was assessed on lentiviral vector production from 293SF-LVP cells. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in sub-screens 1-4 as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-29). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result (black square) set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained and screen controls results are represented by dark red circles. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart.

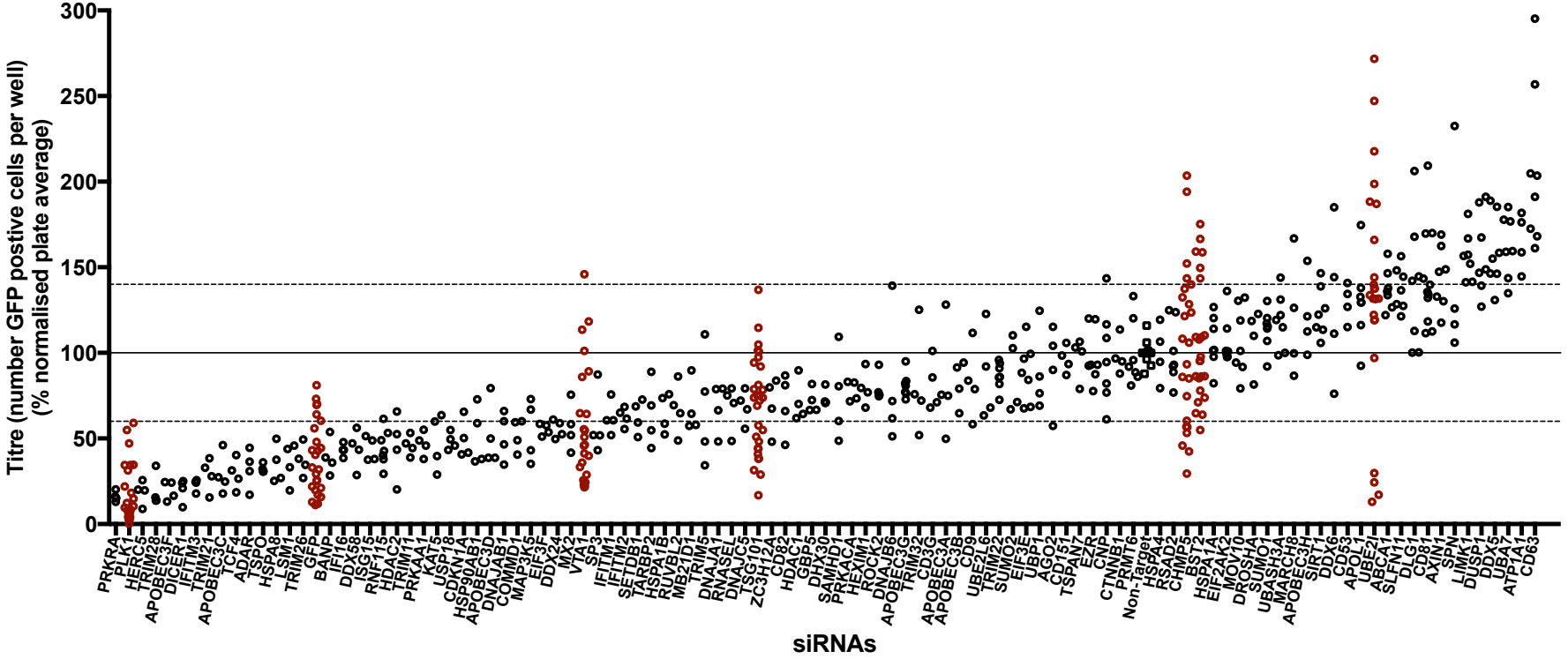
Table 7.3 Ranked average results of the whole siRNA screen

Rank	Gene	Screen Result
1	***CD63***	**206.68**
2	***ATP1A1***	**165.40**
3	***UBA7***	**162.40**
4	***DDX5***	**158.77**
5	***DUSP1***	**158.31**
6	***LIMK1***	**156.69**
7	***SPN***	**145.30**
8	***AXIN1***	**144.04**
9	***CD81***	**143.33**
10	*DLG1*	139.14
11	*SLFN11*	137.56
12	*ABCA1*	137.20
13	*UBE2I*	135.07
14	*APOL1*	130.44
15	*CD53*	129.32
16	*DDX6*	129.18
17	*SIRT1*	123.99
18	*APOBEC3H*	121.68
19	*MARCH8*	119.88
20	*UBASH3A*	118.54
21	*SUMO1*	113.79
22	*DROSHA*	108.28
23	*MOV10*	106.91
24	*EIF2AK2*	106.74
25	*HSPA1A*	106.34
26	*BST2*	105.59
27	*CHMP5*	103.27
28	*RSAD2*	100.10
29	*HSPA4*	100.00
30	Non-Target	100.00
31	*PRMT6*	99.53
32	*CTNNB1*	98.39
33	*CNP*	97.67
34	*EZR*	97.67
35	*TSPAN7*	97.39
36	*CD151*	96.26

Rank	Gene	Screen Result
37	*AGO2*	91.75
38	*UBP1*	89.13
39	*EIF3E*	88.57
40	*SUMO2*	87.83
41	*TRIM22*	86.70
42	*UBE2L6*	86.60
43	*CD9*	83.18
44	*APOBEC3B*	82.45
45	*APOBEC3A*	82.18
46	*CD3G*	81.53
47	*TRIM32*	81.33
48	*APOBEC3G*	81.16
49	*DNAJB6*	81.03
50	*ROCK2*	80.05
51	*HEXIM1*	79.53
52	*PRKACA*	77.78
53	*SAMHD1*	74.72
54	*DHX30*	73.78
55	*GBP5*	71.89
56	*HDAC1*	71.58
57	*CD82*	70.05
58	*ZC3H12A*	69.78
59	*TSG101*	69.48
60	*DNAJC5*	68.55
61	*RNASEL*	68.45
62	*DNAJA1*	68.15
63	*TRIM5*	67.67
64	*MB21D1*	67.40
65	*RUVBL2*	67.39
66	*HSPA1B*	65.13
67	*TARBP2*	64.38
68	*SETDB1*	62.90
69	*IFITM2*	62.63
70	*IFITM1*	62.20
71	*SP3*	58.55
72	*VTA1*	57.91

Rank	Gene	Screen Result
73	*MX2*	56.85
74	*DDX24*	55.55
75	*EIF3F*	55.25
76	*MAP3K5*	54.59
77	*COMMD1*	52.30
78	*DNAJB1*	51.88
79	*APOBEC3D*	51.70
80	*HSP90AB1*	51.63
81	*CDKN1A*	49.55
82	*USP18*	48.40
83	*KAT5*	48.05
84	*PRKAA1*	46.98
85	*TRIM11*	45.95
86	*HDAC2*	45.48
87	*RNF115*	44.86
88	*ISG15*	44.00
89	*DDX58*	43.91
90	*IFI16*	43.25
91	*BANP*	39.25
92	*GFP*	38.79
93	*TRIM26*	37.30
94	*LSM1*	35.68
95	*HSPA8*	34.90
96	*TSPO*	32.45
97	*ADAR*	32.28
98	*TCF4*	29.18
99	*APOBEC3C*	29.00
100	*TRIM21*	28.73
101	*IFITM3*	23.10
102	*DICER1*	20.00
103	*APOBEC3F*	19.60
104	*TRIM28*	19.25
105	*HERC5*	18.60
106	*PLK1*	17.91
107	*PRKRA*	16.13

Legend. Rank: rank of the siRNA target when the 'Screen Result' column is sorted in reverse numerical order, **Gene:** targets of the siRNA pools used in the screen, **Screen Result**: mean titre of virus (number of Green Fluorescent Protein positive cells per well) produced in sub-screens 1-4 as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-25), the titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set at 100% (bold lines). The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Screen controls results are indicated in dark red and 'hits' in bold.


Titre (number GFP postive cells per well)
(% normalised plate average)
300
250
200
150
100
50
0
siRNAs
PRKRA
PLK1
HERC5
TRIM28
APOBEC3F
DICER1
IFITM3
TRIM21
APOBEC3C
TCF4
ADAR
TSPO
HSPA8
LSM1
TRIM26
GFP
BANF1
IFI16
DDX58
ISG15
RNF115
HDAC2
TRIM11
PRKAA1
KAT5
USP18
CDKN1A
HSP90AB1
APOBEC3D
DNAJB11
COMMD1
MAP3K5
EIF3F
DDX24
MX2
VTA1
SP3
IFITM1
IFITM2
SETDB1
TARBP2
HSPA1B
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HDAC7
GBP5
DHX30
SAMHD1
PRKACA
HEXIM1
ROCK2
DNAJB6
APOBEC3G
TRIM32
CD3G
APOBEC3A
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C19
UBE2L6
TRIM22
SUMO2
EIF3E
UBP1
AGO2
CD151
TSPAN7
EZR
CNP
CTNNB1
PRMT6
Non-Target
HSPA4
RSAD2
CHMP5
BST2
HSPA1A
EIF2AK2
MOV10
DROSHA
SUMO1
UBASH3A
MARCH8
APOBEC3H
SIRT1
DDX60
CD53
APOL1
UBE2J1
ABCA1
SLFN11
DLG1
CD81
AXIN1
SPN
LIMK1
DUSP1
DDX5
UBA7
ATP1A1
CD63

Figure 7.8 Individual results of the whole screen evaluating the effect of siRNA pools-mediated knockdown of host factors and GFP on lentiviral vector production in activated in 293SF-LVP cells. The effect of siRNA-mediated knockdown of 105 host cell factors and GFP was assessed on lentiviral vector production from 293SF-LVP cells. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in sub-screens 1-4 as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-29). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result (black square) set at 100% (solid line) ± SEM. Symbols represent normalised mean result for each of the independent target host factor studies with dark red open circles representing screen controls results. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained and screen controls results are represented by dark red circles. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart.

7.3 Discussion

Experiments in this chapter aimed to evaluate candidate inhibitory factors of lentiviral vector production emerging from a systematic investigation of the literature conducted in Chapter 5 to identify genes constituting possible inhibitory factors of HIV-1 in the late phase of its life cycle. The experiments were performed using the siRNA screening protocol developed in Chapter 6. Here, a targeted approach was used rather than a genome-wide screen, or screens focussing on single genes or pathways.

Overall, out of the 152 candidate inhibitory factors identified in Chapter 5, 89 were evaluated in four sub-screens. It was decided to not test the remaining 63 genes due to time and financial constraints and also because the evidence supporting their candidate inhibitory factor status was not as strong. Out of the total 105 human genes screened (including controls and candidate essential/auxiliary factors) (Table 7.3) nine were identified as hits increasing virus production when knocked-down 40% over Non-Target siRNA transfection. This represented 8.5% of the genes screened, a high proportion compared to many genome-wide screens (Table 6.2) demonstrating the power of the targeted approach used here.

7.3.1 The identified ‘hits’

As established in Section 6.2.1.8, the nine ‘hits’ were identified when knock-down of a particular gene using the corresponding siRNA pool led to a 40% increase in vector production compared to the Non-Target siRNA pool in the averaged results of the whole screen (Figure 7.9). The experimental designed was powered at 80% to detect such an increase and this was also two standard deviations of the values obtained from a complete plate transfected with Non-Target siRNAs. ‘Near-hits’ were also identified when

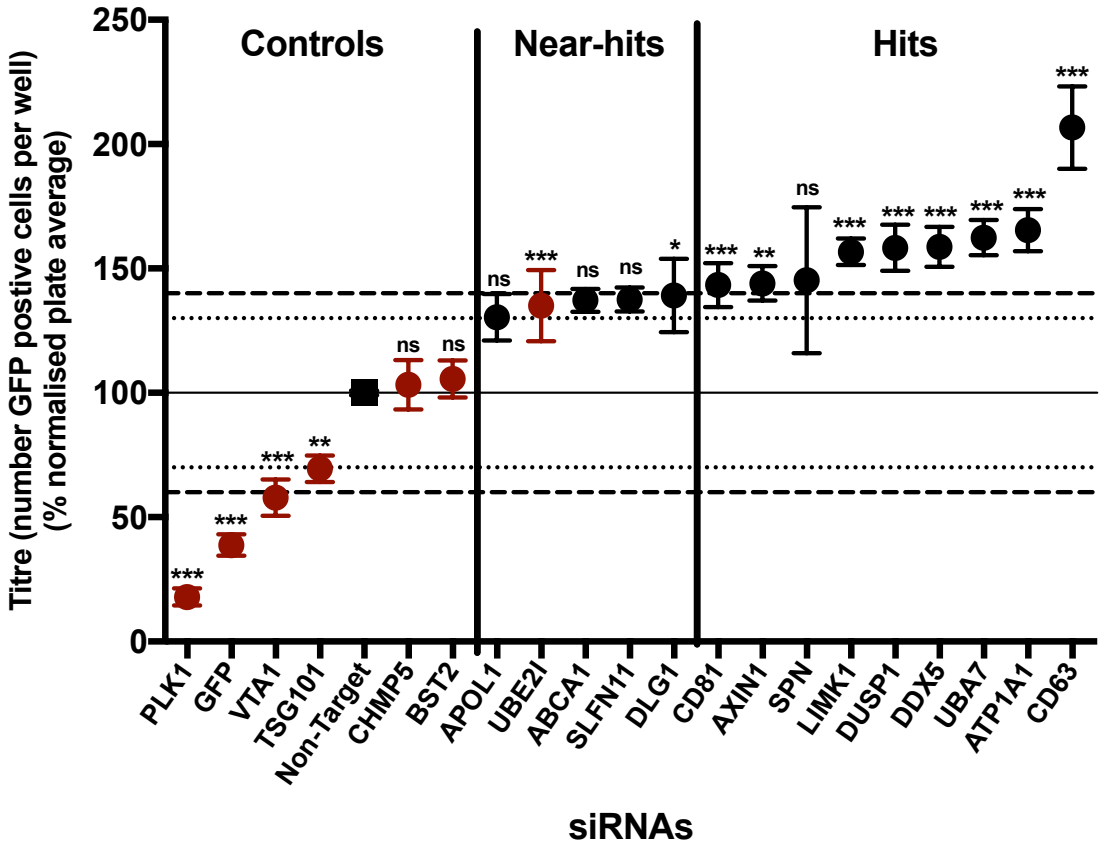
Titre (number GFP postive cells per well)
(% normalised plate average)
250
200
150
100
50
0
Controls
Near-hits
Hits
***
***
***
**
ns
ns
ns
***
ns
ns
*
***
**
ns
***
***
***
***
***
PLK1
GFP
VTA1
TSG101
Non-Target
CHMP5
BST2
APOL1
UBE2I
ABCA1
SLFN11
DLG1
CD81
AXIN1
SPN
LIMK1
DUSP1
DDX5
UBA7
ATP1A1
CD63
siRNAs

Figure 7.9 Selected results from sub-screens 1-4 showing the effect of siRNA pools targeting genes identified as 'hits' and 'near-hits' as well as the eight screen controls on lentiviral vector production in activated 293SF-LVP cells. The knockdown of nine host factors was found to increase lentiviral vector production from 293SF-LVP cells by ≥40% (termed 'hits'). A further five host factors, when knocked-down, were found to increase lentiviral vector production from 293SF-LVP cells by between <40% and ≥30% (termed 'near-hits'). Also represented is the effect of knock-down of the eight screen controls (Non-Target (black square) and BST2, CHMP5, GFP, PLK1, TSG101, UBE2I and VTA1 (dark red circles)). Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-25). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set at 100% (solid horizontal line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target) while the dotted lines indicate the 'near-hit' threshold (± 30% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05, < 0.01 and < 0.001 were deemed a significant difference as indicated with one (*), two (**) and three (***) respectively on the chart.

knock-down of a particular gene using the corresponding siRNA pool led to an increased virus production between 30-40%.

It is important to note that these hits will need to be validated in further experiments. The mRNA or protein level of expression of knocked-out targets could be evaluated by RT-qPCR or Western blots respectively to assess the efficiency of the knock-down. Additional validation of the 'hits' could include using siRNAs with different sequences or using a different technology such as shRNAs or CRISPR/Cas9. Further work could also assess the impact of these knock-downs on virus particle production through an evaluation of the non-functional titre. This would allow to better understand whether more vectors were produced, if they were more functional or a combination of the two through changes in the P:I ratio.

7.3.1.1 CD63 & CD81

The tetraspanin family, including specific candidate factors CD63 & CD81, was discussed in Section 7.2.3.3. It is interesting to note that, while many studies support the inhibitory factor role of CD81 (Sato *et al.*, 2008; Grigorov *et al.*, 2009; Krementsov *et al.*, 2009; Bregnard *et al.*, 2013; Haller *et al.*, 2014), the picture is not as clear in the literature for CD63 with four studies reporting it as an essential factor (Chen *et al.*, 2008; Ruiz-Mateos *et al.*, 2008; Li *et al.*, 2014; Fu *et al.*, 2015) and three describing it as an inhibitory factor (Sato *et al.*, 2008; Krementsov *et al.*, 2009; Haller *et al.*, 2014). Importantly, the different designs of these studies incorporated different HIV-1 life cycle steps. Thus, one interpretation of these conflicting observations is that in addition to its role in virus budding, there is an important role for CD63 in virus entry (Von Lindern et al., 2003; Li et al., 2011).

7.3.1.2 ATP1A1

The ATP1A1 gene encodes the alpha 1 (catalytic) subunit of the Na^+/K^+-ATPase. This sodium potassium pump belongs to the family of P-Type cation transport ATPases and is an integral membrane protein (Skou, 1957). It establishes and maintains Na^+ and K^+ ions gradient across the plasma membrane. This allows osmoregulation, sodium-coupled transport in addition to nerve and muscle electrical excitability. It appears to be also implicated in signalling functions. It is known to associate with HIV-1 Gag in the cytoplasm (Milev et al., 2012; Kutluay et al., 2014; Ritchie et al., 2015), but its function in the HIV-1 life cycle is currently unknown. The evidence for ATP1A1 being an essential/auxiliary factor was relatively weak; one study, performed in HEK 293T cells using a transfected pseudotyped provirus, showed that the inhibition of HIV-1 gene expression by cardiac glycosides was dependent upon the inhibition of the human Na+/K+-ATPase of which ATP1A1 is a subunit (Laird *et al.*, 2014).

7.3.1.3 UBA7

UBA7 (UBE1L) was identified as an inhibitory factor for HIV-1 production in a study of another factor: ISG15 (Okumura *et al.*, 2006), which is part of the ubiquitin-like pathway activated by UBA7. The Gag protein in HIV-1 requires ubiquitination for assembly and release of virions from infected cells (Schubert et al., 2000; Strack et al., 2000). ISG15 overexpression in NL4-3 transfected HEK 293T cells inhibited virion release while co-transfection of ISG15 and UBA7 completely blocked HIV-1 replication. This was shown to be due to the fact that Gag ubiquitination is reduced by 40% in cells over-expressing UBA7 alone, and by 55% when both UBA7 and ISG15 were over-expressed. In contrast with that study, the current screen found UBA7 inhibition to increase virus production, while ISG15 inhibition decreased virus production (Table 7.3). This is an example of the difficulty of extrapolating over-expression studies results to inhibition studies. ISG15

might have an additional role in HIV-1 production and require a certain balance between these roles for efficient production.

7.3.1.4 DDX5

DDX5 is an RNA helicase (Ford et al., 1988) member of the DEAD-box family, which are involved in all aspects of RNA metabolism (Reviewed in Fuller-Pace, 2013). During HIV-1 infection, DDX5 interacts with and enhances the function of Rev (Yasuda-Inoue *et al.*, 2013; Zhou *et al.*, 2013). It is known to form heterodimers with DDX17, with which it shares 90% sequence identity (Lamm et al., 1996), and both protein form homodimers with themselves (Ogilvie et al., 2003), a dynamic thought to be important in the HIV-1 life cycle. There is a consensus that DDX17 is an essential factor for HIV-1 production as overexpression raises HIV-1 production while knock-down diminishes it (Naji *et al.*, 2012; Yasuda-Inoue *et al.*, 2013; Williams *et al.*, 2015). As for the effect of DDX5, opposing results have been published (Table 7.4). In one study, knock-down of DDX5 increased cellular Gag levels, p24 release and infectivity (Naji *et al.*, 2012) whilst another study found modest enhancement in virus production after siRNA knock-down of DDX5 (Lorgeoux *et al.*, 2013). In other studies, overexpression of DDX5 increased virus production while siRNA knock-down reduced it (Yasuda-Inoue *et al.*, 2013; Zhou *et al.*, 2013; Williams *et al.*, 2015). These studies were performed using different cell lines, however a study of protein expression in various cell lines (Geiger et al., 2012) showed HEK 293T and HeLa-S3 to express similar levels of DDX5, 633 and 623 ppm respectively, and DDX17, 620 and 646 ppm, while the lymphocytic Jurkat cell line expresses less DDX5 (442 ppm) and more DDX17 (841 ppm). This might, however, not alone explain the differences observed. The use of different viral or vector constructs could lead to different results, but one study (Zhou *et al.*, 2013) covered many types of constructs from provirus to third-generation lentiviral vector plus a Rev/RRE-dependent

Table 7.4 Conditions and results of studies that assayed the effect of DDX5 on HIV-1 production

Target	Study	Cell Line	transfection reagent	Time from siRNA/overexpression transfection to challenge	Virus / Vector	Harvest	Result (% of control)
DDX5	(Naji *et al.*, 2012)	HeLa CD4+ TZM-bl	RNAiMAX & Lipofectamine 2000	siRNA: 72 hours & 24 hours	pNL4-3	48 hours	125-150% Gag 200% p24 400% infectivity
DDX5	(Lorgeoux *et al.*, 2013)	HeLa	Possibly Lipofectamine 2000	siRNA: Twice, unknown times	pNL4-3	40 hours	~150% (ns) (RLU & RT)
DDX5	(Yasuda-Inoue *et al.*, 2013)	293FT	FuGENE 6	Over-expression: 0 hours	pDM628+Rev	24 hours	~450% (RLU)
DDX5	(Zhou *et al.*, 2013)	Over-expression: HEK 293T	Lipofectamine 2000	Over-expression: 0 hours	pNL4-3-ΔEnv-GFP pCMV-ΔR8.2 pNL4-3 pDM628+Rev pDMLg/RRE+Rev pDMLg/CTE+Rev	48 hours	~217% (p24) ~258% (p24) ~207% (p24) ~350% (RLU) ~174% (p24) ~105% (p24)
		siRNA: CD4+ T cells		siRNA: 0 hours	pNL4-3 pNL4-3-ΔEnv-GFP pDM628+Rev pDMLg/RRE+Rev pDMLg/CTE+Rev	5 days 48 hours	70% (p24) 28% (p24) ~30% (RLU) ~70% (p24) ~106% (p24)
DDX5	(Williams *et al.*, 2015)	HeLa	Lipofectamine 2000	siRNA: 24 hours	VSV-g-pseudotyped HIV-1 & WT HIV-1	96 hours	~64%-2% (p24) ~93-10% (RLU)
DDX5	Current screen	293SF-LVP	Lipofectamine 2000	siRNA: 72 hours	LVR2-GFP (rHIV.VSV-g CMV GFP) (production)	72 hours	158.8% (GFP)

Legend: Time from siRNA/overexpression transfection to challenge: in the timeline of the experimental setup, time between when the siRNA or overexpression plasmid is transfected and the cells are challenged with a transfection, infection or activation of virus production, **Virus / Vector**: virus or vector used to challenge the cells (Legend: pNL4-3 (proviral plasmid), pDM628 and pDMLg (Rev/RRE-dependent reporter vectors), **Harvest**: time from challenge to harvest of the vector or assay readout, **Result (% of control)**: effect of the siRNA/shRNA treatment on virus production when compared to the control of the screen (Legend: Gag: Gag protein expression, p24: p24 protein expression, ns: not significant, RLU: relative light unit, GFP: green fluorescent protein).

reporter vector and found broadly similar results. The main difference between all these published studies and the screen used here is the absence of a transfection step to initiate virus production which might explain many of the disparities. DDX5 might directly be implicated in the cell's response to the transfection or there could be a transfection timing issue and DDX5 could have different effects on different steps of the HIV-1 life cycle.

7.3.1.5 DUSP1 & AXIN1

DUSP1 and AXIN1 were identified as inhibitory factors as part of a published siRNA screen of a mini-library of selected genes (Kameoka *et al.*, 2007). DUSP1 is a negative regulator of the mitogen-activated protein kinase (MAPK) signal transduction pathways (Reviewed in Farooq & Zhou, 2004), of innate immune responses (Chi et al., 2006; Salojin et al., 2006) and of negative regulators of pro-allergic responses in airway epithelium (Golebski et al., 2015). AXIN1 is a regulator of the wingless/int (Wnt) signalling pathway (Zeng et al., 1997) which controls many biological processes, such as cell proliferation (Reviewed in Clevers, 2006). Overexpression of AXIN1 promotes the downregulation of CTNNB1 and TCF4 (Yang et al., 2010), two genes also identified in the Chapter 5 literature review (Wortman *et al.*, 2002; Henderson *et al.*, 2012; Narasipura *et al.*, 2012), but which resulted in titres, here, of 98.4% and 29.2% respectively of the Non-Target siRNA control (Table 7.3). In the published screen (Kameoka *et al.*, 2007), cells of the J111 line, a human acute monocytic leukaemia cell line, transfected with various siRNAs were transfected further with a luciferase reporter provirus. This showed that siRNAs against AXIN1 and DUSP1 could enhance this reporter's replication by acting in the late phase of the viral life cycle. A few other genes were initially identified and then rejected in this study (Kameoka *et al.*, 2007), including EZR and ROCK2 which were found not to have an effect on virus production here (Table 7.3) with 97.7% and

80.1% production of the Non-Target control. In a follow-up study, it was shown that Tat-dependent viral reporter gene expression was enhanced in AXIN1-siRNA-transfected J111 cells, as was the viral transcription, but not in HeLa cells (Kameoka *et al.*, 2009).

7.3.1.6 LIMK1

LIMK1 was identified as an essential/auxiliary factor for HIV-1 production in a siRNA screen targeting host membrane trafficking proteins (Wen *et al.*, 2014a). Validation with shRNAs also showed a five-fold reduction in HIV-1 particle release compared to control shRNA-expressing cells. In a follow-up study, particle output and cell-to-cell transmission were reduced by LIMK1 knock-down and rescued by LIMK1 replenishment (Wen *et al.*, 2014b), an effect attributed to disruption of the actin cytoskeleton. LIMK1 was also found to have a role in the early portion of the HIV-1 life cycle, facilitating viral entry, DNA synthesis and nuclear migration through actin polymerisation (Vorster et al., 2011; Xu et al., 2012). Furthermore, a closely related kinase, LIMK2, was also identified as a potential essential factor in the early phase of the life cycle in one of the genome-wide screens (Konig *et al.*, 2008). These results contrast with the 56.7% increase in virus production observed here (Table 7.3), probably due to the many differences in production protocols.

7.3.1.7 SPN

SPN, also known as sialophorin or CD43, is a protein on the surface of lymphocytes involved in immune response (Park et al., 1991) and was identified as a possible inhibitory factor (Mclaren *et al.*, 2015). When overexpressed, it inhibited HIV-1 production in HEK 293T cells by more than 90%. In the current screen, CD43 inhibition increased virus production by 45.3%, but with a large inter-plate variability. APOL1, a ‘near hit’ at 130.4% of Non-Target control production, was also identified in this study and another

(Taylor *et al.*, 2014). Other genes identified in the same study as SPN (GBP5 and IFI16) were not identified as hits in the current screen instead leading to reduced virus titres, with 71.9% and 43.3% of the Non-Target control value respectively (Table 7.3). Again, this could demonstrate differences in results from overexpression and knock-down studies, and/or be due to the different experimental format (transient transfection instead of a stable producer cell line).

7.3.2 'Near-hits'

Knock-down of DLG1, a very near-hit in this study (139.1% of Non-Target control) was shown to upregulate HIV-1 Env expression and accumulation of both Gag and Env CD63+ intracellular compartments (Perugi *et al.*, 2009).

SLFN11 was found to act by selectively inhibiting the expression of viral proteins based on codon-usage (Li *et al.*, 2012). HIV-1 infection modulates the tRNA pool to improve its translation efficiency (Van Weringh et al., 2011). SLFN11 binds tRNAs and counteracts HIV-induced changes in the tRNA pool (Li *et al.*, 2012). Interestingly, in the same study, it was shown that while HEK 293 cells express SLFN11, HEK 293T cells do not. Knock-down of SLFN11 in HEK 293T might, therefore, not have the same effect than it had in the current screen which was performed on cells of the 293SF lineage.

ABCA1 is involved in cholesterol efflux from cells (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999), which, as discussed in Section 3.1, reduces virion infectivity. Lentiviral accessory protein Nef was shown to impair cholesterol efflux in infected cells via its action on cholesterol regulatory efflux protein ABCA1 (Mujawar *et al.*, 2006).

7.3.2.1 UBE2I

Interpretation of UBE2I results was problematic. While sub-screen 1 identified it as an essential/auxiliary factor, supporting the published literature, when subsequently used as a control (albeit from an independent preparation of the commercial siRNA pool), it repeatedly scored within the highest genes (as shown by the spread of data in Figure 7.8). TSG101 was also tested from two independent preparations of the commercial siRNA pool but, as shown in Figure 7.2E, no significant difference was observed between the preparations.

When the data from all sub-screens is averaged, UBE2I qualifies as a 'near-hit', but if the data from sub-screen 1 was considered as an anomaly and disregarded, it would qualify as a 'hit'. Identification of UBE2I as an essential factor was based on two studies (Jaber *et al.*, 2009; Bohl *et al.*, 2013), where UBE2I co-localised with Gag and where knock-down of UBE2I, while not decreasing titre, reduced infectivity 10-fold. While this affected Gag trafficking, the most important effect was attributed to decreased Env stability and incorporation into virions. This was shown to not be applicable after VSV-g pseudotyping, where a slight increase in infectivity compared to the control siRNA was observed (Jaber *et al.*, 2009). This latter observation is consistent with the majority of the results obtained here.

7.3.3 siRNA knock-down of restriction factors

Figure 7.10 shows the whole screen results for four of the restriction factors (APOBEC3G, BST2, SAMHD1 and TRIM5). SERINC3 and SERINC5 were not included here as they have been reported not to affect VSV-g pseudotyped virus production (Rosa *et al.*, 2015; Usami *et al.*, 2015). Since APOBEC3G and BST2 are expressed in very low amounts in HEK 293 cells (< 1 ppm) (Tanner *et al.*, 2007), it was not unexpected that

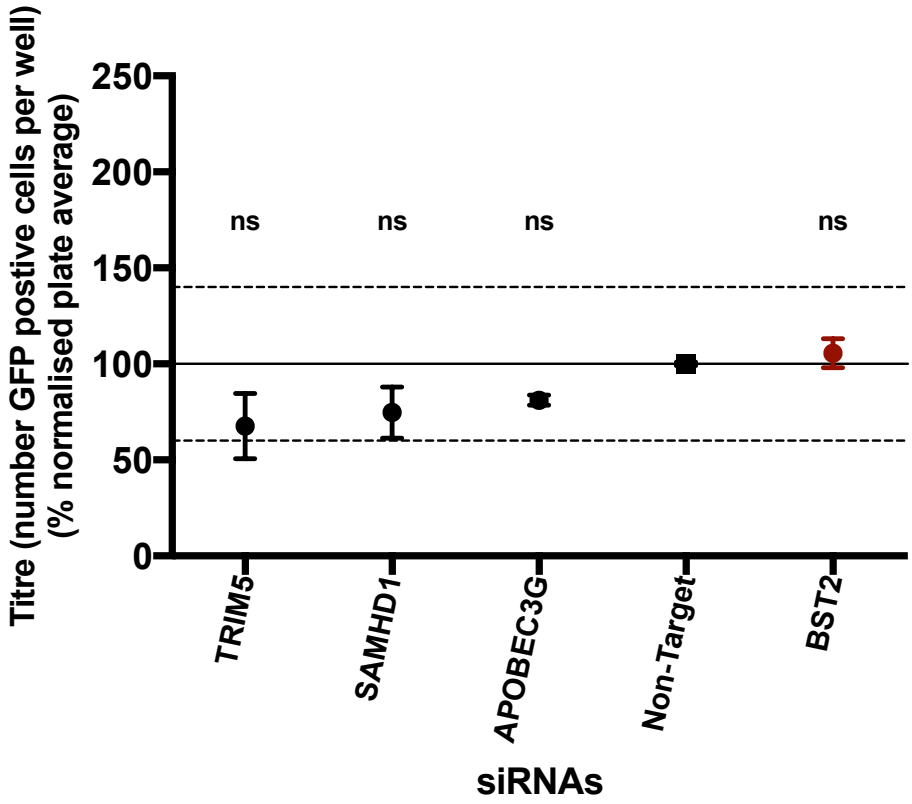

Figure 7.10 Selected results from sub-screens 1-4 showing the effect of siRNA pools targeting four restriction factors on lentiviral vector production in activated 293SF-LVP cells. The knockdown of four restriction factors was found to have no effect on lentiviral vector production from 293SF-LVP cells. The BST2 siRNA result is represented as a dark red circle as it was also a screen control. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-25). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result (black square) set at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart.

knocking-down these genes had little effect on virus production. The same was true for SAMHD1 and TRIM5, which were outside the scope of this current study as they do not act in the late phase of the HIV-1 life cycle (Section 5.1.1.3). Interestingly, the knock-down of other early inhibitory factors MX2 and DDX58, significantly reduced virus production (56.8% and 43.9% respectively) even though their effects are described only as being early in the virus life cycle.

7.3.4 siRNA knock-down of PKR and its inhibitors

Figure 7.11 shows selected results of the whole siRNA screen for PKR and its inhibitors, which were previously discussed in Chapter 4. While overexpression of PKR had a strong negative effect on virus production yields (Figure 4.2), its knock-down did not have a significant effect. The observed titre (106.7%) is in line with published data in primary CD4+ T cells where a non-significant increase (120%) was also observed (Sanghvi & Steel, 2011). This discrepancy is important because a number of studies that identified candidate genes used over-expression, which is not as reliable as knock-down to assess the impact of expression of a gene. For example, the study that identified PACT as an HIV-1 inhibitor (Clerzius *et al.*, 2013) found that its over-expression alone raised RT activity by 20% while it reversed the effect of PKR over-expression 5.3-fold. However, shRNA inhibition of PACT had a strong effect reducing RT activity 55-75%. Interestingly, knocking-down the three PKR inhibitors resulted in significant effects on virus production yields, which correlates with published studies identifying these as auxiliary factors (Christensen *et al.*, 2007; Clerzius *et al.*, 2009; Clerzius *et al.*, 2013). Furthermore, PACT was in Chapter 4 the only over-expressed PKR inhibitor that showed a trend towards improving titres (Figure 4.6) and, here, of all the genes evaluated in sub-screens 1-4, knock-down of PACT (PRKRA) resulted in the greatest reduction of titres. This result, as that of the many other candidate genes that scored below 60% of the Non-Target control,

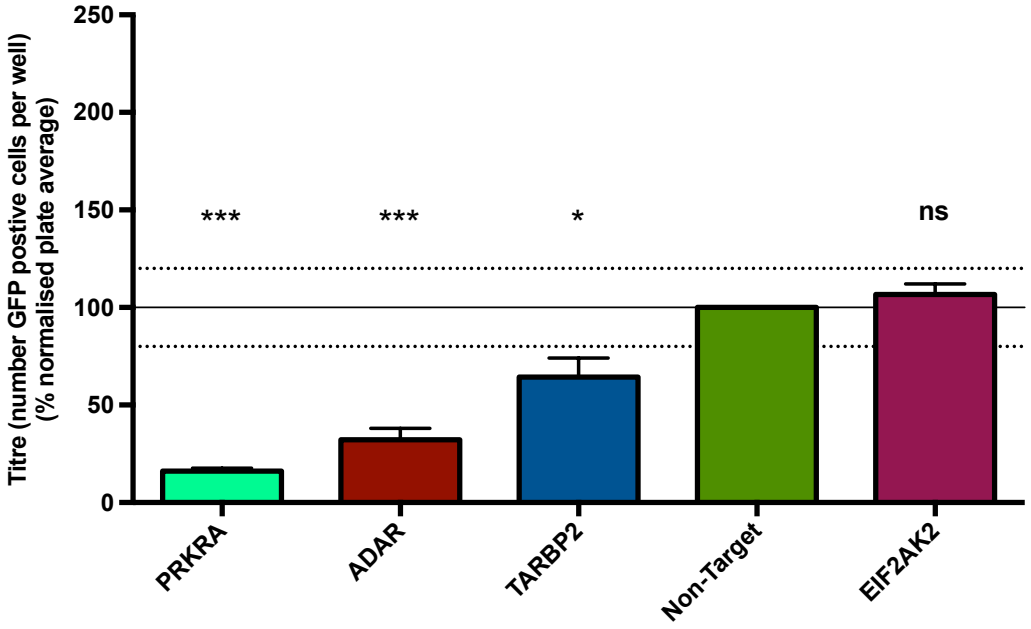

Figure 7.11 Selected results from sub-screens 2 and 3 showing the effect of siRNA pools targeting PKR and its inhibitors on lentiviral vector production in activated in 293SF-LVP cells. A selection of results of sub-screens 2 and 3 showing the impact of PKR (EIF2AK2) and its inhibitors TRBP1 (TARBP2), ADAR1 (ADAR) and PACT (PRKRA) on lentiviral vector production in 293SF-LVP producer cells using siRNA pools. Mean titre of virus (number of Green Fluorescent Protein (GFP) positive cells per well) produced in sub-screens 2 and 3 as described in Section 2.4.4 in the presence of the indicated siRNAs (n=4-7). Titres were assayed as described in Section 2.5.2 and are presented normalised to the Non-Target control siRNA result set (dark green) at 100% (solid line) ± SEM. The dashed lines indicate the 'hit' threshold (± 40% value of Non-Target). Results are sorted (left to right) in ascending order of the average value obtained. Statistical significance was determined by one-way ANOVA ($F(106,565) = 14.93$, $p = < 0.001$) on the entire siRNA screen's data followed by Dunnett's post-test. A calculated p value of > 0.05 was deemed non-significant as indicated with ns on the chart. Calculated p values of < 0.05 and < 0.001 were deemed a significant difference as indicated with one (*) and three stars (***) respectively on the chart.

could provide leads for future knock-in (permanent over-expression) studies where additional production of the gene product might boost virus production. Alternatively, these genes might be interesting in the study of HIV-1 as a disease-causing agent and/or provide a novel class of target for anti-HIV-1 drugs.

7.3.5 Screen Limitations

7.3.5.1 Scope limitation

In Section 6.1.1, a number of published genome-wide screens were described. These mostly focussed on essential/auxiliary factors with the only published genome-wide screen targeted specifically at inhibitory factors focussing only on factors acting early in the HIV-1 life cycle. A major limitation of the screen developed in this thesis was its reliance on published literature to provide the primary gene list. It seems probable that some inhibitory factors have simply not been characterised in the literature yet, or were not found by the search terms used, and could not, therefore, be included. This could be due to a tendency for genes to be studied under a selective pressure with a modest number of well-characterised genes being studied and quoted with increasing frequency while the rest remain essentially unstudied (Hoffmann & Valencia, 2003). Approximately half of the candidate inhibitory factors (72/152, 47.4%) were identified in papers published since 2010, including a high number of candidate genes that were extracted from papers from 2015, the last year for which the literature review was conducted (18/152, 11.8%). Rather than an investigation of the published literature, an approach with less bias would have been a genome-wide screen, which would have had the potential to identify genes not yet known to be involved in that part of the HIV-1 life cycle. Regrettably, the time and resources required to perform such a genome-wide screen (incorporating ~200 times more genes) were constrained. Nevertheless, these current

efforts provide a compelling argument for such a screen and will provide essential assay conditions and valuable know-how should such a screen be conducted in the future.

7.3.5.2 Screen characteristics limitations

A crucial limitation of the screen developed in this thesis, and probably of other similar published screens, is that it can always be argued that the results obtained may be specific to the cell line, virus, pseudotype and/or production technique used. This is exemplified by the poor overlap in the genes identified in the genome-wide siRNA screens for both HIV-1 (Reviewed in Bushman *et al.*, 2009; Cherry, 2009) and influenza (Reviewed in Meliopoulos et al., 2012; Chou et al., 2015) presented in Table 6.1. It is also well known that protein expression levels vary widely between cell lines. HEK 293-based cells employed in the current study are a good choice for lentiviral vector production, they are extensively used in the field, readily transfected and have a relatively low expression of key restriction factors (Table 5.1). Similarly, some inhibitory factors identified here, in HEK 293-based cells, might have low expression in other cell lines and their inhibition might, therefore, have a reduced impact. Even between cells of the HEK 293 lineage, there are differences in expression with some candidate inhibitory factors (APOBEC3B, APOBEC3F, CD63, CD9, CDKN1A, DUSP1 and IFI16) being downregulated when compared to expression levels in the parental HEK 293 cell line (Lin et al., 2014). Indeed, this may explain the instances where the findings observed here are at odds with other published studies which used other HEK 293 derivatives, other cell lines or primary cells. Furthermore, some genes expressed at low levels in 293SF-LVP cells might not have been identified by this screen, but may still act as inhibitory factors in other cell lines.

The overall study design may also explain the surprising results obtained for some candidate *inhibitory* factors revealed here as possible *essential/auxiliary* factors, or as having no impact on virus replication. Several published studies used virus infection to assess the effect of candidate genes, involving the virus proceeding through steps of the life cycle not assessed here. Importantly, HIV-1 itself incorporates all the so-called accessory proteins that are removed from third-generation lentiviral vectors used here and, therefore, may be more equipped to counteract inhibitory as well as restriction factors, again potentially confounding the results. Furthermore, in many other studies, proviral DNA was transfected, in contrast with the screen used here, where virus production is initiated by the addition of doxycycline and cumate to the culture media. A producer cell line, such as that used here, might behave differently from transiently transfected cells, which undergo additional stress during transfection. Furthermore, some cellular factors might have a direct impact on the transfection efficiency itself rather than on the viral production specifically. Finally, the virus pseudotype might impact production and the role of inhibitory factors, as mentioned for the candidate essential factor UBE2I (Section 7.3.2.1). This could also be true for inhibitory factors, as described in Section 5.1.1.3, the most recently discovered restriction factors SERINC3 and SERINC5 had little impact on the production of viruses pseudotyped with VSV-g (similar to the virus produced in the current screen) (Rosa *et al.*, 2015; Usami *et al.*, 2015). Therefore, although SERINC3 and SERINC5 are recognised as genuine restriction factors in the late phase of the HIV-1 life cycle, they would have probably not been identified as a 'hit' if tested here.

7.3.5.3 siRNA-based screen limitations

A further limitation of the siRNA screen used in the current study is that it depended upon a specific library of siRNAs, which lacked coverage of some non-protein coding genetic

components. Examples of siRNA coverage gaps include microRNAs (miRNAs) and long non-coding RNA (lncRNA), some of which have been shown to impact HIV-1 replication (Reviewed in Barichievy et al., 2015; Rice, 2015). For example, miR-17-5p and miR-20a are suppressed during HIV-1 replication and their knockdown enhanced HIV-1 production while their over-expression reduced it substantially (Triboulet *et al.*, 2007). In the case of miR-29, inhibiting miR-29a enhanced HIV-1 viral production and infectivity, whereas expressing a miR-29 mimic suppressed viral replication (Nathans *et al.*, 2009). The same was true of miR-29b & miR-29c at much lower levels. It was also demonstrated that miR-29a downregulates the expression of Nef and HIV-1 replication (Ahluwalia et al., 2008). In the case on lncRNA, knock-down of NEAT1 enhanced virus production by increasing nucleus-to-cytoplasm export of Rev-dependent instability element-containing HIV-1 mRNAs (Zhang *et al.*, 2013). It was also shown that HIV-1 infection reduces the lncRNA NRON expression levels which acts as a non-coding repressor of the Nuclear Factor of Activated T cells, modulating HIV-1 transcription and replication (Imam *et al.*, 2015). Some libraries allow testing of the effect of these two sets of RNAs, and these could be evaluated in the future.

Finally, a meaningful limitation is that siRNA exerts only a reduction of a gene's protein production, rather than a complete knock-out. At the start of the screen, the cells have a certain level of the target protein already present but, at any point during virus production, some level of the protein might still be present in the cell. The manufacturer of the siRNA (GE Dharmacon) guarantees at least 75% knock-down in mRNA levels 24-48 hours after transfection. This leads to a gradual decrease in the presence of the protein in the cell, depending on its half-life, as it is degraded and its levels are not fully replenished. The extent of depletion by siRNAs of their target proteins varies with the target's half-life; short-lived proteins may be diminished to 10% or less of their normal abundance within 3–5 days, whereas longer-lived proteins may show little or no depletion which would hide

their real effect on lentiviral vector production in this screen (Reviewed in Sharma & Rao, 2009). Also, the effect of the siRNA eventually wanes allowing targeted protein levels to increase. These competing effects are shown in Figure 7.12 representing a stylised hypothesis of the effect of siRNA transfection on protein levels during virus production, in the current siRNA knock-down screen. The area under the curve (in grey) represents the hypothetical level of a protein present during virus production. Although the level is greatly reduced, the protein might still exert its effect on virus production, fully or partially, depending on the concentration required to exert its effect in the cell.

In addition, as demonstrated in Figure 6.4, the knock-down efficiency of the GFP siRNA was lower than 100% with some cells in the GFP siRNA transfected wells still expressing high levels of GFP. The same can be expected of the other siRNA used and these cells, therefore, express normal levels of the gene product being targeted. The titration results obtained in this screen are therefore a composite of the production of cells where the targeted gene product is knocked-down (although some gene products might still be present during production) and cells where it has not been knocked-down, skewing the results towards no change relative to the Non-Target control value.

This is in opposition to a knock-out cell line where the gene would be completely absent. It is, therefore, possible that some genes would only have been identified in a complete knock-out screen. However, the technology currently available for knock-out was not suitable here. Such knock-out screens usually require a direct readout after the knock-out event (rather than the secondary titration approach adopted here) in order to identify positive hits within a pool, or would necessitate growth of specific clones for each gene, an immensely time-consuming process.

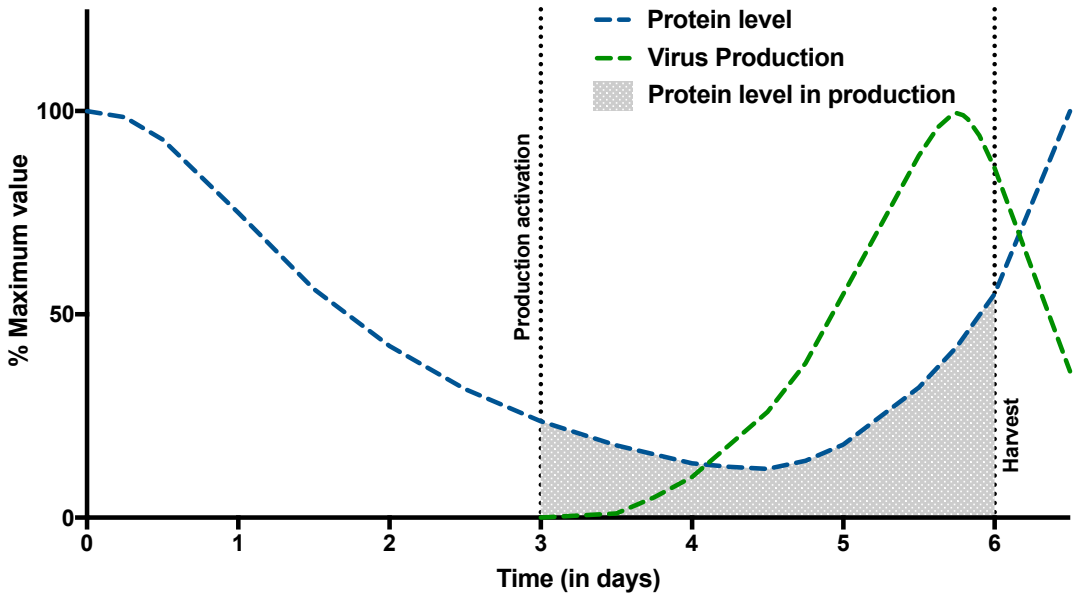

Figure 7.12 Stylised hypothesis of the effect of siRNA transfection on protein levels during virus production in the siRNA knock-down assay. This hypothetical graph shows how protein levels decrease following transfection with siRNA. This is based on the manufacturer's guarantee of knock-down of ≥ 75% at the mRNA level 24 to 48 hours post-transfection. The effect is expected to wane, but the actual kinetics are unknown. The hypothetical timing of virus production is estimated from experience with virus production in the laboratory. The shaded area indicates the protein levels present during virus production in a knock-down experiment compared to no expression in a knock-out experiment.

In conclusion, this chapter used the screening protocol developed in Chapter 6 to assess 89 of the 152 candidate inhibitory factors identified in Chapter 5. The nine 'hits' identified in this screen could be used in further studies to fully understand their effect on lentiviral vector production and ultimately increase titres of clinical vectors. Most of the 'hits' and 'near-hits' identified in these studies have been identified as acting in either the budding or the infectivity stage of the HIV-1 life cycle (Figure 7.9), highlighting how critical these steps are in virus production. However, various other steps of the HIV-1 life cycle are also implicated (Figure 7.13), suggesting that the simultaneous targeting of more than one gene might be an interesting prospect, resulting in a more profound effect. However, double siRNA transfections might be difficult and cause interference. Alternatively, this could be attempted via the generation of knock-out cell lines, followed by an additional siRNA screen.

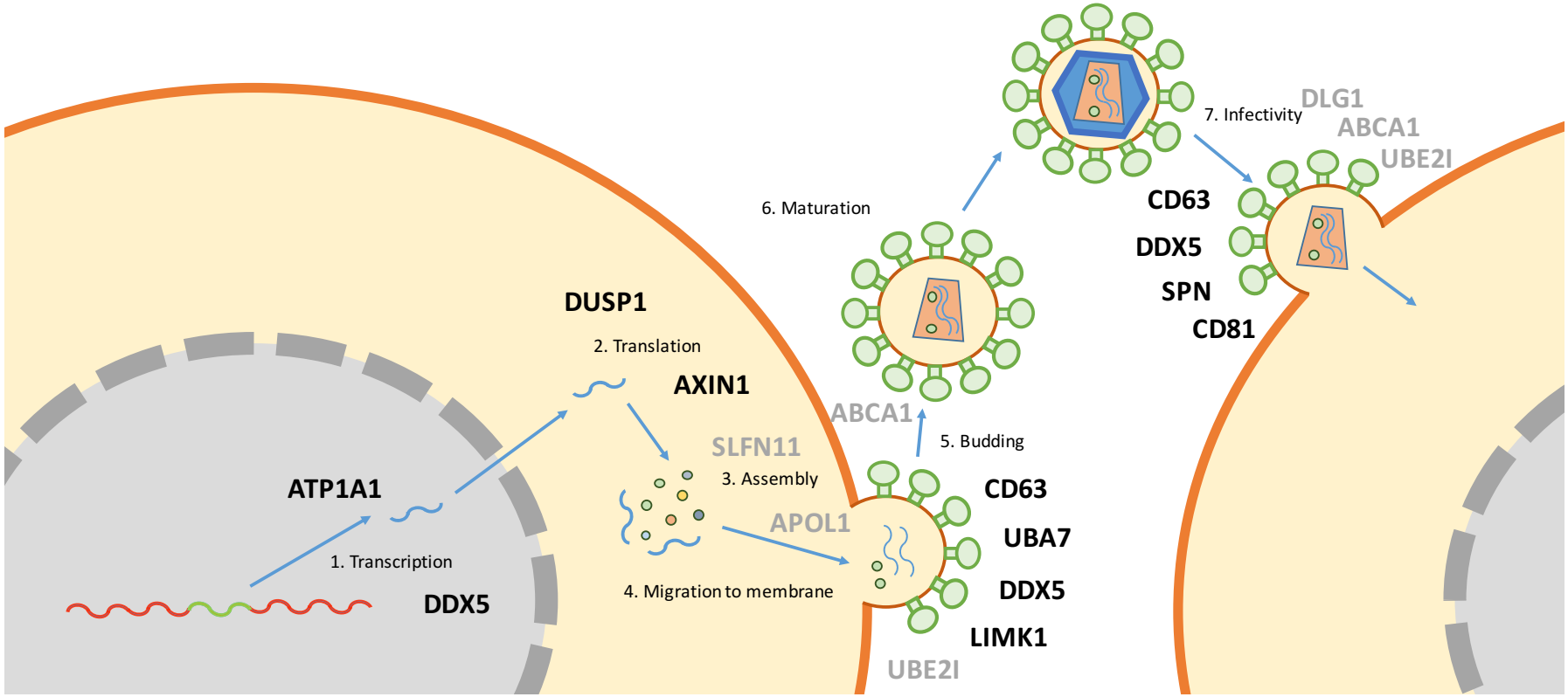

Figure 7.13 'Hits' and 'near-hits' identified as inhibitory factors in the siRNA screen and their inferred site of action in the late phase HIV-1 life cycle. The genes identified as 'hits' (black) or 'near-hits' (grey) in the siRNA screen are indicated on the diagram adjacent to the step in the late steps of the HIV-1 life cycle in which they are presumed to be active, as derived from the literature review in Chapter 5. Life cycle steps depicted: (1) transcription (2) translation, (3) assembly, (4) migration to the cell membrane, (5) budding, (6) maturation and (7) infectivity of the produced virions.

Chapter 8 – Discussion

The natural ability of lentiviruses to transduce non-dividing cells has been exploited in the development of vectors for gene therapy. An additional advantage inherent to lentiviruses, is the relatively straightforward ability to pseudotype the vectors with novel envelope proteins in order to facilitate targeted delivery to specific cell types (Section 1.3.1). Lentiviral vectors have been successfully used in *ex vivo* clinical trials, in the treatment of haematopoietic stem cell disorders and for CAR-modified T cell therapies (Section 1.4.1). However, they suffer from substantially lower titres compared with the more widely-used rAAV vectors and therefore have limited applicability for *in vivo* gene therapy. Although direct injection of lentiviruses into the eye for treatment of age-related macular degeneration (Reviewed in Pecen & Kaiser, 2015) and into the brain for treatment of Parkinson's (Palfi *et al.*, 2014) are feasible, treatment of larger organs would generally require much greater quantities of virus. This thesis aimed to investigate strategies to improve lentiviral vector productivity during manufacture, focusing mainly on the upstream process (Section1.5.1 and Figure 1.5), with a view to ultimately increasing the likelihood that lentiviral vectors could be adopted for widespread disease treatment.

To generate a successful vector for gene therapy, every aspect of the vector design needs to be carefully taken into consideration, not only the choice of vector and its tropism via serotype or pseudotype selection, but also the selection of the encoded transgene and its accessory and expression sequences. This allows the best vector to be selected for the disease application. Subsequently, and sometimes in parallel, attention is paid to the manufacture of the vector and the ability to deliver highly concentrated and purified vector for use in the clinical setting. In this thesis, different aspects of the interactions between viruses and their host cells have been considered,

and in particular to apply knowledge learned from the virology literature to vector production.

8.1 Effect of media supplementation on production of lentiviral vector in FreeStyle 293

Optimisation and supplementation of cell culture media for increased cell growth is frequently used to boost vector production yields. Studies in this thesis were initiated in the context of lentiviral vector production in serum-free, suspension cell culture, using the proprietary FreeStyle 293 (Section 2.2). Based on the hypothesis that enveloped vectors budding from cells might ultimately deplete the cell membrane of essential lipid and protein constituents, a number of supplements were assayed using a simple protocol for small-scale (20 mL) lentiviral vector production, using transient transfection of HEK 293T cells in suspension. While some supplement additions had a tendency to result in higher titres (Figure 3.2B & 3.4B), none allowed significant augmentation (Figures 3.2-3.4). In the case of Pluronic F-68 supplementation, its addition to cell growth media in order to reduce foaming of culture media is recommended in large scale bioreactors (Kilburn & Webb, 1968a). While supplementation did not result in increased vector titres (Figure 3.4), neither did it negatively affect titres in small-scale production and this was, therefore, implemented in the large-scale pre-clinical production of UKCFGTC vectors.

The observed lack of effect of the supplements tested here is possibly due to titres being too low overall for the anticipated depletion of cell membrane components. Supplementation might be beneficial, however, during the late-stage optimisation of vector production. In this stage of process development, producer cell lines will already be generating very high titres and cell membrane components may become rate-limiting. Similarly, the late-stage use of a producer cell line would avoid the need for transfection

of producer plasmids, and its accompanying toxicity, a potential confounding factor in interpreting some of the studies.

8.2 Effect of PKR inhibition on lentiviral vector production

A cell undergoes many changes in response to virus infection, therefore it is possible that understanding and manipulating intrinsic responses to virus entry could offer potential opportunities to increase vector production. A major pathway of intrinsic response to viruses, the PKR pathway (Figure 4.1), was investigated as such an opportunity. As observed in published studies which used proviral DNA, the overexpression of PKR was shown here to negatively impact titres in third-generation lentiviral vector production (Figure 4.2). The overexpression of cellular PKR inhibitors, which were shown in published work using proviral or second-generation lentiviral vector systems to increase titres (Section 4.1.2), was however not as successful here in the context of a third-generation vector (Figures 4.3-6.6). One possible reason is the abundance of transfected plasmids required (six here compared with two in published studies using proviruses) and also possible interactions between the different components of the transfection process, negatively impacting production.

Interestingly, results from the siRNA screen (Table 7.3 & Figure 7.11) showed a modest impact, if any, of knocking-down PKR on vector titres. In contrast, the knock-down of the genes that lead to the expression of ADAR1 or PACT had a strong adverse impact on lentiviral vector production in the inducible cell line-based siRNA screen (Table 7.3 & Figure 7.11). In addition to PKR inhibition, these proteins have been shown to have multiple effects on cellular pathways (Section 4.1.2) and could be acting here through one of these other pathways. The siRNA targeting PRKRA (the gene expressing PACT) was observed to have the most adverse impact in the screen (Figure 7.6 & Table 7.3),

an effect that mirrors the results obtained with overexpression, even though these were not significant. The siRNA screen also identified a number of candidate inhibitory factors, the knock-down of which actually greatly decreased lentiviral vector production (Table 7.3 & Figure 7.11). While these were incidental findings, they might yet be of interest for lentiviral vector production, as their permanent overexpression, through gene 'knock-in' using the CRISPR/Cas9 technology for example, or alteration of expression patterns, might still be of relevance in generating a novel cell line for lentiviral vector production. Additionally, these identified auxiliary/essential factors might be interesting targets for HIV-1 therapeutics research.

8.3 Effect of candidate HIV-1 inhibitory factors on lentiviral vector production

Due to the focus in HIV-1 research of preventing infections, genome-wide screens have tended to focus on essential and auxiliary factors (putative druggable targets) and early restriction factors (Table 6.2), leaving the late portion of the HIV-1 life cycle largely unexplored. In order to better assess the spectrum of virus-host cell interactions, a systematic review of the literature was performed to identify all the published inhibitory factors active in the late phase of the HIV-1 life cycle. The identification of such factors is relevant in the context of lentiviral vector production, which covers the stages in the life cycle from transcription to subsequent infectivity of the produced vectors (Figure 5.3). This search identified 152 candidate inhibitory factors (Table 5.1), representing a more realistic reflection of the complexity of the virus-cell interactions compared with the four canonical restriction factors that are often cited (APOBEC3G, BST2, SAMHD1 and TRIM5α). This, in turn, led to the development of a targeted screen to identify which of these candidate restriction factors might be important in lentiviral vector production (Chapter 6). This screen, when performed in quadruplicate, was powered at 80% to

detect a 1.4-fold increase in titre. Nine of the 92 candidate inhibitory factors experimentally evaluated in this study, when knocked-down using siRNAs, led to such an increase in titre and were therefore termed ‘hits’.

This high-throughput screen design could readily be applied to encompass the entire genome, an approach that would allow the identification of previously unknown restriction factors. Such a screen could also include additional targets missing from the ON-TARGETplus siRNA library. For example, GE Dharmacon also provides products targeting lncRNAs and miRNAs allowing assessment of the impact of non-protein coding RNAs on lentiviral vector production. Such additional reagents might require further optimisation before use, especially in the selection of appropriate controls. In fact, an essential modification before performing a genome-wide screen, based on the results of the screen performed here, would be to adjust the screen controls to include validated inhibitory factors selected from the ‘hits’ identified here, as well as repeated non-target controls to minimise plate-to-plate variation.

8.4 Further development of siRNA screen results

HEK 293 is a permanent cell line of primary human embryonal kidney transformed by exposing cells to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977). The HEK 293 cell line and its derivatives are the main cell lines used for lentiviral vector production (Section 1.5.1.1). The expression profile of some of the restriction factors relevant to the late phase of the HIV-1 life cycle (APOBEC3G, BST2, SERINC3 and SERINC5) (Section 5.2.1) suggests that they are expressed at very low levels (> 1.5 ppm) in HEK 293 cells (Table 5.1). HEK 293-derivative cell lines have been described as growing faster than the parental cell line indicating a selection through culture for rapid growth. In a study of differential genome expression between different cells of the HEK

293 lineage (Lin *et al.*, 2014), a pattern of upregulation and downregulation of genes involved in the cell cycle activation and in proliferation was observed in HEK 293-derivative cell lines compared to the parental cell line. HEK 293 were transformed with the SV40 large tumour antigen to generate the HEK 293T cell line (DuBridge et al., 1987) (Section 1.5.1.1). These have been shown to produce higher lentiviral vector titres than the parental HEK 293 when using transient transfection (Ausubel *et al.*, 2012).

The widely-used HEK 293T/17 clone is a high-performing clone isolated from the parental HEK 293T cell line and has a good track record of expressing genes following transient transfection of the relevant plasmids (Pear *et al.*, 1993). Nevertheless, except for the aforementioned modifications, the HEK 293T cell line has not been extensively optimised for lentiviral vector production. Based on the results presented in this thesis, the genetic modification of HEK 293T to completely remove one or more of the inhibitory factors identified in Chapter 7 could potentially enhance vector production titres. This would require the generation of knock-out cell line clones with one or more of the identified genes removed, using genome editing technologies.

The generation of producer cell lines edited using genome editing technologies such as CRISPR/Cas9 has already been suggested in order to reduce the immunogenicity of the produced vectors (Cantore et al., 2015). As presented in Figure 1.3, when viruses bud out of the producer cells, they take out a portion of the membranes and the associated proteins. This includes the highly polymorphic and antigenic MHC Class-I expressed on lentiviral vector packaging cells and subsequently incorporated on the vector's envelope. It has been suggested that it could be useful to knock-down the β-2 microglobulin gene, a component required for the assembly and trafficking of the MHC-I to the plasma membrane (Cantore *et al.*, 2015). The producer cells would therefore not have the MHC Class-I on their surface and would produce a less immunogenic viral product.

A similar approach, where specific genes are permanently removed from the producer cell, could aim at increasing titres rather than reducing the immunogenicity of the produced virions. Capitalising on the results of the current study, the different 'hits' could be knocked-out of producer cells with a view to improving lentiviral vector titres. Furthermore, it is possible that the knock-out of certain genes, such as members of the APOBEC family, while not shown to have an impact on titres in the current siRNA screen, might be relevant for safety purposes preventing possible editing mutation in the final product. When knock-out cell lines are generated to confirm 'hits', the impact of this removal on vectors with alternative pseudotypes or different lineage (EIAV, FIV, SIV or retroviral) could be assessed. The utility of multiple knock-outs could then easily be explored, starting with a single knock-out clone and then assessing secondary inhibitory factors using the siRNA screen developed in Chapter 6, to identify the best combination of knock-out clones.

Modifying the producer cells to improve lentiviral vector production might nonetheless only be half of the solution. Another aspect to focus on when considering virus-cell interaction is the vector restriction upon entry into the target cell. Restriction and inhibitory factors acting early in the HIV-1 life cycle, while they do not affect lentiviral vector production, might affect their capacity to deliver the transgene into host cells. Removing selected genes from the producer cell might increase infectivity and, therefore, impact on this part of the lentiviral vector life cycle, but the target cells themselves have a full set of inhibitory factors. While it is not desirable to modify the host cell to make it more susceptible to transduction by the lentiviral vector, the vector could be improved to resist the host's phenotype. A good example of this is a novel vector that introduces SIV_{mac}'s accessory protein Vpx to protect lentiviral vectors against the action of SAMHD1 from target cells in human dendritic cells (Tareen et al., 2014). As this

example demonstrates, while accessory proteins have been removed from lentiviral vectors in order to reduce their virulence (Section 1.3.1), this might have been detrimental to the infectivity of the vectors in patients and would benefit from being fully assessed in terms of safety and efficacy.

8.5 Relevance and conclusion

The exploration of the benefits of media supplementation and the identification and manipulation of virus restriction in producer cells may be relevant to other viral gene therapy vectors. A targeted screen for inhibitory factors, such as the one employed here, however, might be difficult to perform for viruses without the extensive literature readily available for HIV-1; thus, a simple genome-wide screen, without prior knowledge, might be more successful. For example, a genome-wide screen has identified several host restriction factors for AAV transduction (Mano *et al.*, 2015), but a literature search for restriction factors involved in vector production yielded only one result: APOBEC3A (Chen et al., 2006). For other lentiviruses, such as EIAV, FIV and SIV, HIV-1 inhibitory factors might also be active, however, some might reasonably be expected to behave differently. For example, APOBEC3B and APOBEC3C have been shown to restrict SIV while they had only a mild effect on HIV-1 (Yu *et al.*, 2004). Nevertheless, in the case of enveloped viruses, the tetraspanin protein family might be widely-active. They are potent inhibitors as they are involved in membrane budding not only of viruses but, also, in the case of CD63, of exosomes. As such, cell lines where CD63 has been knocked-out might be of interest in the study of exosomes. Additionally, these could lead to increased titres in the context of a novel type of gene therapy vector termed ‘vector-exosomes’. These are constituted of a non-enveloped vector (rAAV) packaged into an exosome (Maguire et al., 2012) and, therefore, bud from the cell membrane like lentiviral vectors.

In conclusion, the experiments performed in this thesis accomplished the early steps in identifying a way to improve upstream lentiviral vector production, which was the main aim of this thesis. Focussing on a less-studied aspect of lentiviral vector production, virus-cell interaction, allowed the identification of key factors that could influence production titres. This type of approach and the findings arising from this study are of fundamental importance to the advancement of gene therapy. The development of novel gene therapy vectors and their increasingly creative application to new disease targets continue to attract media attention and biotech investment. However, for the genuine application of gene therapy in the health care arena, vector production and large-scale manufacturing needs to be prioritised. This is particularly true for lentiviral vectors, where *ex vivo* gene therapy performed to treat leukaemia in cell therapy trials has been a huge success, but this is not (yet) an option for *in vivo* gene therapy applications in large organs. Thus, in order to remain competitive with other gene therapy systems, lentiviral vector production titres need to be increased significantly – even a doubling of virus titre would significantly reduce overall costs. Finally, careful consideration of the health economics of developing costly gene therapy products will be required to ensure gene therapy fulfils its potential and reducing the overall cost of vector manufacturing is a key strategy to its long-term success.

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