DIFFERENTIAL TIMING OF TRANSLOCATION OF HIV-1 SUBTYPE B AND C VPU TO THE ER/GOLGI AND PLASMA MEMBRANE COMPARTMENTS

Catherine Macdonald Bell

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of

Master of Science in Medicine

Johannesburg, 2009

DECLARATION

I, Catherine Macdonald Bell declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine, in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Catherine M. Bell

27 August 2009

Date

For

Lawrence Bell

1974-1980

PUBLICATIONS AND PRESENTATIONS

Some aspects of the work conducted for this dissertation have been presented elsewhere as a poster:

- Bell, C.M., Capovilla, A., Stevens, W., and Papathanasopoulos, M.A. (2007). **"Differential timing of translocation of HIV-1 subtype B and C Vpu to the ER/Golgi and plasma membrane compartments".** The 4th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention. Sydney, Australia. 22-25 July 2007.

Also:

- Bell, C.M., Capovilla, A., Stevens, W., and Papathanasopoulos, M.A. (2009). **"Differential timing of translocation of HIV-1 subtype B and C Vpu to the ER/Golgi and plasma membrane compartments".** Manuscript in preparation.

ABSTRACT

The HIV-1 Vpu protein functions largely to target CD4 molecules for proteasomal degradation, and to enhance virion release. The subcellular localisation of Vpu is related to these functions. Previous studies showed subtype B Vpu localisation at the ER/Golgi complex, while subtype C Vpu localised to the plasma membrane (PM) at 48 hours post-transfection. To determine if subtype C Vpu can localize to the ER/Golgi, we evaluated the cellular localisation of Vpu from two South African subtype C isolates as compared to subtype B Vpu, over time. Codon optimized *vpu* genes from subtype C isolates FV5 and FV15 (which have a six and two amino acid insert in the N-terminal domain, respectively) and a representative subtype B *vpu* were TA cloned into the pcDNA6.2/C-emGFP expression vector. The three Vpu-emGFP recombinant plasmids were cotransfected with pDsRed-ER, pDsRed-Golgi, or pDsRed-Mem into HEK 293T cells, and observed at 24, 48, and 60 hours post-transfection under a confocal microscope to confirm the presence of Vpu at different subcellular compartments. Cotransfection and microscopy conditions were methodically optimised. At 24 hours post-transfection, the subtype C FV5 Vpu had ER/Golgi localisation, but none at the PM. The subtype C FV15 Vpu had weaker ER/Golgi localisation and no PM localisation. In contrast, the subtype B Vpu had strong PM localisation. At 48 hours, FV5 and FV15 Vpu showed PM localisation, while subtype B Vpu was clearly localised at the ER/Golgi. At 60 hours, FV5 Vpu was observed at the PM, whereas FV15 and subtype B Vpu showed ER/Golgi localisation. These findings illustrate the efficient translocation of Vpu between different cellular compartments and for the first time, the difference in timing between subtype B and C Vpu, as well as íntrasubtype differences. This difference in shuttling suggests implications for the timing of viral assembly and release. Further investigations may clarify the impact of this timing on the difference in disease pathogenesis noted between infections with the different subtypes.

ACKNOWLEDGMENTS

To my supervisor, Dr Maria Papathanasopoulos, thank you for all your input, constructive criticism, and patience on this project.

To Ms Carol Crowther, Dr Clem Penny, and Ms Samantha Barichievy, thank you for the assistance with transfections and confocal microscopy. Also, thank you to Professor Patrick Arbuthnot and the Antiviral Gene Therapy Research Unit for use of their confocal microscope.

The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pcDNA-Vphu from Dr. Stephan Bour and Dr. Klaus Strebel.

This research was funded by the Department of Molecular Medicine and Haematology, the University of the Witwatersrand, South Africa, the Poliomyelitis Research Foundation (PRF), and the National Research Foundation (NRF).

To Gareth Jennings and Sue Aitken: I am so grateful for your meticulous proofreading of various drafts, and for making valuable suggestions.

To my dear family and friends, Mum, Dad, Alex, Josie and Bruce, Meryn and Billy, Sheena, Gill, Bridgette, Alexia, Emma, Sue, Michelle, and Gareth: Thank you for supporting me through thick and very thin.

To Shivi and Di, Sue, Annie Eckhart, and the Bongers family: Thank you for your big hearts and for keeping a roof over my head.

TABLE OF CONTENTS

Chapter 2: Materials and methods

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
AGM	African green monkey
AICD	Activation Induced Cell Death
AIDS	Acquired Immunodeficiency Syndrome
AP	Activator Protein
APC	Antigen presenting cell
ART	Antiretroviral therapy
ATF4	Activating Transcription Factor four
ßTrCP	β-Transducin repeats-containing protein
bp	Base pair
BST2	Bone marrow stromal antigen two
CAML	Calcium-modulating cyclophilin ligand
CAT	Chloramphenicol acetyltransferase
CCD	Closed circuit detection
CCR5	Chemokine C-C motif receptor five
CD	C-terminal domain
CD4	Cluster designation four
$CD4^+$ T-cell	CD4-positive T-cell (T-helper cell)
$CD8^+$ T-cells	CD8-positive T-cell (Cytotoxic T-cell)
CK II	Casein kinase II
CLIP	Class II-associated Ii peptide
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CRM1	Chromosome region maintenance protein one
CXCR4	Chemokine C-X-C motif receptor four
DNA	Deoxyribonucleic acid
ddNTP	Dideoxy-nucleoside triphosphate
delVpu	Vpu-deleted mutant
dNTP	deoxy-nucleoside triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
E3	Enzyme 3 ligase in the ubiquitin-dependent proteolysis pathway
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EIAV	Equine infectious anaemia virus
emGFP	Emerald green fluorescent protein

Env	Envelope protein or external glycoprotein of HIV-1
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ESCRT	Endosomal Sorting Complex Required for Transport
EtBr	Ethidium bromide
FCS	Fetal calf serum
FITC	Fluorescein iso-thiocyanate
Gag	Capsid protein of HIV-1 including membrane anchoring (MA), core capsid (CA), and nucleocapsid (NC) proteins
GAP-43	Growth associated protein 43
GC	Guanosine-cytosine
GPI	Glycosyl phosphatidylinositol
Group M	Major group of HIV-1
Group N	Non-M or -O group of HIV-1
Group O	Outlier group of HIV-1
HEK 293 T	Human embryonic kidney 293 T cells
HeLa	Cervical cancer cell line originating from patient, Henrietta Lacks
HIV-1	Human Immunodeficiency Virus type 1
HRP	Horseradish Peroxidase
IE	Immediate-early
IFNα	Interferon-α
IFNγ	Interferon-gamma
IκBα	Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
KSHV	Kaposi's sarcoma-associated herpes virus
IL-6	Interleukin-6
LB	Luria Bertani broth
LTNP	Long-term non-progressor
LTR	Long terminal repeat
MHC	Major Histocompatibility Complex
MIF	Macrophage inhibitory factor
MLV	Murine leukaemia virus
mRNA	Messenger RNA
MVB	Multivesicular body
MWM	Molecular weight marker
Nef	Regulatory factor for HI-1 expression
NFκB	Nuclear factor kappa B
NK	Natural killer
NTD	N-terminal domain
PBS	Phosphate buffered Saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin

PM	Plasma membrane
PMT	Photo-multiplier tube
Pol	Polymerase enzyme of HIV-1 including Protease (PR), Reverse transcriptase (RT), RNase H, and Integrase (IN)
POP	Performance optimised polymer
RNA	Ribonucleic acid
RER	Rough endoplasmic reticulum
Rev	RNA transport, stability, and utilisation factor of HIV-1
SARS	Severe Acute Respiratory Syndrome
SCF	Skp I-Cullin-F-box
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHIV	Simian-Human Immunodeficiency chimeric virus
siRNA	Short interfering RNA
SIV	Simian immunodeficiency virus
SIV_{cpz}	Simian Immunodeficiency Virus from chimpanzees
SIV_{gsn}	Simian Immunodeficiency Virus from greater spot-nosed monkeys
SOC	Super optimal broth medium
TA	Thymidine-adenosine
TASK-1	TWIK-related acid-sensitive potassium channel 1
Tat	Viral transcriptional transactivator of HIV-1
TCR	T-cell antigen receptor
TGN	Trans-Golgi network
TM	Transmembrane
TMD	Transmembrane domain
TNFα	Tumour necrosis factor alpha
TPR	Tetratricopeptide repeat
TRAF1	TNF receptor-associated factor 1
TRITC	Tetramethyl rhodamine iso-thiocyanate
UBP	Vpu binding protein
Vif	Viral infectivity factor of HIV-1
Vpr	Viral protein R of HIV-1
Vpu	Viral protein U of HIV-1

Chapter 1

Introduction

1.1 The Human Immunodeficiency Virus and the global AIDS epidemic

1.1.1 The global AIDS epidemic

The Human Immunodeficiency Virus type 1 (HIV-1) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) (Barre-Sinoussi, Chermann et al. 1983; Blattner, Gallo et al. 1988), and is responsible for the pandemic affecting between 30 and 36 million people worldwide (www.unaids.org 2008). Sub-Saharan Africa is the most seriously affected by the HIV pandemic, accounting for 67% of all infections and 72% of AIDS deaths in 2007 (www.unaids.org 2008). This number continues to rise although the prevalence of HIV in sub-Saharan Africa, currently ranging between 10% and 12%, has stabilised due to increased access to antiretroviral therapy (ART) (www.unaids.org 2008). Outside Africa, infections are on the rise in a number of countries, with global adult HIV prevalence ranging between 3% and 4% (www.unaids.org 2008). Despite the benefits of ART and improvements in access to therapy, there are approximately three million AIDS-related deaths per year worldwide, including an estimated 490 000 children under the age of fifteen (www.unaids.org 2008).

1.1.2 Classifications of HIV

HIV-1 and the closely related simian immunodeficiency virus (SIV) are classed as retroviruses, and belong to the subgroup known as lentiviruses. Retroviruses are viruses that contain ribonucleic acid (RNA) genomes, able to reverse transcribe their genome into a deoxyribonucleic acid (DNA) copy (cDNA), and thus replicate within the host cell nucleus. Primate lentiviruses are grouped into five distinct lineages, including HIV-1, HIV-2, and SIV_{CPZ} (Osmanov, Heyward et al. 1995). HIV-1 is divided into group M (major), N (non-group M or O) and O (outliers). Group M accounts for over 90% of all HIV-1 infections worldwide, and phylogenetic analysis of the envelope (e*nv*) and core (*gag*) genes has identified at least eight genetic subtypes (A, B, C, D, F, G, H, J, K) (http://www.hiv.lanl.gov/ 2009). Numerous circulating recombinant forms (CRFs) also exist within group M and continue to be identified, with a total of 43 CRFs described to date (http://www.hiv.lanl.gov/ 2009). HIV-1 is considerably more virulent than HIV-2, while HIV-1 group M subtype C accounts for over 45% of new infections worldwide (Oliveira and Cassol 2009). The reasons for the pervasiveness of this subtype are still unknown, but some differences do exist between this subtype and others within this group. Notably, syncytia- (CXCR4-tropic) and non-syncytia-inducing (CCR5-tropic) subtype C isolates are less fit in peripheral blood mononuclear cell (PBMC) competition assays than the nine subtype B isolates tested (Ball, Abraha et al. 2003). The replicative fitness of subtype C strains is also reduced in PBMCs when compared to other group M isolates, but is still 100 fold

higher than HIV-2 or group O isolates (Arien, Abraha et al. 2005; Salim and Ratner 2008).

1.1.3 Pathogenesis of HIV-1

HIV-1 infection is characterised by a gradual deterioration in immune function and ultimately AIDS (Levy 1993). Pathogenesis studies of HIV-1 explore the diverse mechanisms that lead to this immune system destruction. Understanding how the virus establishes infection is essential to the identification and development of effective therapeutics and vaccines. HIV infects cells expressing the surface glycoprotein, cluster designation four (CD4) (Lyerly, Matthews et al. 1987). CD4-positive ($CD4^+$) cells include T-helper lymphocytes and macrophages. Viral tropism is determined by the binding of the viral envelope protein (Env, gp120) to its primary receptor, CD4, in conjunction with a chemokine co-receptor, CCR5 or CXCR4 (Dejucq 2000). Cytotoxic T-cells ($CD8^+$ T-cells) and B-cell-produced antibodies act to moderate viral levels in acute infection, but the virus persists in the form of escape mutants and viral reservoirs (Greene 1993). Infection of follicular dendritic cells, and therefore accumulation of virus within lymphoid germinal centers, causes tumour necrosis factor alpha (TNFα) and interleukin-six (IL-6) secretions (Schnittman, Denning et al. 1990). This in turn initiates T-cell activation allowing further HIV dissemination (Ho, Neumann et al. 1995). The lymph node architecture then breaks down preventing normal immune system function. Infection eventually leads to

depletion of $CD4^+$ T-cells by poorly understood mechanisms (Connor, Mohri et al. 1993). Speculation around $CD4^+$ T-cell loss includes: direct killing by viral budding (Garry 1989), apoptosis of infected and uninfected cells (Ameisen and Capron 1991; Laurent-Crawford, Krust et al. 1991; Finkel, Tudor-Williams et al. 1995; Pantaleo and Fauci 1995), antibody-dependent cellular cytotoxicity (ADCC) (Ahmad, Yao et al. 1994), anergy (Sousa, Carneiro et al. 2002), and damage to immune system precursor cells (Schnittman, Denning et al. 1990). In due course, severe immunosuppression arises, distinguished by idiopathic $CD4^+$ lymphocytopenia (a CD4 count of less than 300 cells per mm^3 blood) (Aledort, Operskalski et al. 1993; Busch, Valinsky et al. 1994). The result is vulnerability to opportunistic infections, with AIDS patients typically manifesting infections of the lungs, brain, eyes and intestinal tract (www3.niaids.nih.gov 2008).

1.1.4 The structure and life cycle of HIV-1

The replication cycle of HIV involves reverse transcription of the viral RNA, integration of the viral cDNA into the host genome, transcription of viral messenger RNA (mRNA), and translation of mRNA into viral proteins (Frankel and Young 1998). These proteins assemble into progeny virions, before budding from the host cell membrane. The final maturation step, involving protease cleavage of specific sites within the complete Gag protein, leads to the production of fully infectious virions

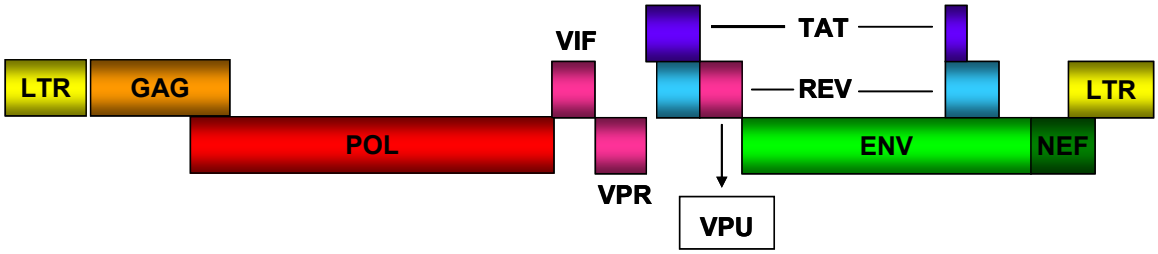


Figure 1.1: Schematic of the HIV-1 genome. The approximately 9.7 kb genome is comprised of three structural genes (*gag, pol, env*), two regulatory genes (*tat, rev*), and four accessory genes (*vif, vpr, vpu, nef*). The nine genes are flanked by a 5' and 3' LTR regions. The focus of this study, Vpu, is outlined. This image was adapted from the HIV Sequence Compendium 2003, Landmarks of the HIV-1 genome, page iv (Leitner, Foley et al. 2003).

capable of initiating further rounds of infection (Frankel and Young 1998; Schubert, Ott et al. 2000). Viral genes act in concert with host cellular machinery to control these processes. HIV-1 is a particularly efficient virus, able to perform a multitude of functions with a genome comprising of just nine genes (Figure 1.1), encoding at least sixteen proteins (http://www.hiv.lanl.gov/ 2009). Three of the nine genes encode structural proteins (*gag, pol, env*), two have regulatory functions (*tat, rev*), and four are dubbed accessory genes (*vif, vpr, vpu, nef*) (http://www.hiv.lanl.gov/ 2009). The accessory genes of HIV are unique in that most other retroviruses only encode the *env, gag,* and *pol* genes (Hout, Mulcahy et al. 2004). The accessory proteins were initially thought to be dispensable for viral replication, but have now been shown to function as crucial enhancers of viral pathogenesis. They act as versatile adaptor molecules that connect viral and cellular pathways, and lead to efficient viral replication, assembly, and release. Therefore, although the accessory proteins are

dispensable for replication *in vitro*, they allow the virus to establish and persist within the host. The viral protein U (Vpu) is the smallest of the accessory proteins, and performs a central role in viral budding, thus enhancing viral pathogenesis and infectivity.

1.2 The Viral Protein U

The Viral Protein U is an oligomeric, type I integral membrane protein of approximately 16 kDa (Cohen, Terwilliger et al. 1988). It ranges from 81 to 86 amino acids in length depending on the viral subtype (http://www.hiv.lanl.gov/ 2009). It is expressed late in the viral life cycle from bicistronic mRNA that also encodes the HIV-1 envelope glycoprotein, ensuring synchronous expression (Schwartz, Felber et al. 1990; Bour and Strebel 2003) (Figure 1.2). It is found only in the infected cell and is not incorporated into virions (Strebel, Klimkait et al. 1988). Interestingly, this protein is a distinguishing characteristic, unique to HIV-1 and the simian strains, SIV_{cpz} and SIV_{gsn} (Huet, Cheynier et al. 1990; Courgnaud, Salemi et al. 2002). Vpu is generally well conserved in isolates from AIDS patients (Lee, Schwartz et al. 1997), with premature stop codons noted in long term non-progressors (LTNPs) (Yamada and Iwamoto 2000).

Vpu is a multifunctional protein, whose effect on efficient viral replication and CD4+ T-cell depletion in HIV-1 infected individuals is now irrefutable. Two major and separate

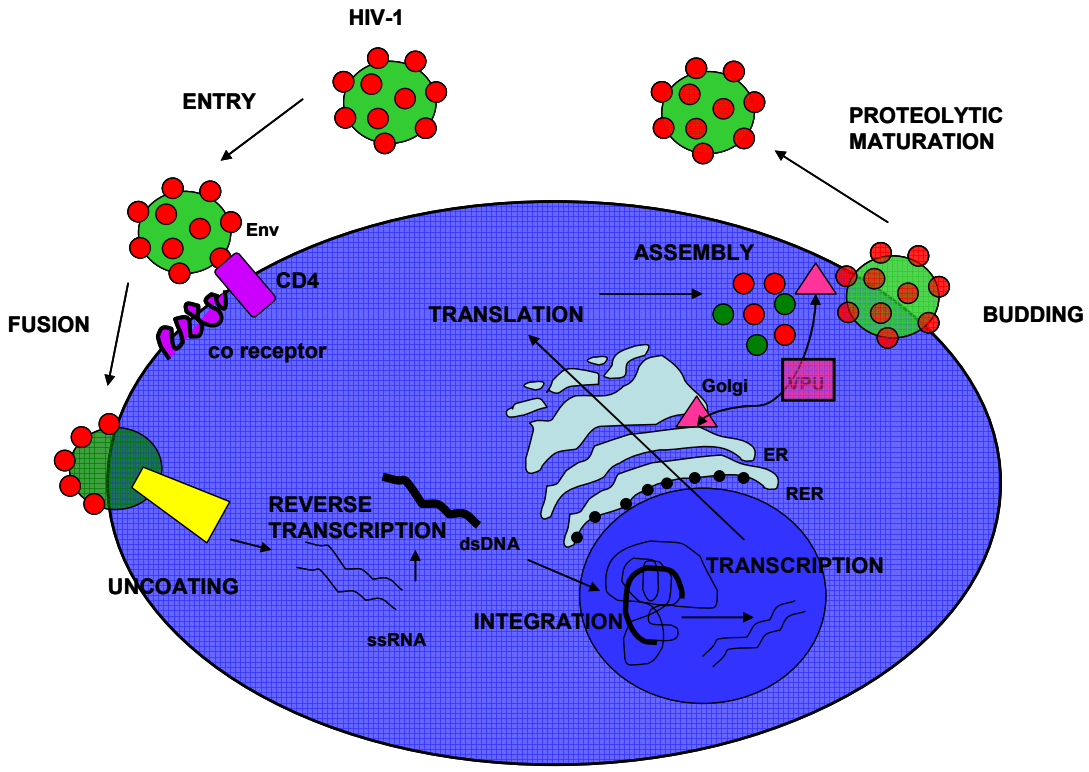


Figure 1.2: Overview of the HIV-1 replication cycle highlighting the expression of Vpu. Entry involves binding of viral envelope spikes (gp120 in red) to the CD4 receptor on the surface of host cells. Conformational changes in gp120 allow for engagement of the coreceptor, CCR5 or CXCR4, and fusion via envelope gp41 and internalisation of the viral core. Uncoating of the core releases the two single-stranded viral RNAs (ssRNA) for reverse transcription into double-stranded DNA (dsDNA). Proviral DNA is transported into the cell nucleus and integrated into the host chromosomal DNA. Full-length and spliced viral mRNA, as well as progeny virion RNA, are transcribed by host polymerases. Viral proteins are translated on the ribosomes of the rough endoplasmic reticulum (RER), and processed via the endoplasmic reticulum (ER) and the Golgi apparatus. New viral proteins and genomic RNA are assembled into immature virions. The new virus particles bud from the cell via the plasma membrane, and become infectious by proteolytic cleavage of the entire Gag protein by viral Protease. Vpu acts specifically at the ER/Golgi interface to bind newly synthesised CD4 molecules, and at the cell plasma membrane to down-regulate the host antiviral proteins, tetherin and Calcium-modulating cyclophilin ligand **(CAML), and to form cation-specific ion channels. This illustration was adapted from (Greene, Debyser et al. 2008).**

biological functions have been identified and unambiguously demonstrated. These functions are linked to the protein's two major structural domains: the transmembrane domain (TMD) functions in the enhancement of virion release, and the cytoplasmic domain (CD) functions in the degradation of newly synthesized CD4 molecules at the endoplasmic reticulum (ER) (Strebel, Klimkait et al. 1988; Maldarelli, Chen et al. 1993). Lesser-defined roles for Vpu include ion channel activity, the downregulation of major histocompatibility complexes (MHC) type I and II, and the induction of apoptosis (Hout, Mulcahy et al. 2004). The long held conviction that Vpu overcomes a host restriction factor to virion release has recently been confirmed in the guise of the newly characterised protein, tetherin (Neil, Zang et al. 2008). Accordingly, Vpu contributes to viral pathogenesis by overcoming this newly identified antagonist to virus release.

1.2.1 Vpu structural characteristics

The Vpu protein is organised into three domains: a short N-terminal domain (NTD), a hydrophobic TMD, and a relatively hydrophilic CD. Interestingly, the NTD of subtype C Vpu contains a one to five amino acid insertion accounting for the variation in subtype C-protein length. This insertion is absent in all other subtypes and non-subtype C CRFs (http://www.hiv.lanl.gov/ 2009). The functional relevance of this has not yet been investigated. The TMD anchors the protein within host cell membranes (Cohen, Terwilliger et al. 1988; Strebel, Klimkait et al. 1988), and it has been well

demonstrated to act as a monomer of a pentameric ion channel favouring monovalent cations (Lemaitre, Willbold et al. 2006). Pore radius profiles show that a homomeric pentamer bundle has the lowest potential energy profile, and molecular modelling studies confirm that five Vpu transmembrane (TM) spanning α-helices oligomerize to act as an ion-selective channel (Cordes, Kukol et al. 2001; Lemaitre, Ali et al. 2004). The TMD is connected to the CD via a highly conserved salt bridge region, 36-**E**Y**RK**IL-41 (Figure 1.3). Molecular dynamics simulations show that the glutamate (E) is involved in salt bridge formation with the lysine (K) and arginine (R), forming a flexible kink between the TMD and CD (Sramala, Lemaitre et al. 2003). The CD is therefore free to bind proteins within the cytoplasm. This region contains two amphipathic alpha helices (α-helix 1 and 2) flanking an especially conserved motif with two crucial serines (S), 58-ED**S**GNE**S**EG-66 (Figure 1.3) (Willbold, Hoffmann et al. 1997). These serines are subject to phosphorylation by casein kinase II (CKII) allowing for binding of Vpu to the human host factor, β-transducin repeats-containing protein (ßTrCP), an essential step in CD4 proteasomal degradation (Margottin, Bour et al. 1998). Alpha-helix two, as well as the C-terminal residues physically interact with CD4 (Margottin, Benichou et al. 1996). The concurrent binding of Vpu to CD4 and ßTrCP results in the degradation of CD4 via the ubiquitin-dependent proteolytic apparatus (Terwilliger, Cohen et al. 1989; Willey, Maldarelli et al. 1992; Margottin, Benichou et al. 1996).

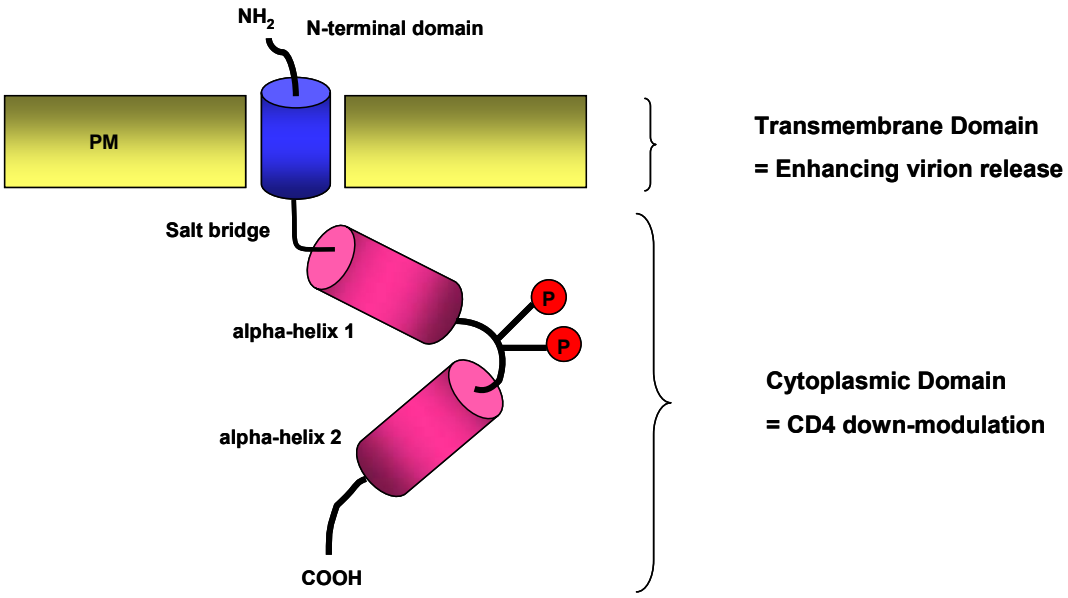


Figure 1.3: Schematic diagram showing the major structural domains of Vpu and their orientation in the cellular membrane. The hydrophobic transmembrane domain inserts into cellular membranes, while the hydrophilic cytoplasmic domain is free to bind proteins within the cell. The transmembrane domain enhances virion release by oligomerising into ion-channels, and by binding to tetherin. The crucial serines positioned between the cytoplasmic α-helices, are phosphorylated (in red) by casein kinase II, and allow for the binding of ßTrCP and CD4. This leads to the ubiquitination and degradation of CD4 via the E3-ligase complex. This illustration was adapted from (Bour and Strebel 2003).

1.2.2 Vpu functional characteristics

Vpu interacts with a diverse range of host proteins to perform its function (summarized in Table 1.1). The structural domains of Vpu are linked to its role in the viral life cycle. Studies on the Vpu protein from laboratory-adapted subtype B virus have shown that the TMD functions in enhancing virion release, and the CD is involved in CD4 degradation (Terwilliger, Cohen et al. 1989; Klimkait, Strebel et al. 1990; Willey, Maldarelli et al. 1992; Montal 2003). The trafficking and subcellular localization of Vpu is related to these functions. Its presence at the rough endoplasmic reticulum (RER)/trans-Golgi network (TGN) is linked to its ability to down-modulate CD4, while its presence at the plasma membrane (PM) is correlated to its ability to enhance virion release. A Vpu-enhanced green fluorescent protein (EGFP) construct showed subcellular localization of subtype B Vpu predominantly to the ER/Golgi complex, while subtype C Vpu was more efficiently transported to the plasma membrane (PM) at 48 hours post transfection (Pacyniak, Gomez et al. 2005). Further experiments with chimeric subtype B/C Vpu proteins where the CD regions were exchanged, determined that the difference in localisation was a factor of the second α-helical region of the CD (Pacyniak, Gomez et al. 2005). Subtype B Vpu-retention at the Golgi was accordingly narrowed down to carboxyl 13-23 amino acids (Pacyniak, Gomez et al. 2005).

Golgi proteins have tyrosine-, dileucine-, or phenylalanine-based retention or retrieval signals. Subtype C Vpu has two of these dileucine-based motifs (RK**LL** and LR**LL**), as well as another potential CKII site (TMVD). Notably, LRLL and TMVD both reside in the carboxy-terminal of the protein. The overlapping tyrosine-based (YXXф) and dileucine-based ([D/E]XXXL[L/I]) motifs in the salt bridge region of subtype C Vpu were found to be involved in the trafficking of Vpu (Ruiz, Hill et al. 2008). It was concluded that this membrane proximal, cytoplasmic, tyrosine-based motif is essential for efficient virus release, and that the dileucine motif is a sorting signal affecting the intracellular trafficking, cytopathology and the enhanced release function of subtype C Vpu (Ruiz, Hill et al. 2008). Interestingly, both subtype B and C Vpu were functional for CD4 down-modulation in these experiments (Pacyniak, Gomez et al. 2005). The ability of subtype C Vpu to down-modulate CD4 in its apparent absence in the ER/Golgi complex poses numerous questions that require further investigation.

1.3 CD4 downregulation

CD4 is a type I transmembrane glycoprotein that functions, along with the T-cell antigen receptor (TCR), to recognise antigens presented by the MHC II molecules on antigen presenting cells (APCs) (Bowers, Pitcher et al. 1997). The adaptive immune response requires $CD4^+$ T-cells to initiate B-cell and $CD8^+$ T-cell differentiation (Bowers, Pitcher et al. 1997).

Table 1.1: Overview of all HIV-1 Vpu and human protein interactions cited in the HIV-1, Human protein Interaction database* (www.ncbi.nm.nih.gov; last updated in November 2007) (Ptak, Fu et al. 2008; Fu, Sanders-Beer et al. 2009; Pinney, Dickerson et al. 2009), including the newly described interactions with tetherin (Neil, Sandrin et al. 2007) and CAML (Varthakavi, Heimann-Nichols et al. 2008).

Interaction	Protein
Activates	Caspase 3 preproprotein ßTrCP isoform 2
Antagonises	Tetherin/CD137/BST-2/HM1.24 CAML
Degrades	CD4 antigen precursor
Downregulates	BCL2-like isoforms 1 BCL2-related protein A1 CD4 antigen precursor MHC 1, A precursor MHC 1, B MHC 1, C precursor TNF receptor-associated factor 1
Inhibited by	CD4 antigen precursor
Inhibits	ßTrCP isoform 2 NFKß, subunit 1
Interacts with	FBXW11: F-box and WD-40 domain protein 11; ßTrCP 2 Potassium channel, subfamily K, member 3 Small glutamine-rich tetratricopeptide TNF-receptor superfamily, member 6, isoform 1 precursor
Phosphorylated by	CK 2, α-prime polypeptide CK 2, ß-polypeptide CK 2, α-1 subunit isoform A
Recruits	ßTrCP isoform 2
Regulates	CD4 antigen precursor
Upregulates	CD40 isoform 1 precursor Vascular cell adhesion molecule 1 isoform a preursor
Stabilizes	ATF4 Catenin (cadherin-associated protein), beta 1, 88 kDa NFκ light polypeptide gene enhancer in B-cells inhibitor, alpha

*For individual references, refer to the website, or within the text.

This process depends on the recognition by $CD4^+$ T-cells of antigens presented on the surface of APCs by MHC II α and ß chains (Bowers, Pitcher et al. 1997). CD4 binds to the Lck non-receptor tyrosine kinase via its cytoplasmic tail, before trafficking together along the biosynthetic pathway to the plasma membrane (Bijlmakers and Marsh 1999). These proteins remain a stable complex by interaction with an APC, allowing contact with the TCR and consequently T-cell activation (Bowers, Pitcher et al. 1997). CD4 is also found in small amounts on all endomembranous systems. These include the endocytic, lysosomal, and secretory pathways (Bour, Schubert et al. 1995)

This ubiquitous nature of CD4 poses several problems for HIV-1 replication. CD4 binds the Env precursor, gp160 at the TGN, effectively preventing protease processing of the viral protein, and its trafficking to the PM (Willey, Maldarelli et al. 1992). There is also a degree of CD4-retention within the budding virion, impeding normal virion function (Cortes, Wong-Staal et al. 1998). The presence of CD4 on the cell surface also contributes to superinfection, a state that results in an increase in cytopathic effects on host cells. Protection against superinfection is speculated to decrease apoptosis caused by accumulated, un-integrated viral DNA (Daniel, Katz et al. 1999), and to slow the rate of viral recombination (Lama 2003). CD4 down-modulation thus enhances viral release and infectivity (Cortes, Wong-Staal et al. 1998; Bour, Perrin et al. 1999). Since CD4 interacts with MHC II on APCs, and a vital

co-stimulatory factor of TCR-mediated T-cell activation, down-modulation may also reduce the host's antiviral response (Wildum, Schindler et al. 2006).

The virus has therefore developed multiple and redundant mechanisms to down-modulate CD4 and in so doing enhance pathogenesis. HIV-1 CD4 downregulation is carried out through the actions of Nef, Vpu, and to a lesser extent, Env (Wildum, Schindler et al. 2006). Nef is expressed early in the viral lifecycle, and downregulates CD4 at the cell surface with subsequent lysosomal degradation (Aiken, Konner et al. 1994; Craig, Pandori et al. 1998). In contrast, both Env and Vpu are expressed later, and are involved in preventing the transport of *de novo* synthesized CD4 molecules to the cell surface, as well as the targeting of CD4 molecules for ubiquitin-dependent proteasomal degradation at the RER (Willey, Maldarelli et al. 1992; Geleziunas, Bour et al. 1994; Montal 2003).

Vpu specifically functions early in the secretory pathway by binding to the cytoplasmic tail of CD4 via its CD α-helices (Bour, Schubert et al. 1995; Tiganos, Yao et al. 1997). The host cell factor, ßTrCP is thus recruited to the ER membrane (Margottin, Bour et al. 1998). This action depends on the phosphorylation of the two crucial serines in the Vpu CD by CK II (Schubert, Henklein et al. 1994), and its subsequent binding to the WD-repeat domain of ßTrCP (Tiganos, Yao et al. 1997). ßTrCP then mediates the docking of the Skp I-Cullin-F-box (SCF) complex and transferal of ubiquitin to the cytoplasmic tail of CD4 (Margottin, Bour et al. 1998). This action adapts CD4 to the

E3 ubiquitin ligase complex, resulting in the proteasomal degradation of CD4 and the subsequent release of gp160 (Bai, Sen et al. 1996; Margottin, Bour et al. 1998) (Figure 1.4). Thus, gp160 is released for processing into gp120 and gp41, proteins necessary in large amounts for virion assembly. Remarkably, Vpu itself, unlike other ßTrCP-interacting proteins, is not targeted for degradation by proteasomes (Lindwasser, Chaudhuri et al. 2007). This process resembles the ER-associated degradation (ERAD) pathway, which recognises and clears misfolded proteins and unassembled protein subunits from the ER (Meusser and Sommer 2004).

1.4 Enhancement of virion release

Vpu is involved in the detachment of newly assembled HIV-1 virions from the cell surface. Several mechanisms of Vpu-mediated release are apparent (Figure 1.4). First, Vpu is able to direct cation-specific channel activity via its TMD, scrambling of which abolishes its ability to increase membrane conductance and virion release (Schubert, Ferrer-Montiel et al. 1996). Secondly, Vpu is also able to interact with a number of host factors that act normally to inhibit virus budding. Initially these proteins were identified as the TWIK-related acid-sensitive potassium channel 1 (TASK-1) (Hsu, Seharaseyon et al. 2004), and the Vpu binding protein (UBP) (Handley, Paddock et al. 2001). More recently however, tetherin (also known as bone marrow stromal cell antigen 2 or BST-2, HM1.24 and CD317) (Neil, Zang et al. 2008) and

calcium-modulating cyclophilin ligand (CAML) (Varthakavi, Heimann-Nichols et al. 2008) have been identified (detailed in section 1.5).

The Vpu TM domain directs weak ion-selective channel formation, possibly modifying the micro-environment to enhance virion release from infected cells (Lakos, Somogyi et al. 1990). Protein oligomerization results in hydrophilic pores at the membranes of virus-infected cells (Gonzalez and Carrasco 1998). Hence, virion release may be influenced by changes in host cell membrane protein conformation, membrane fluidity, and ion homeostasis (Gonzalez and Carrasco 1998; Gonzalez and Carrasco 2001). Viral ion channels, or viroporins, are characteristic of enveloped viruses, and are formed by the proteins of influenza A (M2 protein), influenza B (NB protein), influenza C (CM2 protein), poliovirus (2B protein), Sindbis virus (6K protein) and more recently, the Paramecium bursaria chlorella virus and the hepatitis C virus (p7 protein) (Fischer and Sansom 2002; Premkumar, Wilson et al. 2004). The E protein from the Severe Acute Respiratory Syndrome (SARS) coronavirus is also able to fashion cation-selective ion channels in lipid bilayers (Wilson, Gage et al. 2006).

Viroporins are all associated with membrane permeability, influencing viral entry, glycoprotein trafficking, replication, and budding (Ciampor 2003; Gonzalez and Carrasco 2003). Vpu specifically interacts with TASK-1 ion channel via the first 40 amino acids of TASK-1 that are also homologous to Vpu (Hsu, Seharaseyon et al. 2004; Strebel 2004). Vpu is able to abolish TASK-1 current, while overexpressing

TASK impairs Vpu-enhanced viral particle release (Hsu, Seharaseyon et al. 2004). This suggests Vpu may have evolved from this mammalian protein, and has hijacked its function. Conspicuously, viroporins form a target for amiloride analogues, traditionally used as antivirals and to inhibit calcium channel blockers (Garcia, King et al. 1990). The Vpu TM domain is targeted by the amiloride derivatives, 5-(N,N-hexamethylene)amiloride and 5-(N,N-dimethyl)amiloride, but not by amiloride or amantadine itself (Ewart, Mills et al. 2002). This ability of a variety of enveloped viruses to form ion channels suggests its importance in the viral life cycle, and advocates the effectiveness of amiloride derivatives as potential anti-HIV agents (Premkumar, Wilson et al. 2004).

Vpu stimulates the release of an array of retrovirus particles from cells. This list includes cells infected by murine leukaemia virus (MLV), equine infectious anaemia virus (EIAV), Visna virus, as well as virus-like particles based on the structure of the Ebola virus (Neil, Sandrin et al. 2007). The integrity of the Vpu TMD is essential for progeny virus release (Tiganos, Friborg et al. 1998), with budding structures accumulating and unable to detach from the cell surface in the absence of Vpu (Klimkait, Strebel et al. 1990). This accumulation of virions, observed by electron microscopy, is the result of endocytic uptake of nascent particles from the cell surface in $CD63^+$ endosomes (Neil, Eastman et al. 2006; Neil, Sandrin et al. 2007). In the event that internalisation is blocked, virions form characteristic tethers at the cell

surface, and these chains can be released by protease treatment (Neil, Eastman et al. 2006).

This mechanism of Vpu-enhanced virus release is distinct from Endosomal Sorting Complex Required for Transport (ESCRT)-Gag interaction that allows for interaction with proteins involved in sorting via multivesicular bodies (MVBs) (Schwartz, Geraghty et al. 1996; Neil, Eastman et al. 2006). Alternatively, Vpu is involved in interactions with the pericentriolar recycling endosome (Vasundhara 2006). Vpu inhibits the internalisation of Env and Gag into late endosomes, leading to viral assembly at the PM (Van Damme and Guatelli 2008). Vpu colocalises with markers for the pericentriolar recycling endosome, and must exit this compartment to exert its effect (Varthakavi, Smith et al. 2006). Disruption of protein sorting by the recycling endosome, using Rab11a and myosin Vb mutants, inhibits Vpu particle release (Varthakavi, Smith et al. 2006). Vpu therefore facilitates the amassing of Gag at the PM, allowing virion assembly (Neil, Eastman et al. 2006).

Vpu-mediated virus detachment is also cell-type-dependent and species specific (Sakai, Tokunaga et al. 1995; Jouvenet, Neil et al. 2009; McNatt, Zang et al. 2009). It is required for efficient virion release in non-permissive cells, such as HeLa cells (Strebel, Klimkait et al. 1989; Terwilliger, Cohen et al. 1989; Klimkait, Strebel et al. 1990; Gottlinger, Dorfman et al. 1993). Permissive cells, however, do not need Vpu for effective virion release, and include the African green monkey (AGM) cell line,

COS-7 (Geraghty, Talbot et al. 1994; Neil, Eastman et al. 2006). Remarkably, tetherin proteins from old world monkey species (AGMs and rhesus macaques), and from mice, inhibit HIV-1 release, but are resistant to Vpu (McNatt, Zang et al. 2009). Thus, viral antagonists may have driven the selection of the tetherin variants that exist in chimpanzees and humans (McNatt, Zang et al. 2009).

Vpu may also function to enhance virus release by causing the dissociation of Gag-UBP complexes and the redistribution of Gag to the cell periphery (Callahan, Handley et al. 1998; Handley, Paddock et al. 2001). UBP is a member of the tetratricopeptide repeat (TPR) protein family (Chen, McPartlin et al. 1994). These proteins play diverse roles including organelle targeting, mitosis, immunophilin-, and phosphatase-protein activity (Hase, Riezman et al. 1983; Sikorski, Boguski et al. 1990; Ratajczak, Carrello et al. 1993; Chen, McPartlin et al. 1994). Importantly, UBP also interacts with HIV-1 Gag, and its overexpression results in a reduction in virus release (Handley, Paddock et al. 2001). This state is rescued by the co-expression of Vpu, which causes Gag-UBP dissociation and renders Gag unable to rebind to UBP, allowing Gag to move to the PM (Callahan, Handley et al. 1998).

Taken together, this evidence suggests that Vpu antagonises a host cell restriction factor that acts non-specifically to disrupt HIV-1 particle release in some human but not simian cells (Varthakavi, Smith et al. 2003). This function may in turn be related to its facility for ion channel formation. The pericentriolar endosome represents a

possible site for host restriction factor interaction (Varthakavi, Smith et al. 2006). This host cell factor was initially proposed to be the background ion channel, TASK-1 (Hsu, Seharaseyon et al. 2004), or the Gag-binding protein, UBP (Handley, Paddock et al. 2001). Recently, though, the novel host-restriction factor, tetherin has been shown to antagonise the budding of virus particles by causing the internalisation from and accumulation of virions at the cell surface (Neil, Zang et al. 2008). Accordingly, Vpu may enhance virion release by blocking tetherin activity.

1.5 Identification of novel host-restriction factors overcome by Vpu

1.5.1 Tetherin

Vpu blocks the accumulation of virions in intracellular vacuoles at the PM, allowing viral budding (Klimkait, Strebel et al. 1990). The factor that causes this accumulation of virions was concluded to act after virion assembly, at the cell surface, and to induce virion internalisation. The requirement for Vpu is also enhanced by the treatment with interferon-α (IFN-α) (Neil, Sandrin et al. 2007). Neil *et al*. therefore concluded that the inhibitor of retrovirus release is an IFN-α-induced, cell surface protein (Neil, Sandrin et al. 2007; Neil, Zang et al. 2008). They identified this protein by microarray analyses of mRNAs expressed by untreated and IFN-α-treated permissive and non-permissive cells (Neil, Zang et al. 2008). Tetherin/CD137/BST-2/HM1.24 was identified and confirmed by transfection of these cells with wild-type

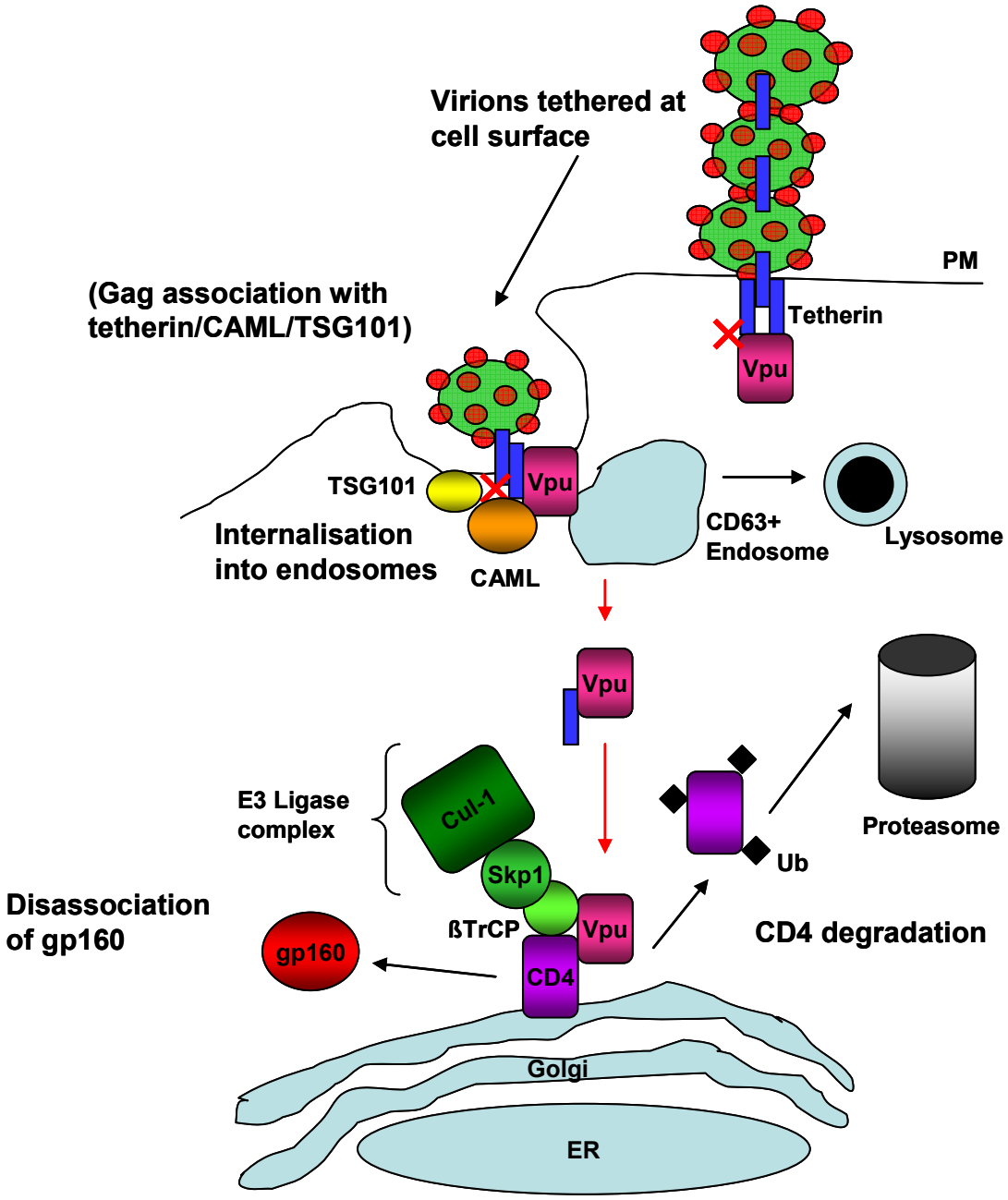


Figure 1.4: Schematic representation of the functions of Vpu within the infected cell. Vpu acts late in the viral life cycle to bind newly synthesised CD4 at the ER/Golgi interface. Concurrent binding to ßTrCP adapts CD4 to the E3-ligase complex causing the ubiquitination and subsequent degradation of CD4 in the proteasome. This process effectively releases bound gp160 from CD4 and allows for gp160 to traffic to the cell membrane to act in virion assembly. Vpu also traffics to the cell surface to function in enhancing virion release. In the absence of Vpu, virus particles are tethered at the cell surface in the form of long chains, and then internalised into early endosomes via TSG101/tetherin/CAML. The trapped virions are then transported to lysosomes where they are digested. Vpu binds tetherin via its TM domain and prevents the accumulation of virions at the cell surface. Vpu also prevents the association of Gag with TSG101 and the internalisation of virions via the action of tetherin and CAML.

and Vpu-deleted (delVpu) HIV-1 proviral plasmids (Neil, Zang et al. 2008; Van Damme, Goff et al. 2008). Single-cycle HIV-1 replication assays in CD4-negative cells expressing CD137 constitutively, showed >100 fold reduction in delVpu virion yield (Neil, Zang et al. 2008). Viral Gag expression in infected cells remained unchanged in the absence or presence of tetherin demonstrating that viral assembly remains unaltered and tetherin acts only at the level of viral release (Neil, Zang et al. 2008). Short interfering RNA (siRNA) targeted against tetherin also enhances the yield of delVpu virions from HIV-1 infected cells (Neil, Sandrin et al. 2007).

Tetherin colocalised with Vpu, and in the absence of Vpu, virions were associated with tetherin at the cell surface and in endosomes (Neil, Sandrin et al. 2007). Notably, tetherin has a complex glycosylation pattern, a N-terminal TM anchor, as well as a C-terminal glycosyl phosphatidylinositol (GPI) membrane anchor (Kupzig 2003). It is able to localise to multiple membrane compartments and removal of this GPI anchor eradicates its ability to inhibit delVpu virion release, suggesting that this anchor allows the protein to retain nascent virions (Neil, Zang et al. 2008). Tetherin is therefore likely to localise to cholesterol-rich domains in the PM, where it may encounter assembling retrovirus particles (Kupzig 2003).

Miyagi *et al.* analysed endogenous tetherin and virus release in HeLa cells, T-cells and macrophages (Miyagi, Andrew et al. 2009). This group showed that while Vpu reduces tetherin expression in transfected HeLa cells, and infected macrophages, it

does not decrease cell surface tetherin levels in CEMx174 and H9 T-cells (Miyagi, Andrew et al. 2009). Efficient HIV-1 replication in these cell lines does require Vpu, however. Also, tetherin was not present in virions physically sheared from the cell surface (Miyagi, Andrew et al. 2009). Overall, this suggests that the Vpu-tetherin interaction is not the only function required for virion release.

1.5.2 Calcium Modulating Cyclophilin Ligand

An additional host restriction factor, CAML was also identified recently by another group (Varthakavi, Heimann-Nichols et al. 2008). CAML is a cyclophilin B-binding integral membrane protein with a vital role in the survival of developing T cells, and for the regulation of TCR-activated tyrosine kinase $p56^{Lck}$ (Bram and Crabtree 1994; Tran 2005). Notably, it is required for recycling of epidermal growth factor receptor (EGFR) to the PM (Tran, Russell et al. 2003). In the absence of Vpu, simian cells expressing CAML accumulated virions at the cell membrane. Varthakavi *et al.* revealed that Vpu and Env rescued delVpu-virion release, and depletion of CAML by targeted interfering RNA eliminated the need for Vpu to elicit HIV-1 and MLV virion release (Varthakavi, Heimann-Nichols et al. 2008).

CAML was identified by yeast two-hybrid screening of human HeLa cell DNA using full-length codon-optimised Vpu as bait. The hydrophilic N-terminus of human CAML (using truncated recombinant CAMLs) was sufficient for Vpu interaction, confirmed by

co-immunoprecipitation experiments using antibodies to endogenous CAML in human cells (Varthakavi, Heimann-Nichols et al. 2008). Colocalisation experiments showed significant overlapping fluorescence at the perinuclear region, with lesser amounts on peripheral vesicular structures. CAML elicits the characteristic delVpu phenotype in HIV-1 infected cells of accumulated and tethered virions that is recovered by protease treatment (Varthakavi, Smith et al. 2003; Varthakavi, Heimann-Nichols et al. 2008).

HIV-1 Vpu was concluded to offset the block to particle release mediated by CAML by a hitherto unknown mechanism. The authors contemplate the role of CAML in EGFR recycling and whether it interacts with Vpu along the endosomal recycling pathway (Varthakavi, Heimann-Nichols et al. 2008). Vpu may prevent CAML from reaching the cell surface, and this may in turn alter the surface expression of the newly described protein, tetherin.

1.6 Additional functions of Vpu

1.6.1 Downregulation of the major histocompatibility complex class I and II

Nef acts early in the HIV-1 viral life cycle by promoting the endocytosis of MHC I from the surface of APCs, while Vpu acts later to interfere with MHC I heavy chain synthesis retaining them at the ER (Schwartz, Marechal et al. 1996; Kerkau, Bacik et al. 1997). Yeast two-hybrid assays and co-immunoprecipitation experiments confirm

the interaction of Vpu with MHC II invariant chains, or CD74 (Hussain, Wesley et al. 2008). CD74 is a type II transmembrane protein that binds the α and ß chains of MHC II in the ER (Becker-Herman, Arie et al. 2005). Peptide loading of MHC II occurs in the Golgi by displacement of the class II-associated Ii peptide (CLIP) domain of CD74 (Ploegh 1998). This is followed by antigen presentation at the cell surface. CD74 is also expressed as a trimer on the cell membrane where it mediates signalling initiated by the macrophage inhibitory factor (MIF, also known as macrophage migration inhibitory factor; glycosylation-inhibiting factor; phenylpyruvate tautomerase) (Leng, Metz et al. 2003). The interaction between HIV-1 Vpu and CD74 occurs specifically between α-helix 1 of Vpu and the 30 amino acid C-terminal of CD74 (Hussain, Wesley et al. 2008). The surface expression of mature MHC II was observed to be downregulated in human monocytic U937 cells transfected with delVpu constructs, and this correlated to decreased Ova peptide antigen presentation to Ova-specific MHC II restricted-T cells (Hussain, Wesley et al. 2008).

Natural killer (NK) cells are able to kill virus-infected cells where MHC I is downregulated (reviewed in (Fauci, Mavilio et al. 2005). Interestingly, Env, Pol, and Vpu stimulate the NK cell-mediated ADCC response (Stratov, Chung et al. 2008). This study used overlapping peptides to detect HIV-specific NK cell-recognition responses (Stratov, Chung et al. 2008). Vpu-specific interferon-gamma (IFNγ) expression by the non-T cell immune response was observed (Stratov, Chung et al. 2008). Epitope mapping demonstrated that this response was directed against the

Vpu CD sequence EMGHHAPW**D**V**D** in subtype B, and that the C-terminal aspartic acid residues (highlighted) were essential for this response (Stratov, Chung et al. 2008).

1.6.2 Induction of tumour formation and apoptosis

Vpu acts as a strong competitive inhibitor of βTrCP, impairing the proteasomal degradation of cellular SCF-βTrCP substrates (Orian, Gonen et al. 2000). βTrCP is redistributed to the cytoplasm from the nucleus in the presence of full-length Vpu, resulting in the cytoplasmic accumulation of βTrCP substrates (Besnard-Guerin, Belaidouni et al. 2004). These substrates include β-catenin, the inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), and Activating Transcription Factor 4 (ATF4, also known as tax-responsive enhancer element B67) (Besnard-Guerin, Belaidouni et al. 2004; Salim and Ratner 2008) (Table 1.1). These proteins play a role in innate immunity, cancer and several autoimmune disorders (Li 2000). Vpu may therefore contribute to tumour formation in AIDS patients, an opinion underscored by the high levels of β-catenin found in Kaposi's sarcoma (Locher, Fujimura et al. 2003). Notably, β-catenin also promotes the maturation of thymocytes, implicating Vpu in the renewal of CD4+ T-cells, and the maintenance of the viral life cycle (Gounari, Aifantis et al. 2001).

This inhibition of IκBα degradation, in the presence of TNFα, suppresses nuclear factor kappa B (NFκB) activity (Bour, Perrin et al. 2001). This in turn sensitizes cells to Fas-mediated apoptosis (Akari, Bour et al. 2001). Vpu-induced apoptosis is specific and independent of other viral proteins and depends upon Vpu-βTrCP binding (Akari, Bour et al. 2001). Vpu also reduces the levels NFκB-dependent anti-apoptotic proteins, Bcl-xL, A1/Bfl-1, and TNF receptor-associated factor I (TRAF I) (Akari, Bour et al. 2001). Moreover, caspase-3 levels are increased, promoting caspase-mediated apoptosis (Akari, Bour et al. 2001).

1.7 Biological relevance of HIV-1 Vpu

1.7.1 Simian-Human Immunodeficiency Virus (SHIV)/Macaque models

Information in the literature regarding Vpu function *in vivo* is limited but SIV/HIV-1 (SHIV) chimeric viruses that are able to infect monkeys offer a useful model. Studies of monkeys infected with pathogenic SHIV provide the strongest evidence for the role of Vpu in HIV-1 pathogenesis (Li, Halloran et al. 1995). Vpu is definitively required for pathogenicity in infected macaques (Mackay, Niu et al. 2002), and CD4 down-regulation is crucial for infectivity (Bour, Perrin et al. 1999; Lama, Mangasarian et al. 1999). Deletion of *vpu* causes decreased viral loads, while *vpu*+ SHIV contributes to $CD4^+$ T cell loss during infection (Stephens, McCormick et al. 2002). Remarkably, SHIV constructs expressing subtype C Vpu had a more gradual rate of $CD4^+$ T-cell

loss and decreased viral loads following inoculation of macaques, when compared to inoculation with a subtype B construct (Hill, Ruiz et al. 2008). This group also inoculated C8166 cells with chimeric SHIV expressing subtype C Vpu proteins with a mutated tyrosine and dileucine in the salt bridge region (Ruiz, Hill et al. 2008). The SHIV with an altered dileucine motif showed increased replication kinetics and was more cytopathic than the parental virus, and the altered tyrosine motif caused a decreased rate of replication (Ruiz, Hill et al. 2008).

1.7.2 CD4 down modulation by Vpu and pathogenesis

The down-modulation of CD4 is crucial for the pathogenesis of HIV-1. CD4 interacts with MHC II on APCs and is a costimulatory factor of TCR-mediated T-cell activation (Weiss and Littman 1994). CD4 down-modulation also enhances the release and infectivity of HIV-1 particles (Bour, Perrin et al. 1999; Lama, Mangasarian et al. 1999; Levesque, Zhao et al. 2003). The virus must counteract the effects of CD4 to prevent its accumulation in the budding virion and the result of superinfection. High levels of CD4 on the surface of HIV-1 infected cells reduces the infectivity of released virions by the sequestering of the viral envelope by CD4 (Lama, Mangasarian et al. 1999). HIV-1 is also able to prevent superinfection by Vpu-mediated down-modulation of CD4 at the ER. As discussed, superinfection is an immune state that leads to enhanced levels of apoptosis and viral recombination. Decreasing CD4 may thus reduce cell killing, enhance viral spread and reduce frequency of viral recombination

(Lama 2003). Recombination is beneficial to escape the host immune response, but hypothetically detrimental late in the life cycle as it promotes cell death reducing the time for productive virion assembly and release (Lama 2003). Vpu-induced downregulation of MHC I and II molecules also plays a role in the pathogenesis of HIV-1 by contributing to the inability of $CD8^+$ T-cells to eradicate the virus from infected individuals (Hussain, Wesley et al. 2008).

1.8 Vpu as a therapeutic target

Overall, this multifunctional protein represents an attractive target for therapeutic and preventative interventions. However, there are currently no drugs targeting Vpu in human clinical trials. Some options do present themselves. Investigators have shown that derivatives of the Na^+-K^+ antiporter amilorides can prevent Vpu-mediated virion release (Ewart, Nasr et al. 2004). Further, the Vpu-ßTrCP binding motif appears to be unique, presenting a distinctive point for binding of a small molecule (Lindwasser, Chaudhuri et al. 2007). Such an inhibitor may reduce Vpu-induced CD4 degradation, allowing CD4 to bind and retain gp160. Vpu-tetherin also represents an attractive complex to target. Tetherin forms part of the bone marrow stromal cell factors that are involved in the development of B cells (Ishikawa, Kaisho et al. 1995). The specific function of human tetherin/BST-2 in uninfected individuals has yet to be resolved. Speculation involves a role in pre-B-cell growth, and possibly rheumatoid arthritis

(Kupzig 2003). An antiviral agent would need to target specific contact points between the TMD of Vpu and tetherin to avoid further immune system dysfunction.

1.9 Rationale for the study

Structure/function studies of the *vpu* gene have focused on laboratory-adapted subtype B isolates, with limited studies detailing the biological characteristics of subtype C *vpu* (Scriba 2001). HIV-1 subtype C represents over 56% of all new HIV-1 infections worldwide, and predominates in countries of East Africa, Southern Africa, India, and Brazil. Of particular interest, HIV-1 subtype C is the predominant subtype circulating within the heterosexual population of South Africa (van Harmelen, Williamson et al. 2001). The relative worldwide pervasiveness of this subtype points to an enhanced ability to evade the host immune response and establish infection. Vpu modulates a diverse range of host cell processes, and may therefore influence the increased viral infectivity observed in subtype C isolates at multiple levels. Thus, further biological characterization is required as a first step in understanding Vpu's contribution to HIV-1 subtype C pathogenesis, particularly with respect to its ability to down-modulate CD4 expression. We hypothesise that the South African subtype C Vpu will behave similarly to the previously characterised Indian subtype C Vpu (Pacyniak, Gomez et al. 2005). A greater understanding of the underlying mechanisms of HIV-1 subtype C pathogenesis will allow for a more rational approach to designing novel antiviral drugs.

Previous work has described the genotypic characterisation of *vif, vpr* and *vpu* from 20 primary HIV-1 isolates available in our laboratory, and extensive analysis and comparison of known functional motifs (Bell, Connell et al. 2007). This study aims to determine the subcellular localization of selected South African HIV-1 subtype C Vpu proteins over time, as compared to subtype B Vpu. Two subtype C isolates were selected based on differences in their overall length, with 05ZAFV5 and 05ZAFV15 containing a six and two amino acid insertion in the N terminal domain, respectively.

Specific Study objectives:

1. To construct vectors expressing codon-optimized HIV-1 subtype B and C Vphu-emGFP fusion proteins.

2. To determine the subcellular localisation of the subtype B and C Vphu-emGFP constructs in HEK 293T cells cotransfected with pDsRed-ER, pDsRed-Mem, and pDsRed-Golgi markers (Living Colors® Reef Coral Fluorescent Proteins, Clontech Laboratories, Inc., Mountain View, CA), over time, by confocal microscopy.

Chapter 2

Materials and methods

2.1 Viral isolates and plasmids used in this study

2.1.1 Viral isolate selection

A comparison of the predicted amino acid sequences derived from twenty primary viral isolates available in our laboratory lead to the selection of the two subtype C isolates used in this study (Bell, Connell et al. 2007). The two isolates, 05ZAFV5 and 05ZAFV15, were chosen based on their predicted Vpu protein length and the presence of conserved and significant sequence motifs. No ethics clearance was required for this study (Appendix A) as viral isolates were obtained from a previously approved study (M041002).

2.1.2 Plasmids

The plasmids described below were used as template, cloning, or expression vectors. Plasmid maps are outlined in Appendix B.

2.1.2.1 pcDNA-Vphu and pcDNA™6.2/C-emGFP-GW/TOPO® mammalian expression vectors

The codon-optimised pcDNA-Vphu vector (5779 bp) encodes the Vphu protein from the HIV-1 subtype B strain, NL4-3 under the constitutive control of a cytomegalovirus

(CMV) promoter in a pcDNA3.1 (-) backbone (Nguyen 2004). Two microlitres of 1 µg/µl plasmid was used to transform competent *E. coli* cells and to make glycerol cell stocks, as described in section 2.2.6 below. This vector was used as a template to PCR amplify the v*phu* gene for subcloning into the pcDNA™6.2/C-emGFP-GW/TOPO® mammalian expression vector (Vivid Colors™, Invitrogen, Carlsbad, CA). This subtype B Vpu expressing plasmid was used as a positive control in all experiments.

The pcDNA™6.2/C-emGFP-GW/TOPO® mammalian expression vector (5814 bp) from the Vivid Colors™ pcDNA™6.2/C-emGFP-GW/TOPO® Kit (Invitrogen) was used for cloning the subtype B and C codon optimised *vphu* in frame with the emerald green fluorescent protein (emGFP) (Zhang G 1996; Tsien 1998). This vector contains a CMV immediate-early (IE) promoter for strong constitutive gene expression, as well as a thymidine-adenosine (TA) cloning site. It is provided linearised, with a single 3' thymidine overhang, as well as a covalently bound topoisomerase enzyme for TA cloning. An ampicillin resistance gene allows for selection of positive clones in *E. coli*. The positive control vector, pcDNA™6.2/C-emGFP/GW/CAT (6471 bp), allows for the expression of C-terminally emGFP-tagged chloramphenicol acetyltransferase (CAT). This vector was employed as an expression control in transfection experiments.

2.1.2.2 pDsRed-ER, pDsRed-Mem, and pDsRed-Golgi subcellular localisation vectors

The three subcellular marker plasmids, pDsRed-ER, pDsRed-Mem, and pDsRed-Golgi (Living Colors® Reef Coral Fluorescent Proteins, Clontech Laboratories, Inc., Mountain View, CA) were used in cotransfections of mammalian cells with the pVphu-emGFP vectors. This allowed for the visualisation of requisite organelles, as well as the subcellular localisation of the Vphu proteins under investigation. The marker plasmids contain the CMV IE promoter, and an upstream Kozak consensus sequence to enhance translation efficiency as well as a gene encoding kanamycin resistance in *E. coli* (Clontech Laboratories July 2003). Importantly, they also express the monomeric protein, DsRed (Matz 1999) fused to a specific targeting signal peptide. The ER-localising vector, pDsRed-ER encodes the ER targeting sequence of calreticulin (Fliegel 1989) fused to the 5' end of DsRed2 (Matz 1999); and the ER retention sequence, KDEL (Munro 1987; Pelham 1996), fused to the 3' end. The Golgi-localising vector, pDsRed-Golgi encodes the N-terminal 81 amino acids of human ß-1,4-galactosyltransferase (Watzele 1990), a membrane anchoring signal peptide targeting DsRed to the trans-medial region of the Golgi apparatus (Gleeson 1994; Yamaguchi 1995; Ilopis 1998). Lastly, pDsRed-Mem encodes the N-terminal 20 amino acids of neuromodulin (GAP-43) that acts as a palmityolation signal, targeting DsRed monomer (Matz 1999) to cellular membranes. Two microlitres of each 1 µg/µl plasmid was used to transform competent *E. coli* cells, cultured in Luria Bertani broth

(LB) containing 50 mg/ml kanamycin (Roche Diagnostics, Mannheim, Germany), and to make glycerol cell stocks, as described in section 2.2.6 below.

2.2 Construction of *Vphu-emGFP* for fusion protein expression

2.2.1 Codon optimisation of the native *vpu* gene

The *vpu* genes for the selected South African subtype C isolates, 05ZAFV5 and 05ZAFV15 were codon optimised (Operon Biotechnologies GmbH, Cologne, Germany) based on the previously described sequences (Bell, Connell et al. 2007) and are encoded by FV5_pCR Script and FV15_pCR Script (Appendix A). Codon optimisation adjusts the codon frequency of the foreign protein (namely, HIV-1 Vpu) to match the host expression system by changes to secondary structure, GC content and repetitive codons (Kyper 1987), allowing for enhanced expression in a mammalian system. These genes were designated humanised *vpu*, or *vphu*. The PCR Script vectors encode for ampicillin resistance for selection of positive clones in *E. coli*.

2.2.2 PCR optimisation

2.2.2.1 Primer design

Polymerase chain reaction (PCR) primers were designed so that the subtype B and C *vphu* genes could be amplified (~300 bp) and directionally cloned into the pcDNA™6.2/C-emGFP-GW/TOPO® vector (Invitrogen), in frame with the C-terminal *emGFP* tag. Primer design ensured the inclusion of an upstream Kozak consensus sequence for proper initiation and increased efficiency of translation, as well as the removal of the native stop codon for cloning in frame with *emGFP*. Sequence data from the FV5_pCR Script and FV15_pCR Script vectors (Operon) provided the basis of subtype C *vphu* PCR primer design. The subtype B *vphu* gene was sequenced (as described in section 2.2.9 below) from the pcDNA-Vphu vector using a T7 primer, and the sequence data used to design subtype B *vphu* PCR primers. The PCR and sequencing primers are detailed in Table 2.1.

2.2.2.2 PCR amplification of *vphu*

A PCR protocol to amplify the ~300 bp sub B and C *vphu* was developed and optimised as recommended by the Vivid Colors™ pcDNA™6.2/C-emGFP-GW/TOPO® Kit (Invitrogen). The gene fragments (246 bp, 249 bp and 261 bp for subtype B, 05ZAFV5, and 05ZAFV5 *vphu* respectively) were amplified using the

Expand High Fidelity^{PLUS} PCR System (Roche Applied Science GmbH, Mannheim, Germany), as per the manufacturer's instructions. The PCR reaction mix is outlined in Table 2.2a. Reaction mixtures without template DNA (water blank) were set up as negative controls. The PCR reactions were carried out in a total volume of 50 µl, made up with sterile PCR-grade water.

Thermal cycling conditions were optimised on the GeneAmp PCR Systems 2700 thermocycler (Applied Biosystems, Foster City, CA), and were based on the suggested profile for the Expand High Fidelity^{PLUS} PCR System (Roche). Annealing temperatures and times were selected according to the relevant primer characteristics (Table 2.1). The PCR cycling parameters are outlined in Table 2.2b.

Table 2.1: PCR and sequencing primer sequences and specifications for *vphu* amplification

Reaction type	Name	Sequence 5' to 3'	Tm (°C)	Length (bp)	GC content (%)
PCR	hvpu B F	CAGCCGCCATGGTGCCCATTATTG	63	24	58.3
	hvpu B R	CAGGTCGTCAATGTCCC	52.8	17	58.8
	hFV5 F	GAAGAGTGATGGTAGACTTG	58.4	20	45
	hFV5 R	CAGATCGGCGGCGTCCAGC	68.8	19	74
	hFV15 F	GAAGAGTGATGGTTACGCTCG	62.6	21	52
	hFV15 R	TAGATCATTCACGTCCAGG	58	19	47
Sequencing	T7 Promoter F	TAATACGACTCACTATAGGG	56	20	40
	FP2 R	TCACCATGTTAACAGCATCAA	58	21	38

Table 2.2a: PCR Reaction mix

Reagent	Reaction Volume (µl)	Final Concentration
5X Buffer	10	1.5 mM ($MgCl_2$)
10mM dNTPs	1	200 µM
Sense primer	2	0.4 µM
Antisense primer	2	0.4 µM
Template DNA	1	0.1 – 10 ng/µl
Taq polymerase enzyme mix (5U/µl)	0.5	2.5 U
Distilled water	33.5	/
TOTAL	**50**	**/**

Table 2.2b: PCR thermal cycling conditions

Phase	Temperature (°C)	Duration	Cycles
Initial denaturation	94	2 min	1
Denaturation	94	20 s	
Annealing	51	30 s	10
Elongation	72	30 s	
Denaturation	94	20 s	
Annealing	51	30 s	25
Elongation	72	30 s*	
Final elongation	72	10 min	1
Hold	4	infinite	/

* Plus 10 seconds on each elongation step.

2.2.3 Visualisation of PCR amplicon

PCR products were resolved on a 1.5 % Tris actetate ethylenediaminetetraacetic acid (EDTA) (TAE) agarose gel (Appendix C1), containing 0.5 µg/ml ethidium bromide (EtBr) solution (Promega Corporation, Madison, WI, USA), at 80 V for 1 hour. Results were visualized on a UV transilluminator and photographed using Quantity One V4.6.1 1D Analysis software (BioRad, Laboratories, CA, USA). All gels were run with the DNA Molecular Weight Marker X (Roche), which covers the fragment range from 0.07 to 12.2 kb, for sizing purposes (Appendix D).

2.2.4 Purification of PCR products from TAE agarose gels

The GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA) was used to purify the PCR amplicon from an agarose gel. This was done because a discrete band was not previously obtained, and hence contamination by unrelated DNA sequences was observed. This kit uses a chaotropic agent to denature protein, dissolve agarose, and promote the binding of double-stranded DNA to a glass fibre matrix. Proteins and salt contaminants are washed away using an ethanolic buffer, and DNA is eluted in a low ionic strength buffer. Briefly, the PCR product was run on a fresh 1.5 % TAE agarose gel. The DNA band aligning with the correct size restriction fragment of the molecular weight marker was visualised by UV transillumination, and a ~300 mg gel band carefully sliced out of the gel using a sterile surgical blade. Three hundred microlitres capture buffer was added to the gel band in a 1.5 ml microcentrifuge tube (Eppendorf, Hamburg, Germany). The agarose was melted at 60 ° C for 15 min before transferring the sample to a GFX column, and incubating at room temperature for 1 min. The tube was centrifuged for 30 s at 15 000 x g, and the flow-through discarded. The column was then washed with 500 µl Wash buffer at 15 000 x g for 30 s (Eppendorf centrifuge 5417). The DNA was incubated in 50 µl elution buffer (Tris-EDTA, pH 8.0) for 1 min at room temperature, before centrifuging at 15 000 x g for 1 min to recover purified DNA. Purified DNA was used in all sub-cloning reactions.

2.2.5 TA cloning reactions

The purified amplicons were used in TA cloning reactions in order to insert the *vphu* gene in frame with the *emGFP* gene into the pcDNA™6.2/C-emGFP-GW/TOPO® mammalian expression vector (Invitrogen). The cloning reaction was performed according to the manufacturer's instructions. Briefly, two microlitres purified PCR product was mixed with 1 µl salt solution (200 mM NaCl, 10 mM $MgCl_2$), 1 µl TOPO vector, and sterile water to a total reaction volume of 6 µl. The reaction was mixed gently and incubated at room temperature for 10 minutes. The reaction was then placed on ice before proceeding to the transformation step. A negative, PCR product-free control was included in each experiment.

2.2.6 Bacterial transformations and selection of recombinant clones

The TA cloning reaction products were used to transform One Shot® TOP10 chemically competent *E. coli* (Invitrogen), as per the manufacturer's instructions. Briefly, two microlitres of the cloning reaction was added to a vial of competent *E. coli* (kept on ice) and mixed gently. The mixture was incubated on ice for 30 minutes, heat shocked in a 42 °C water bath for 60 s, and immediately transferred back to ice. Two hundred and fifty microlitres of room temperature Super Optimal (S.O.C.) broth medium (Invitrogen) was added before shaking the tube horizontally at 200 rpm in a 37 °C incubator for one hour. Aliquots of the bacterial cell mix from each

transformation (50 µl and 200 µl), including the negative control, were spread on pre-warmed selective LB plates, containing 100 µg/ml ampicillin (Roche) (Appendix C1), and incubated overnight at 37 °C.

The next day, ten discrete colonies from each transformation were selected and used to inoculate 5 ml aliquots of LB containing 100 µg/ml ampicillin (Roche) (Appendix C1), and cultured overnight at 37 °C in a shaking incubator at 225 rpm. Overnight cultures were used to make glycerol stocks and for plasmid MiniPrep DNA-isolations (see section 2.2.7). Briefly, 0.85 ml bacterial cell culture containing the required clone was mixed with 0.15 ml filter-sterilised glycerol (Saarchem, Merck Chemicals, Modderfontein, SA). This mix was transferred to a 1.8 ml cryovial (Nalge Nunc Int., Rochester, NY, USA), and frozen overnight in a "nest" box at -80 °C, before transferring to liquid nitrogen storage.

2.2.7 Recombinant plasmid DNA isolation

Recombinant plasmid DNA was isolated using the GenElute™ HP Plasmid MiniPrep and MaxiPrep Kits (Sigma-Aldrich Inc., St Louis, USA). The binding columns in these kits were used in the spin purification format. For the MiniPrep kit, the overnight recombinant *E. coli* culture was harvested by centrifugation at 12 000 x g for 1 min (Eppendorf centrifuge 5415D) and subjected to the modified alkaline-SDS lysis procedure, as per the manufacturer's instructions. Briefly, the cell pellet was

resuspended in 200 µl Resuspension Buffer and lightly vortexed before addition of 200 µl Lysis Buffer and gentle inversion. The mixture was allowed to clear before adding 350 µl Neutralisation Solution and inverting six times. The cell debris was pelleted at 12 000 x g for 10 min, during which time 500 µl Column Prep Solution was added to the binding column, centrifuged at the maximum speed and the flow-through discarded. The lysate was cleared with a filter syringe by centrifuging for 1 min, leaving the DNA bound to the silica-based column membrane. The remaining contaminants were removed by a wash step where 750 µl Wash Solution was added, centrifuged for 1 min, and the flow-through discarded. Finally, the bound DNA was eluted in 60 µl Elution Solution at 12 000 x g for 1 min and stored at -20 °C. The clones containing the genes of interest were cultured overnight on a large scale (250 ml ampicillin-containing LB), the plasmid DNA purified by the GenElute™ HP Plasmid MaxiPrep Kit (Sigma-Aldrich) and stored at -20 °C for use in transfection reactions. The GenElute™ HP Plasmid MaxiPrep Kit (Sigma-Aldrich) was also used for the large-scale preparation of the subcellular marker plasmids, pDsRed-ER, pDsRed-Golgi, and pDsRed-Mem (Clontech). Plasmid DNA concentration was determined by spectroscopy on a NanoDrop 1000 (Thermo Fisher Scientific, DE, USA).

2.2.8 Characterisation of recombinant clones by restriction enzyme digest and agarose gel electrophoresis

To confirm the presence of the insert in pcDNA™6.2/C-emGFP, a restriction enzyme digest was performed on the selected clones. An *Eco*RI digest of pcDNA™6.2/C-emGFP-Vphu results in two bands: the excised *vphu* fragment of ~300 bp, and the original 5.9 kb plasmid. The digestion reaction for each clone contained (in order): 11.5 µl sterile water, 2 µl 10X buffer, 5 µl plasmid DNA, and lastly 1.5 µl *Eco*RI enzyme (New England BioLabs Inc., Ipswich, MA, USA). The reaction was mixed gently and incubated in a 37 °C water bath for one hour. The entire twenty microlitre reaction was run on a 0.8%, EtBr-treated, TAE agarose gel at 80 V for one hour.

2.2.9 Characterisation of recombinant clones by cycle sequencing reactions

Recombinant Vpu-emGFP plasmids, as defined by restriction enzyme digests, were sequenced to verify the orientation of the insert, and to ensure the insert was in frame with the *emGFP* gene.

2.2.9.1 Sample preparation

The T7 promoter sequence forward primer and the FP2 reverse primer (Table 2.1) were used to sequence the purified plasmid DNA in both directions using the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems), as per the manufacturer's instructions. Briefly, a cycle sequencing reaction mixture consisting of 4 µl 2.5X Terminator Ready Reaction Mix, 4 µl 5X Sequencing Buffer, 2 µl purified DNA template, and 9 µl reagent-grade water, was added to 1.1 µl of each individual primer (3.2 ρmol working concentration) making up a volume of 20.1 µl.

2.2.9.2 Thermal cycle sequencing

Thermal cycle sequencing reactions were performed in the GeneAmp PCR Systems 2700 thermal cycler (Applied Biosystems). Thermal cycle sequencing conditions were set as recommended for the Big Dye Terminator V3.1 Cycle Sequencing system: a rapid thermal ramp to 96 °C for 1 min, 25 cycles of denaturation for 10 s at 96 °C, annealing for 5 s at 50 °C, and elongation for 4 min at 60 °C, concluding in a rapid thermal ramp to 4 °C. Reaction products were used immediately in purification procedures.

2.2.9.3 Purification of extension products

Cycle sequencing products were purified of unincorporated dye terminators, salts, dNTPs, and ddNTPs by isopropanol precipitation, prior to being run on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). This step was done to avoid excess dye terminators obscuring data in the initial part of the sequence, thereby interfering with base calling. Isopropanol precipitation was adapted from the ViroSeq HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA, USA). Completed twenty microlitre cycle sequencing reactions were transferred to a MicroAmp 96-well Optical Reaction Plate (Applied Biosystems). Eighty microlitres of 80% isopropanol was added to each well, mixed by reverse pipetting, sealed with a plate cover, and incubated in the dark at room temperature for 15 min. The plate was then centrifuged for 45 min at 2000 x g (Eppendorf centrifuge 5810). The plate was immediately uncovered and inverted onto paper towel, and spun in this inverted position for 1 min at 700 x g. The plate was allowed to air dry, protected from light, in an upright position for 3 min, before resuspending the purified DNA pellet in 20 µl HiDi Formamide (Applied Biosystems). Resuspended purified DNA was either stored at -20 °C, or used immediately in automated sequence detection.

2.2.9.4 Sample electrophoresis

Purified cycle sequencing products were run on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). This automated sixteen-capillary sequencer separates fragments according to size on a 6% acrylamide gel. Electrophoresis was configured to a 50 cm capillary running POP-6 polymer gel (Applied Biosystems) on a standard run module. For data analysis, dye set/primer (mobility) files were set as recommended by the manufacturer. The Big Dye Terminator V3.1 Matrix Standard (Applied Biosystems) was used for spectral calibration of dye set Z.

2.2.9.5 Sequence data analysis

Sequence gel analysis and data extraction was performed using the 3100 Genetic Analyzer software suite, specifically the Data Collection V1.0 and Sequencing Analysis V3.3 programs (Applied Biosystems). The resulting overlapping sequence fragments for each isolate were edited and automatically assembled into a single contig by the Sequencher V4.5 program (Genecodes, Ann Arbor, MI). Assembly parameters were set to allow for a minimum homology match of 85%, and a minimum overlap of 20 nucleotides, using the Dirty Data algorithm.

2.3 Transfections of mammalian cell lines

2.3.1 HEK 293T mammalian cell line

Human Embryonic Kidney (HEK) 293T cells were used in all transfection and confocal microscopy experiments. The cells were seeded in 75 cm^3 flasks (Nalge Nunc Int.) and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) (Appendix C2). Cells were passaged as described in section 2.3.2 below.

2.3.1.1 Cell culture conditions and passage

HEK 293T cells were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were passaged every four to six days when subconfluent, using trypsin EDTA solution (Sigma-Aldrich). Briefly, old media was discarded and the cell monolayer washed gently with 1X Phosphate Buffered Saline (PBS) (Sigma-Aldrich). Cells were dislodged by adding 1 ml trypsin EDTA solution, pouring off the excess, and incubating for ~3 minutes at 37 °C. Cells were then tapped off the flask surface and resuspended to a concentration of 4 X 10^6 cells in 20 ml complete DMEM in a 75 cm^3 flask (Nalge Nunc Int.). Cell concentration was determined by staining cells with 0.4% Trypan Blue solution (Sigma-Aldrich), and assessing the viable cell count on an Improved Neubauer haemocytometer (Merck, Darmstadt, Germany). Briefly, ten microlitres cell suspension was added to 90 µl 0.4% Trypan Blue solution and mixed

thoroughly before pipetting onto a clean haemocytometer and allowing diffusion. Viable cells do not take up the dye and appear white under a light microscope. Live cells were counted in all four primary squares and averaged for one square to denote the number of cells in 1 X 10^{-4} ml. By taking into account the dilution factor of ten, the cell count was calculated per ml of cell suspension and adjusted accordingly.

2.3.1.2 Storage of mammalian cell strains

HEK 293T cells were frozen in a 90% fetal calf serum (FCS), 10% dimethyl sulfoxide (DMSO) freezing mix, and stored in liquid nitrogen. Cell stocks were prepared as follows: 1 ml aliquots of cell suspension were centrifuged in 1.8 ml cryovial (Nalge Nunc Int.) at 320 x g for 2 min (Eppendorf centrifuge 5810). The supernatant was carefully pipetted off the cell pellet and the pellet resuspended in 1 ml of the appropriate freezing mix. Cryovials were transferred immediately to a nest box at -80 °C and stored overnight before transferring to liquid nitrogen for indefinite storage.

2.3.2 Vphu-emGFP transfections of HEK 293T cells

2.3.2.1 Confirmation of Vphu expression by fluorescence microscopy

The subtype B and C pcDNA™6.2/C-emGFP-Vphu vectors were transfected singly into HEK 293T cells and the expression of the fusion protein confirmed by fluorescent

microscopy. Cells were seeded the day before transfections at 1.2 x 10^6 cells in 25 cm^3 culture flasks (Nalge Nunc Int.) containing 6 ml complete DMEM. Transient transfections were performed using Polyfect reagent (Qiagen Inc., Valencia, CA, USA). Briefly, the transfection mix containing 4 µg plasmid DNA in 150 µl DMEM without supplements and 40 µl Polyfect, was incubated for 10 min at room temperature to allow complex formation. One millilitre of complete DMEM was added to the transfection mix before the entire mix was added to cells with fresh complete DMEM. Cells were incubated at 37 °C in a 5% CO_2 atmosphere, and prepared for fluorescent microscopy at 24 hours post-transfection. Fluorescence was detected using a Zeiss Carv Axiovert 100M inverted confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a mercury-arc lamp and a closed circuit detection (CCD) camera. The emGFP was excited at 487 nm, the emitted light collected at 509 nm (Tsien, 1998), and detected with a standard fluorescein iso-thiocyanate (FITC) filter set. The pcDNA™6.2/C-emGFP-CAT vector was also transfected as above into HEK 293T cells to act as a positive control for emGFP expression.

2.3.2.2 Confirmation of Vpu expression by SDS-PAGE and Western blot analysis

Expression of recombinant proteins was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of

transfected cell lysates. HEK 293T cells were transfected with 4 µg pcDNA™6.2/C-emGFP-Vphu as described above, and assayed at 24 hours and 48 hours post-transfection. Mock transfected (empty vector) and untransfected cells were also assayed at these time points. Approximately 1.2 x 10^6 cells were washed and scraped into 1 ml 1X PBS (Sigma-Aldrich), before harvesting at 15 000 x g for 5 min (Eppendorf centrifuge 5810). Cells were resuspended in 50 µl cell lysis buffer and vortexed thoroughly. The cell lysate was centrifuged at 10 000 x g for 10 min at 4 °C, the supernatant transferred to a fresh tube and stored at -80 °C until assayed for protein concentration. Twenty micrograms of cell lysate was resolved on a 12.5% SDS-PAGE gel (see Appendix C3 for details of the following solutions and buffers). Briefly, a 4.5% stacking gel and 12.5% resolving gel were prepared in duplicate, set, and assembled into PAGE apparatus (BioRad) with electrophoresis buffer. Fifteen microlitres each of the sample and the 2X loading dye were added to a 1.5 ml microcentrifuge tube (Eppendorf) and boiled for 5 min before transferring to ice. The mix was loaded onto the gel alongside 10 µl Prestained 6-175 kDa Protein Marker (New England BioLabs) (Appendix D). The gel was run at 10 mA per gel for stacking, and then at 25 mA per gel until the dye front was seen a half centimetre from the bottom of the gel (~2.5 hours).

After electrophoresis, one gel was stained with Coomassie Blue dye to visualise all protein and the other was used for immunoblotting as described below. Coomassie staining was carried out by covering the gel in 500 ml of the dye and shaking gently at

room temperature for three to four hours. The stain was then poured off and the gel submerged in 250 ml destain solution. The destain solution was changed three times and the destaining continued until background staining had been removed. The Coomassie gel was visualised on a white light box and the bands compared to the subsequent Western blot image. Gel images were captured using Quantity One V4.6.1 1D Analysis software (BioRad).

The remaining gel was used for transfer of proteins to Hybond-C Extra Nitrocellulose Membrane (Amersham) using a Trans-Blot® SD Semi Dry Transfer Cell (BioRad), as per manufacturer's instructions. Briefly, the gel was equilibrated in transfer buffer for an hour, and then transferred onto pre-equilibrated Hybond C membrane at a constant current of 60 mA for 35 min. The membrane was then blocked for an hour in 100 ml 5% fat free milk powder dissolved in T-TBS to prevent non-specific antibody binding. The membrane was then rinsed three times with T-TBS, prior to primary antibody binding. The membrane was incubated for one hour with 2 ml of the recommended 1:5000 dilution of the primary antibody HIV-1_{NL4-3} Vpu Antiserum, (AIDS Research and Reference Reagent Program, NIH, Bethesda) before washing three times for 5 min in T-TBS. The membrane was then incubated for one hour in 2 ml of a 1:2000 or a 1:4000 dilution of the Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (Zymed Laboratories, San Francisco, CA, USA), and washed three times as before to remove any unbound HRP-conjugate. Enhanced chemiluminescence (ECL) detection of the protein was performed using

the Pierce SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Briefly, working solutions of the substrates (SuperSignal West Pico Stable Peroxide Solution and Luminol Enhancer Solution) were prepared according to the manufacturers' instructions. Equal volumes of the detection solutions (1 ml each) were mixed and added to the membrane for 1 min. The blot was then covered with clear plastic wrap and placed protein side up on a Kodak X-Omatic cassette (Eastman Kodak Company, Rochester, NY, USA). Fuji Super Rx Medical X-Ray film (Africa X-Ray, Industrial and Medical, La Rochelle, SA) was placed carefully over the covered blot and exposed for 1 min. Standard autoradiographic techniques allowed visualisation of the fusion protein using developing solution and fixative.

2.3.3 Cotransfections of HEK 293T cells with Vphu-emGFP and subcellular localisation plasmids

2.3.3.1 Optimisation of cotransfections

The subtype B and C pcDNA™6.2/C-emGFP-Vphu recombinant plasmids were cotransfected with pDsRed-ER, pDsRed-Mem, or pDsRed-Golgi into HEK 293T cells to confirm the presence of Vpu at different subcellular compartments. The plasmid DNA concentration of all clones was measured by spectroscopy at 260nm and 280nm on a Nanodrop 1000 (Thermo Fisher Scientific) prior to transfection reactions. Transfection reactions were optimised with respect to cell number at seeding, ratio of

Vphu-emGFP to DsRed plasmids, ratio of plasmid DNA concentration to transfection reagent volume, transfection reagent type, and slide type.

The amount of cells seeded was optimised so that they could withstand any toxic effects of the transfection reagent, while avoiding becoming excessively confluent by the 60 hours post-transfection time point. The numbers of cells seeded varied from 5 x 10^6 cells in 25 cm^3 flasks, to 250 000 cells in 2 ml chamber slides. The ratio of pcDNA™6.2/C-emGFP-Vphu to pDsRed was optimised to ensure cells expressed both markers. The ratio was also important in ensuring the brightest signal and the least noise are observed under the confocal microscope. A total of 4 µg plasmid DNA was used and split between the dsRed and Vphu-emGFP plasmids 1:1, 1:2, and 1:3 respectively. The efficiency and cytotoxicity of Polyfect (Qiagen) and Lipofectamine 2000 (Invitrogen) transfection reagents were compared. Reagent mixes were made up according to the manufacturer's instructions, and were used as a starting point for optimisation. The ratio of plasmid DNA concentration to transfection reagent volume was then varied 1:1, 1:2, 1:5.

The best possible method to view the transfected HEK 293T cells under the confocal microscope was determined by seeding the cells in various formats. Cells were transfected in 25 cm^3 flasks, six-well plates, and 5 ml or 2 ml Lab-TeK II Chamber Slides (Nalge Nunc Int.). Cells were then transferred onto glass slides after transfection and then fixed, or fixed in the chamber slides. Cells were also viewed

live, by pipetting 100 µl cell suspension directly onto a glass slide. Transfection efficiency and cell cytotoxicity was evaluated visually by fluorescence microscopy as described above in section 2.3.2.1. The optimal cotransfection protocol was employed for all the subsequent cotransfection experiments, and is described below in section 2.3.3.2.

2.3.3.2 Cotransfection experiments

Transient transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Briefly, cells were seeded onto Lab-TeK II Chamber Slides (Nalge Nunc Int.) 24 hours prior to transfection at 65% confluency (~600 000 cells), in media without antibiotics. Two micrograms each of the Vpu-emGFP (05ZAFV5, 05ZAFV15 or subtype B) and DsRed plasmids (ER, Golgi, or Membrane) was diluted in 200 µl Opti-MEM (Invitrogen) while 4 µl Lipofectamine 2000 was diluted in 200 µl Opti-MEM. Samples were incubated for 5 min at room temperature before combining and briefly vortexing. The mixture was then incubated for 20 min at room temperature to allow complex formation. The reaction mix was added to the cells and made up to 3 ml with DMEM without of antibiotics. Cells were processed for confocal microscopy 24, 48, and 60 hours post-transfection. For cells assayed at 48 and 60 hours, the media was replaced with fresh complete DMEM 24 hours post-transfection. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4 Confocal fluorescence microscopy analysis

2.4.1 Optimisation of cell fixation conditions and confocal microscope settings

Cell fixation conditions were optimised with respect to fixation reagent and incubation time. HEK 293T cells were fixed under three different conditions and the results compared. Firstly, 100 µl pre-cooled 3% formaldehyde in PBS was added to the washed cell monolayer and incubated for 15 min at room temperature. Secondly, 100 µl ice cold 100% methanol was added to cells and incubated for 10 min at -20 °C. Finally, 100 µl 3% paraformaldehyde and 0.02% glutaraldehyde in PBS was added to cells and incubated for 15 min at room temperature. Cells were washed three times with 1X PBS (Sigma-Aldrich) after each incubation, and processed for microscopy as described below. Cell viability was then visually determined by microscopy.

Confocal microscopy settings were optimised with respect to magnification and spectral overlap in multichannel mode. The confocal microscope settings were adjusted to reduce cross emission. This was achieved by adjusting the laser intensity, as well as the gain and offset levels of the photo multiplier tube (PMT). The spinning disk was utilised to increase the sharpness of the image. Optimal cell processing and analysis conditions were used for all subsequent experiments, and are described below.

2.4.2 Cell preparation for confocal microscopy

Transfected and untransfected negative control HEK 293T cells were processed for confocal microscopy at 24, 48, and 60 hours post-transfection. Growth media was removed and the cells were washed briefly with 1X PBS (Sigma-Aldrich) at room temperature. Cells were fixed onto the chamber slides using 200 µl freshly prepared 3% formaldehyde solution, for 15 minutes at room temperature. The fixative was removed and the cells gently rinsed with 1X PBS. The nuclei were stained using 200 µl 100ng/ml DAPI solution (Sigma-Aldrich) in the dark for 15 minutes (Appendix C4). The chamber was removed and coverslips were then mounted onto the slides using 80 µl Fluorsave Reagent (Calbiochem, Darmstadt, Germany) and allowed to set in the dark at room temperature for 60 min.

2.4.3 Detection of Vphu-emGFP fusion proteins and subcellular marker proteins by confocal microscopy

Cells were viewed at each time point using a Zeiss Carv Axiovert 100M inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). The emGFP was excited at 487 nm, and the emitted light collected at 509 nm (Tsien, 1998) and detected with a standard FITC filter set, while the DsRed-Monomer was excited 557nm and the emitted light collected at 585 nm and detected with a standard tetramethyl rhodamine iso-thoicyanate/Phycoerythrin (TRITC/PE) filter set. Fluorescent digital images of cells

identified expressing both fluorescent proteins were collected separately and superimposed in multi-track channel mode (sequential excitation/emission) using the Plan-Neofluar 630x objective or 100x /1.30 Oil objective. Images were processed and analysed using Axiovision 2.0 software (Carl Zeiss). All images were collected under the same photomultiplier detector conditions and pinhole diameter. Untransfected cells and cells transfected with an empty vector were used as negative controls and processed for fluorescent microscopy as described above. Differential interference contrast images were collected simultaneously with fluorescence images using the transmitted light detector. Transfection experiments were repeated five or more times for each subtype construct and eight or more images were examined and captured per slide.

Chapter 3

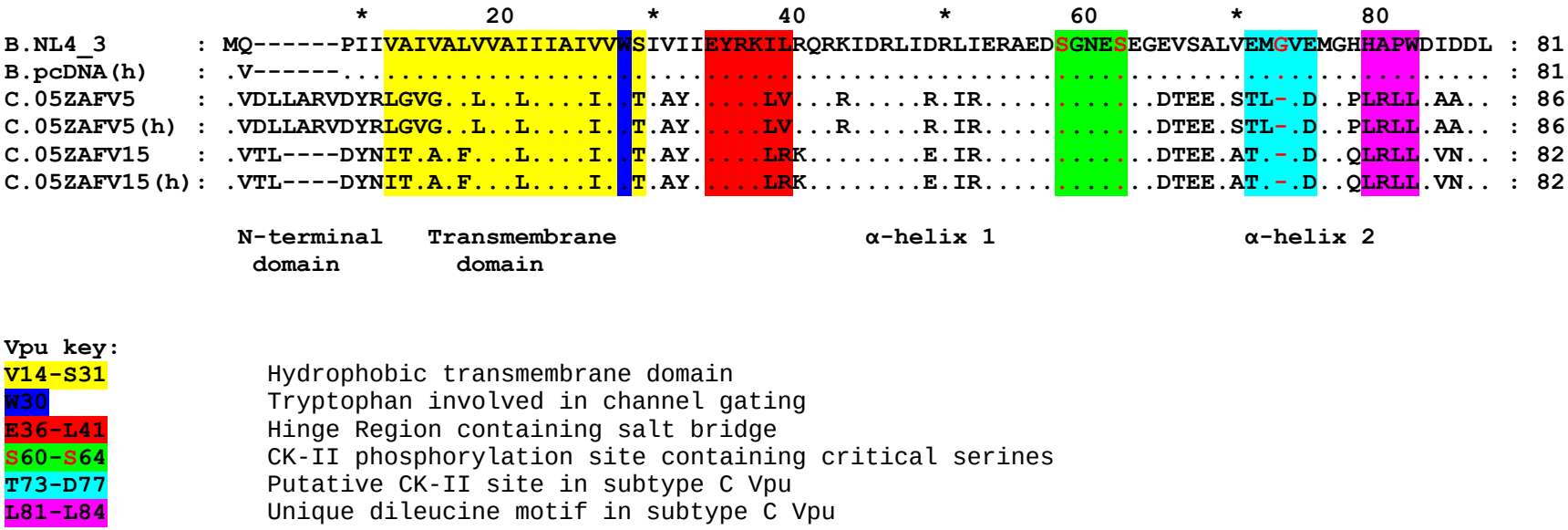
Results

3.1 Viral isolate selection and codon optimisation

Comparison of the previously sequenced HIV-1 subtype C isolates allowed for the selection of the two isolates 05ZAFV5 and 05ZAFV15 used in this study (Bell, Connell et al. 2007). The sequence data distinguished 05ZAFV5 as having a six amino acid insert at the N-terminus and a predicted protein length of 86 amino acids. In contrast, 05ZAFV15 has a two amino acid insert at the N-terminus and is 82 amino acids in length. 05ZAFV5 was therefore selected as a representative subtype C isolate, and 05ZAFV15 was selected because it more closely resembles the 81 amino acid subtype B Vpu in length. The *vpu* genes from these two isolates were codon-optimised for optimal expression in mammalian cells. The subtype B *vphu* is the codon-optimised form of the wild-type *vpu* from the subtype B strain, NL4-3. The predicted amino acid sequences for the native and codon-optimised recombinant clones of subtype B Vphu (pcDNA-Vphu), subtype C 05ZAFV5 (FV5_pCR Script), and 05ZAFV15 (FV15_pCR Script) Vphu are depicted in Figure 3.1. Critical sequence motifs are highlighted in colour.

3.2 PCR optimisation and amplification of *vphu*

PCR primers were designed so that the ~300 bp subtype B and C *vphu* genes could be amplified and directionally cloned into the pcDNA™6.2/C-emGFP-GW/TOPO® vector, in frame with the C-terminal *emGFP* tag. The subtype B and C *vphu* genes



Vpu key:

V14-S31	Hydrophobic transmembrane domain
W30	Tryptophan involved in channel gating
E36-L41	Hinge Region containing salt bridge
S60-S64	CK-II phosphorylation site containing critical serines
T73-D77	Putative CK-II site in subtype C Vpu
L81-L84	Unique dileucine motif in subtype C Vpu

Figure 3.1: An alignment of the deduced amino acid sequences of the wild type and codon-optimised forms of the subtype B NL4-3 Vpu, and the subtype C 05ZAFV5 and 05ZAFV15 Vpu proteins. The sequences are compared to wild type subtype B NL4-3 Vpu (top), and the humanised form is indicated by (h). Dots represent identical residues, and variant residues are shown. The N-terminal domain, transmembrane domain, and cytoplasmic helices are indicated. The critical phosphoserine residues are shown in red lettering. The glycine insertions in the subtype B proteins are also shown in red lettering. Known functional domains and critical residues are highlighted and are annotated in the accompanying key.

were successfully PCR amplified from the pcDNA-Vphu, FV5_pCR Script, and FV15_pCR Script recombinant vectors, respectively, producing an approximately 300 base pair amplicon as seen on a 1.5% agarose gel (Figure 3.2A).

3.3 Construction of Vphu-emGFP fusion proteins

Overall, the subtype B and C *vphu* genes were successfully TA cloned into the pcDNA™6.2/C-emGFP expression vector, and used to transform competent *E. coli* TOP 10 cells. Plasmid DNA from positive clones was purified and characterised by restriction enzyme digestion and sequencing. The pDsRed-Monomer-Golgi, pDsRed2-ER, and pDsRed-Monomer-Mem vectors were successfully transformed into competent *E. coli* TOP 10 cells and the purified plasmid DNA employed in cotransfection experiments.

3.3.1 Characterisation of recombinant clones by restriction enzyme digest and agarose gel electrophoresis

The correct clones were identified by an *Eco*RI restriction enzyme digest. The pcDNA3.1-Vphu was digested by *Eco*RI at position 953 and linearised, identified by a single band at approximately 5.7 kb when resolved on a 0.8% agarose gel (Figure 3.2B, lane 4). The pcDNA™6.2/C-emGFP-Vphu, however, was digested at positions

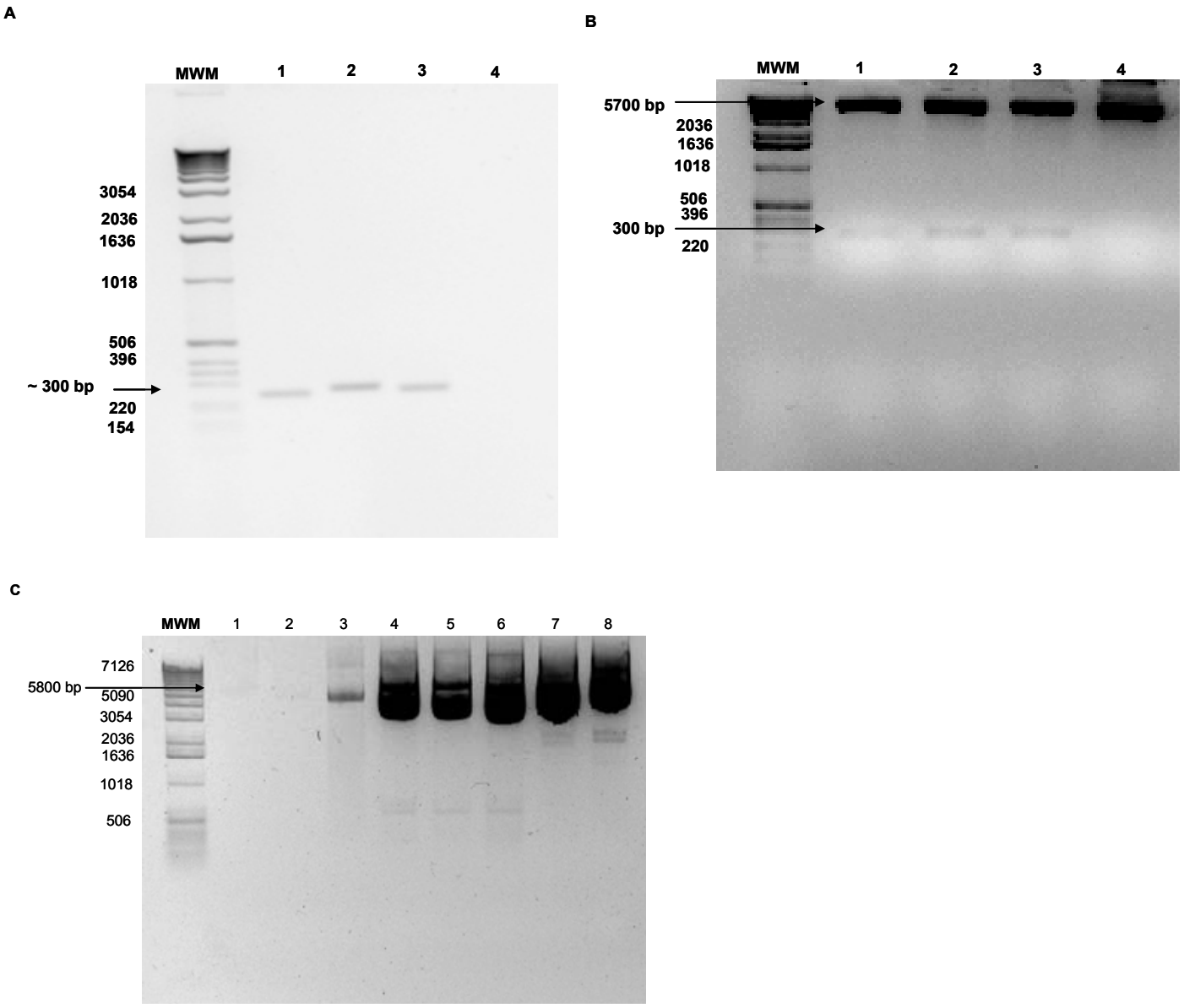


Figure 3.2: Construction of the Vphu-emGFP fusion proteins. (A) Representative image of *vphu* PCR amplicon. Amplicons were resolved by 1.5% agarose gel electrophoresis, at 85 V for 45 min. Lane 1: 05ZAFV5 *vphu*. Lane 2: 05ZAFV15 *vphu*. Lane 3: Subtype B *vphu*. Lane 4: negative control. (B) Confirmation of *vphu* inserts by restriction digest and agarose gel electrophoresis. PcDNA3.1-Vphu and pcDNA™6.2/C-emGFP-Vphu were digested at 37°C for one hour with the restriction enzyme, *EcoRI.* Digests were resolved by 0.8% agarose gel electrophoresis, at 85 V for 1 hour. Lane 1: FV5 pVphu-emGFP. Lane 2: FV15 pVphu-emGFP. Lane 3: SubB pVphu-emGFP. Lane 4: pcDNA3.1-Vphu. (C) Representative image of the plasmids used in this study. Purified plasmid DNA as resolved by 0.8% agarose gel electrophoresis, at 85 V for 1 hour. Lane 1: FV5 pVphu-emGFP. Lane 2: FV15 pVphu-emGFP. Lane 3: Sub B pVphu-emGFP. Lane 4: pDsRed-ER. Lane 5: pDsRed-Goli. Lane 6: pDsRed-Mem. Lane 7: pcDNA6.2-emGFP (empty vector). Lane 8: pCAT-emGFP (positive control). All gel bands were sized in base pairs against Molecular Weight Marker X (MWM) (Appendix D) and viewed by UV transillumination.

882 and 900 into two fragments of approximately 5.9 kb and 0.3 kb in size (Figure 3.2B, lanes 1, 2, and 3).

3.3.2 Characterisation of recombinant clones by cycle sequencing reactions

The purified plasmid DNA for the pcDNA6.2/C-emGFP-Vphu was sequenced using a T7 promoter forward primer and a FP2 reverse primer before extensive analysis. True expression clones were verified as having the *vphu* insert, in the correct orientation, with no stop codon, and in frame with the *emGFP* gene. One clone each for subtype B, and the subtype C 05ZAFV5 and 05ZAFV15 isolates was selected for further studies. Plasmids were resolved on a 0.8% agarose gel and their sizes confirmed, with the subtype B and C pcDNA6.2/C-emGFP-Vphu sitting at approximately 6.1 kb, pDsRed-ER at 4.7kb, pDsRed-Golgi at 4.9 kb, and pDsRed-Mem at 5.3 kb (Figure 3.2C). The positive control plasmid, pcDNA6.2/N-emGFP/GW/CAT (6.5 kb) and the empty vector, pcDNA6.2/C-emFP (5.8 kb) are also shown in Figure 3.2C.

3.4 Transfections of mammalian cell lines

3.4.1 Confirmation of Vphu expression by fluorescence microscopy and western blot analysis

Transfection efficiencies of each expression clone (namely, subtype B pVphu-emGFP, subtype C 05ZAFV5 and 05ZAFV15 pVphu-emGFP, pDsRed-ER/Golgi/Mem) were carried out in HEK 293T cells using Polyfect reagent (Qiagen). The empty vector, pcDNA6.2/C-emGFP was used as a negative control by mock transfection to evaluate the results. Optimum transfection conditions were obtained when overnight cultures of 1.2 x 10^6 cells in a 25 cm^3 culture flask were incubated with a transfection mix of 4 µg plasmid DNA in 150 µl unsupplemented DMEM, and 40 µl Polyfect. Optimised transfection reactions resulted in the successful expression of functional fusion proteins and subcellular localisation proteins in HEK 293T cells. Functional proteins were observed by fluorescence in the cells 24 hours post-transfection, under a Zeiss Carv Axiovert 100M inverted confocal microscope using the 100X oil immersed objective. Single transfections of HEK 293T cells by FV5 Vphu-emGFP (Figure 3.3A), FV15 Vphu-emGFP (Figure 3.3B), or subtype B Vphu-emGFP (Figure 3.3C) resulted in nodes of bright green fluorescence 24 hours post transfection in the perinuclear region. The positive control, pcDNA6.2/C-emGFP/GW/CAT, also showed bright green fluorescence at 24 hours post-transfection (Figure 3.3D), but the expression is seen as a more diffuse pattern

throughout the cell. Transfection with the empty vector, pcDNA6.2/C-emGFP results in no fluorescence (Figure 3.3E). Cell integrity remained intact following transfection and fixing as seen with a transmitted light phase image (Figure 3.3F). Expression of the DsRed vectors was also confirmed by single transfection reactions in HEK 293T cells. The plasmids were observed to successfully localise to the ER (Figure 3.4A), the Golgi apparatus (Figure 3.4B), and cellular membranes (Figure 3.4C) as observed by confocal microscopy 24 hours post-transfection.

Expression was also demonstrated by glycine-containing SDS-PAGE and Western blotting of HEK 293T cell lysates 24 hours and 48 hours post-transfection with subtype B and C pcDNA™6.2/C-emGFP-Vphu (Figure 3.5, Lanes 1, 2, and 3). Mock transfected (empty vector) and untransfected cells were also assayed (Figure 3.5, Lanes 4). Vpu has an apparent molecular mass of 16 kDa in glycine-containing SDS-PAGE gels, or 9 kDa in Tricine containing SDS-PAGE gels (Schubert, Henklein et al. 1994). Blotting with HIV-1_{NL4-3} Vpu rabbit antiserum and subsequent visualisation by ECL resulted in a positive signal from a band at 43 kDa representing the 16 kDa Vphu protein fused to the 27 kDa emGFP protein (Figure 3.5B). Significant background was noted using the Vpu rabbit antiserum at the recommended dilution, and the HRP-conjugated goat anti-rabbit IgG secondary antibody at dilutions of 1:2000 and 1:4000. This is commonly observed when blotting with polyclonal antiserum. The expression of subtype B Vphu-emGFP (Figure 3.5B, Lanes 3) appears to be more efficient than

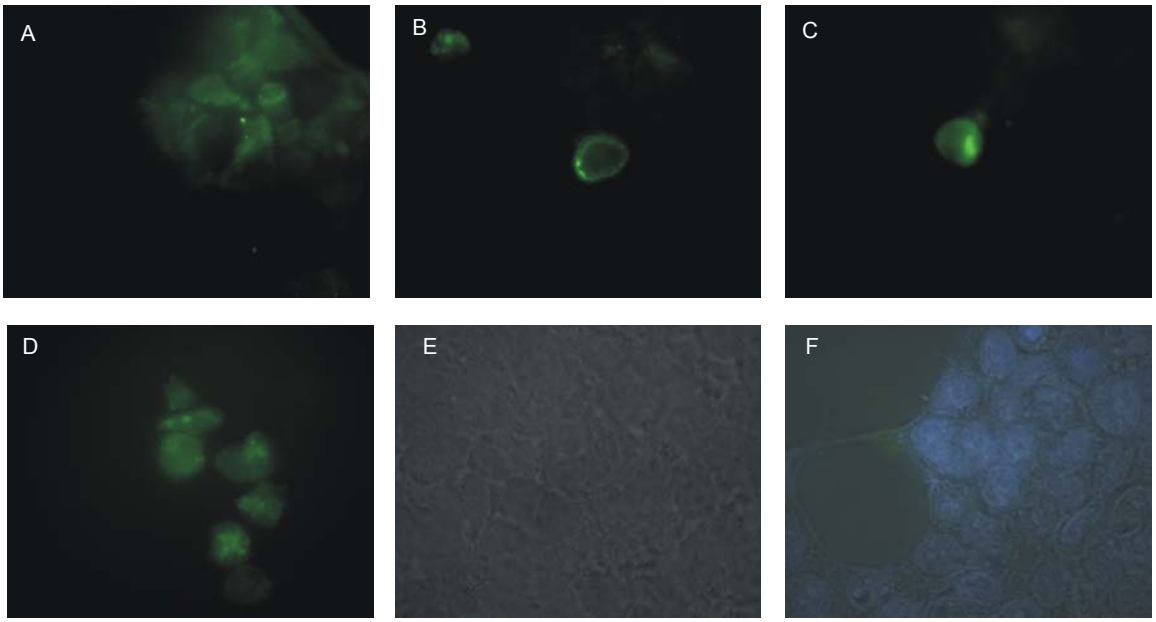


Figure 3.3: Expression of functional Vphu-emGFP subtype B (A) subtype C 05ZAFV5 (B) or 05ZAFV15 pcDNA™6.2/C-emGFP-Vphu (C), positive control plasmid, pcDNA™6.2/C-emGFP/CAT, (D), negative control empty pcDNA™6.2/C-emGFP plasmid (E), and untransfected cells (F), at 24 hours post-transfection of HEK293T cells, as viewed by confocal microscopy. Expressed protein is seen as green. Nuclei are stained blue in (F). Representative fluorescent digital images were collected using the Plan-Neofluor 100x /1.30 Oil objective and analysed using Axiovision 2.0 software. Panel E and F show the transmitted light phase image merged with the fluorescent micrograph to highlight the presence and integrity of the cells.

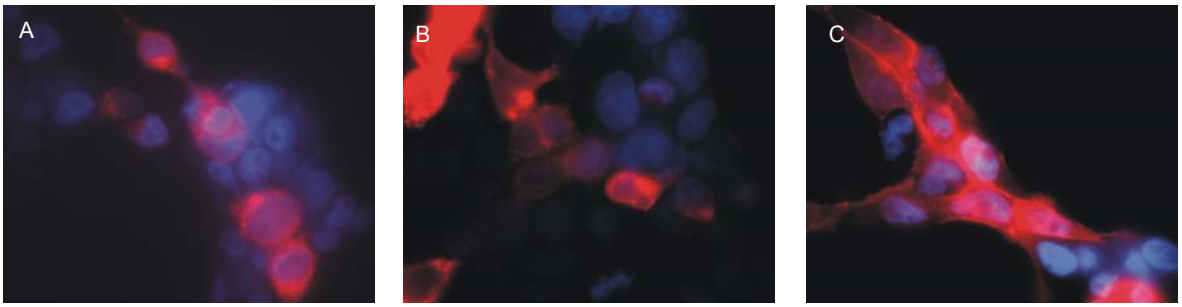


Figure 3.4: Expression of functional DsRed subcellular localisation proteins. HEK 293T cells were transfected with vectors expressing a subcellular localisation protein fused to the fluorescent protein, DsRed. At 24 hours post-transfection, cells expressing DsRed were identified and fluorescence micrographs were collected using a confocal microscope. Images represent cells transfected with either pDsRed-ER (A) pDsRed-Golgi (B) or pDsRed-Mem (C). Expressed DsRed proteins are seen as red. Nuclei are stained blue. Representative fluorescent digital images of DsRed expression were collected using the Plan-Neofluor 100x /1.30 Oil objective and analysed using Axiovision 2.0 software.

the subtype C fusion-proteins (Figure 3.5B, Lanes 1 and 2) at 24 hours and 48 hours post-transfection. Expression also remains relatively stable from 24 hours to 48 hours showing no significant increase or decrease. No protein was detected when cells were transfected with an empty vector (Figure 3.5B, Lanes 4).

3.4.2 Optimisation of cotransfection experiments

The subtype B or C pcDNA™6.2/C-emGFP-Vphu recombinant plasmids were cotransfected with pDsRed-ER, pDsRed-Golgi, or pDsRed-Mem into HEK 293T cells to confirm the presence of Vpu at different subcellular compartments. Cotransfection reactions were optimised with respect to cell number at seeding, ratio of pVphu-emGFP to pDsRed, ratio of plasmid DNA concentration to transfection reagent volume, transfection reagent, and slide type. Transfection efficiency and cell cytotoxicity was evaluated visually by fluorescence and light microscopy respectively, as described in section 3.4.1 (Figure 3.6). Lipofectamine 2000 was the preferred reagent as it caused less cytotoxicity than Polyfect when applied to the cells alone. The amount of cells seeded varied from 5 x 10^6 cells in 25 cm^3 flasks, to 250 000 cells in 2ml chamber slides (Table 3.1). Cells seeded at greater than 60% confluency resulted in excessive confluence by 60 hours post-transfection, with the majority of the cells detached and floating in the media. In contrast, the transfection mix had a detrimental effect on cells seeded at less than 60% confluency. Cells seeded onto 2

ml Lab-TeK II Chamber Slides (Nalge Nunc Int.) demonstrated the best adherence. The ratio of pcDNA™6.2/C-emGFP-Vphu to pDsRed was optimised to ensure a large percentage of cells expressed both markers. The ratio was also important in ensuring that the brightest signal and the least noise were observed under the confocal microscope. A total of 4 µg plasmid DNA was used and split between the dsRed and Vphu-emGFP plasmids at a ratio of 1:1. Optimisation of cotransfections is outlined in Table 3.1.

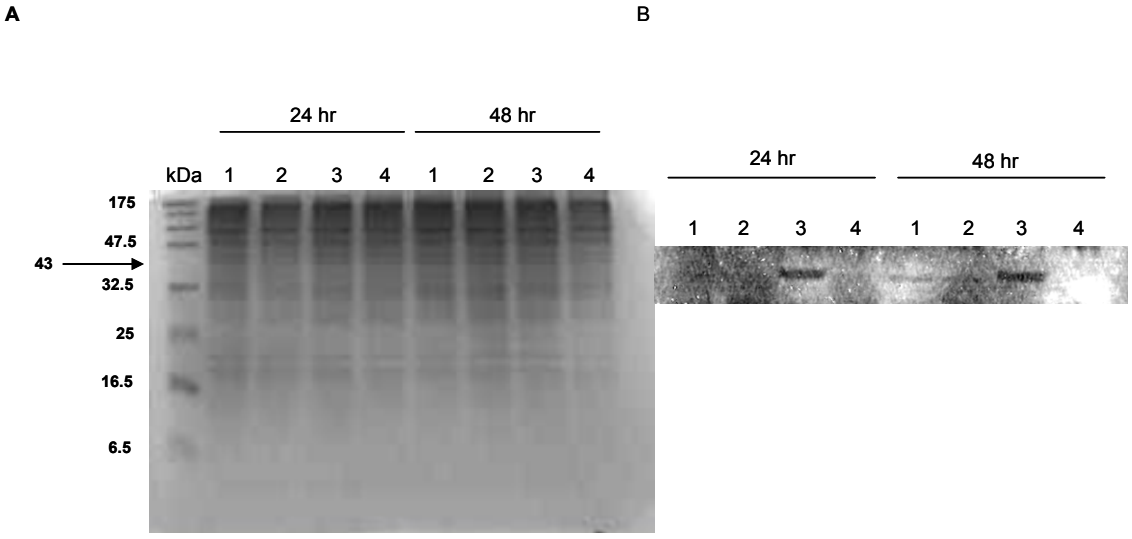


Figure 3.5: SDS-PAGE (A) and Western blot analysis (B) of HEK 293T cell lysates transfected with subtype B, or subtype C pcDNA™6.2/C-emGFP-Vphu, or an empty vector for 24 and 48 hours. Recombinant protein expression (approximately 43 kDa) was detected using HIV-1 NL4-3 Vpu rabbit antiserum. The protein marker in kDa is indicated on the left (Appendix D). Lane 1: FV5 pVphu-emGFP. Lane 2: FV15 pVphu-emGFP. Lane 3: Sub B pVphu-emGFP. Lane 4: Mock transfected.

3.5 Confocal microscopy analysis to determine subcellular localisation

3.5.1 Optimisation of confocal microscopy experimental parameters

Cell fixation conditions were optimised with respect to cell type, fixation reagent, and incubation time and temperature. HEK 293T cells were fixed onto glass slides as well as treated Lab-TeK II Chamber Slides under three different conditions (Table 3.2). The subsequent cell viability and integrity was visually determined by light microscopy. The HEK 293T cells demonstrated a tendency to wash off or shrink when treated with methanol, or incubated with 3% formaldehyde for longer than 15 minutes. Incubation with 3% paraformaldehyde and 0.02% glutaraldehyde preserves external structures, but results in similar stability as 3% formaldehyde, which was chosen as the optimal reagent. Cells were also suspended in a drop of glycerol, which maintained the integrity of both cell types, but caused the cells to float in different planes of view causing difficulty when focussing on a single cell. Figure 3.6 illustrates the difference in cell viability under optimal and suboptimal transfection and fixation conditions.

Table 3.1: Optimisation of mammalian cell cotransfection experiments with subtype B and C pcDNA6.2/C-emGFP-Vpu and pDsRed-ER/Golgi/Mem. The table shows examples of plasmid ratios and transfection reagents used to increase transfection efficiency. Optimal efficiency is shown in bold.

Transfection format	Total pDNA (µg/ml)	pDNA ratio (pEmGFP:pDsRed)	Number of cells seeded (x 10^6)	Transfection reagent	Reaction volume (ml)	Transfection efficiency 24 hr post-transfection (%)*
25 cm^3 culture flask	10	1:1	5.4	100 µl Polyfect	8	20
	10	2:1	5.4	100 µl Polyfect	8	20
Single chamber slide	4	1:1	2.5	40 µl Polyfect	3	40
	2	1:1	2	20 µl Polyfect	3	50
	3	2:1	0.6	40 µl Polyfect	3	50
Double chamber slide	4	1:1	0.25	2 µl lipofect	2	50
	4	1:1	0.25	4 µl Lipofect	2	60
	4	1:1	0.6	2 µl Lipofect.	2	60
	4	**1:1**	**0.6**	**4 µl Lipofect.**	**2**	**80**
6 well plate	2	1:1	0.6	2 µl Lipofect	3	50
	4	1:1	0.6	4 µl Lipofect	3	60
	4	1:1	0.6	20 µl Polyect	3	50
	4	1:1	0.6	40 µl Polyfect	3	10

*Transfection efficiency was determined by manually counting cells with fluorescence as a percentage of the total number of cells on slide.

Table 3.2: Optimisation of fixation conditions for preparation of cotransfected HEK 293T mammalian cells for confocal microscopy

Fixation reagent	Incubation time	Incubation temperature	Cell viability (48 hr post-transfection)*
Ice cold 100 % Methanol	10 min	-20 °C	100% HEK 293T cells were shrunken and internal organelles difficult to distinguish.
Precooled 3% Formaldehyde	15 min	Room temperature	Some cells wash off, but 60% remain fixed to surface.
3% Paraformaldehyde and 0.02% glutaraldehyde	15 min	Room temperature	60% cells wash off the slide.
Glycerol	Indefinitely	Room temperature	All cells are viable but float in different planes of view.

*Cell viability determined by manually counting adherent cells compared to untreated cells.

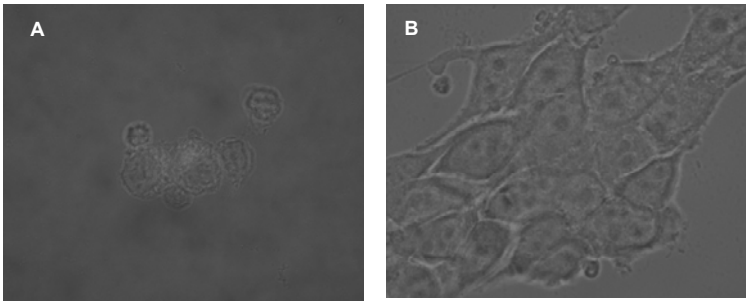


Figure 3.6: HEK 293T cells treated under suboptimal (A), and optimal (B) transfection and fixing conditions, as viewed by confocal microscopy. HEK 293T cells in (A) were transfected using Polyfect reagent, and fixed with 100% methanol. Cells in (B) were transfected with Lipofectamine reagent and fixed with 3% formaldehyde. Transmitted light phase images were collected using the Plan-Neofluor 100x /1.30 Oil objective and analysed using Axiovision 2.0 software.

3.5.2 Subcellular localisation of subtype B and C Vphu proteins

The three Vphu-emGFP recombinant plasmids were successfully cotransfected with pDsRed-ER, pDsRed-Mem or pDsRed-Golgi into HEK 293T cells to confirm the presence of Vpu at different subcellular compartments over time. Cells were fixed with 3% formaldehyde and examined at 24, 48 and 60 hours post cotransfection using a Zeiss Carv Axiovert 100M inverted confocal microscope. The images were sequentially acquired using the Plan-Neofluar 63x or 100x /1.30 Oil objective and superimposed in multitrack channel mode to show colocalisation. Representative digital images were collected and analysed using Axiovision 2.0 software. The cells were viewed immediately after the transfection reaction (0 hours) to ensure cell viability and to note background fluorescence, which was minimal. Transfection

experiments for each subtype construct were repeated five or more times, and eight or more images were examined per slide.

Optimised cotransfection reactions resulted in the successful expression of both subtype B and C Vphu-emGFP fusion proteins, and subcellular localisation proteins in at least 50% of HEK 293T cells (Figures 3.7, 3.8 and 3.9). The single colour channels show green pixels representing Vphu-emGFP expression, and red pixels representing the DsRed subcellular localisation proteins. Figures C, F, I, L, O, R, U, X and ¥ represent the overlay of the green (FITC) and red (PE) channels, which result in a yellow colour when the proteins have colocalised.

Extensive analysis of cotransfections revealed that at 24 hours post-transfection (Figures 3.7.1-3, 3.8.1-3, and 3.9.1-3), the subtype C FV5 Vphu-emGFP protein was in the cytoplasm, with definite localisation at the ER and Golgi (Figure 3.7.1C, and 3.7.2F), but not the plasma membrane (Figure 3.7.3I). The subtype C FV15 Vphu-emGFP protein appeared to have weaker localisation at the ER and Golgi at 24 hours post-transfection (Figure 3.8.1C and 3.8.2F), and no plasma membrane localisation (Figure 3.8.3I). In contrast, the subtype B Vphu-emGFP protein had strong Golgi (Figure 3.9.2F) as well as plasma membrane localisation (Figure 3.9.3I) at 24 hours post-transfection.

At 48 hours post-transfection (Figures 3.7.4-6, 3.8.4-6, and 3.9.4-6), FV5 Vphu-emGFP was still strongly localised at the ER and Golgi (Figure 3.7.4L and 3.7.5O), but some protein was observed at the plasma membrane (Figure 3.7.6R, and arrows in Figure 3.7.5O). FV15 Vphu-emGFP displays ER, Golgi and plasma membrane localisation at 48 hours post-transfection (Figures 3.8.4L, 3.8.5O, and 3.8.6R). Subtype B Vphu-emGFP was clearly localised at the ER at 48 hours post-transfection (Figure 3.9.4L), with some fluorescence seen at the plasma membrane (Figure 3.9.6R). At 60 hours post-transfection (Figures 3.7.7-9, 3.8.7-8, and 3.9.7-9), the Vphu-proteins appear as large accumulations in the shrunken HEK 293T cells. The FV5 Vphu-emGFP was observed at the plasma membrane (Figure 3.7.9¥), while FV15 and subtype B Vphu-emGFP showed ER-localisation at 60 hours post-transfection (Figures 3.8.7U and 3.9.7U). A composite summary of this localisation data is included in Table E1 (Appendix E).

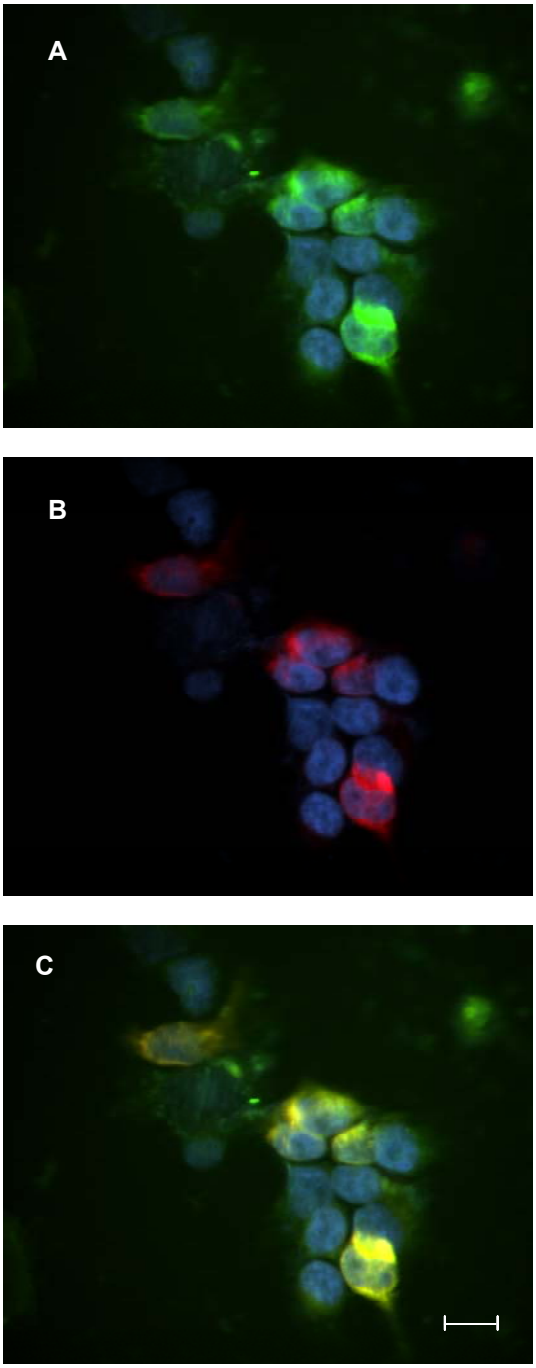


Figure 3.7.1: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 24 hours post co-transfection of HEK 293T cell with dsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (A) and dsRed-ER fluorescence in red (B). An overlay of these images (C) shows colocalisation in yellow.

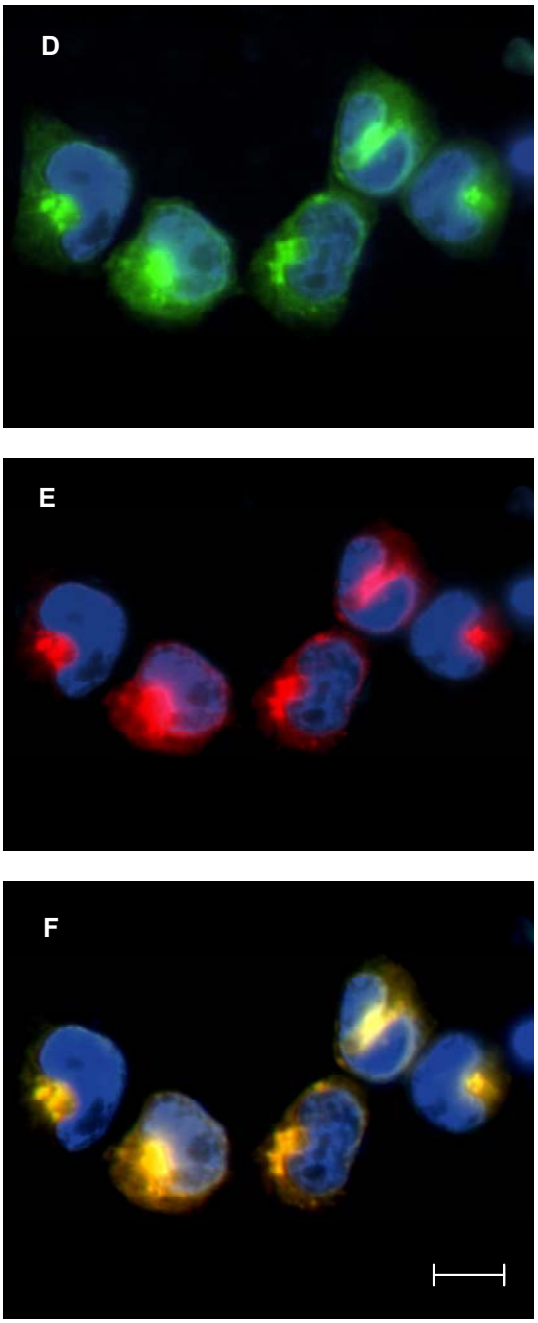


Figure 3.7.2: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 24 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (D) and dsRed-Golgi fluorescence in red (E). An overlay of these images (F) shows colocalisation in yellow.

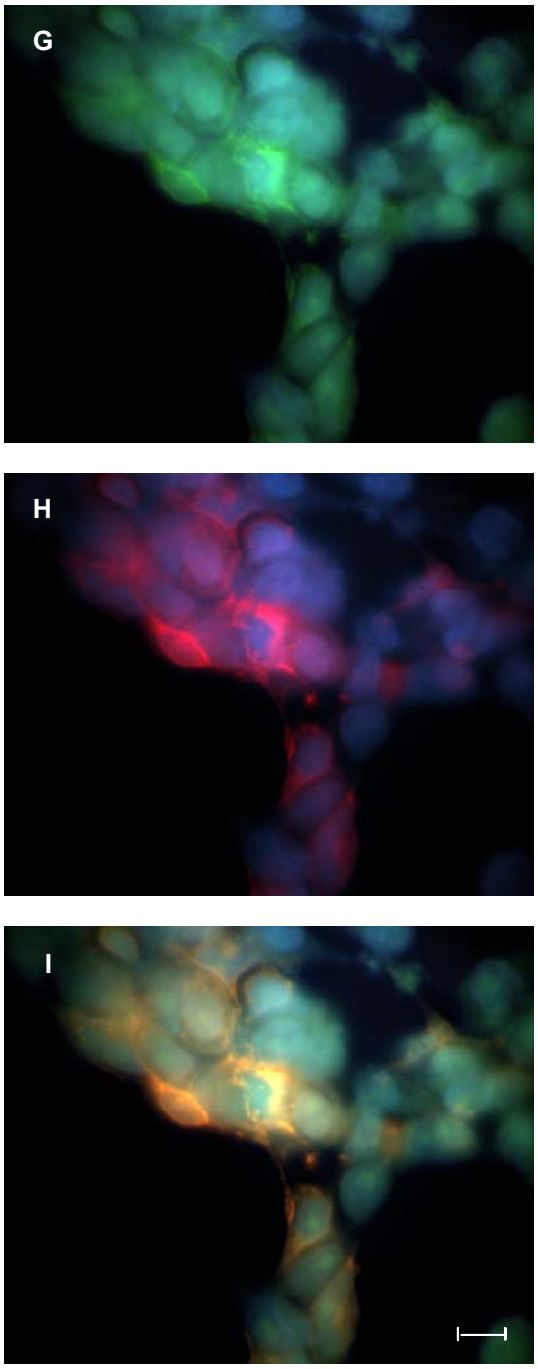


Figure 3.7.3: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 24 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (G) and dsRed-Mem fluorescence in red (H). An overlay of these images (I) shows colocalisation in yellow.

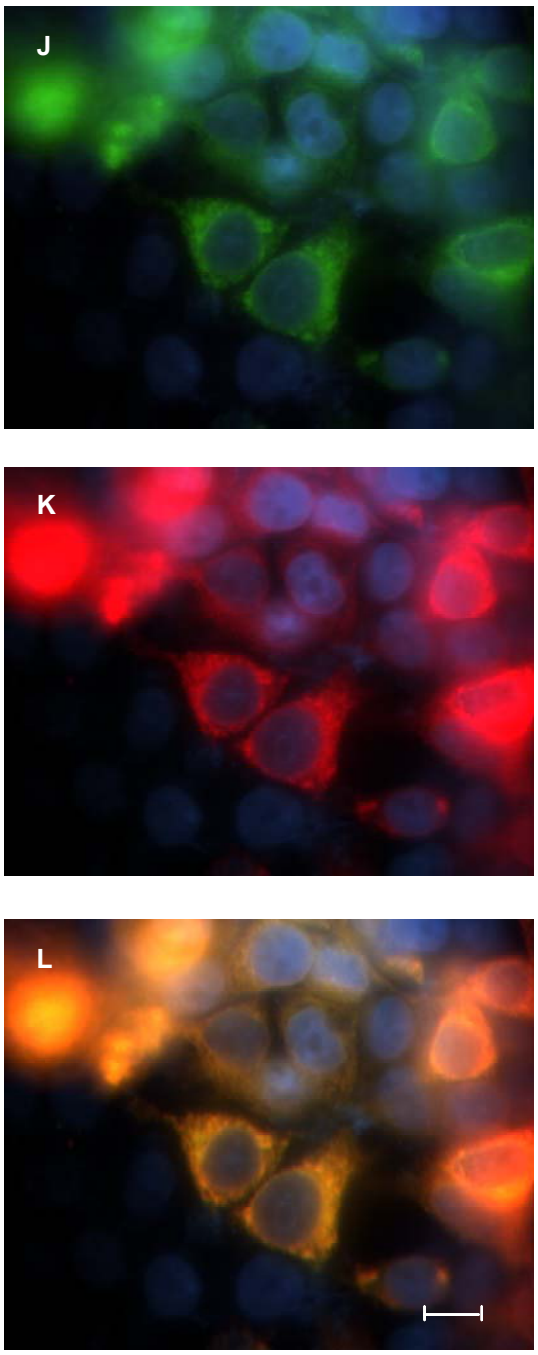


Figure 3.7.4: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 48 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (J) and dsRed-ER fluorescence in red (K). An overlay of these images (L) shows colocalisation in yellow.

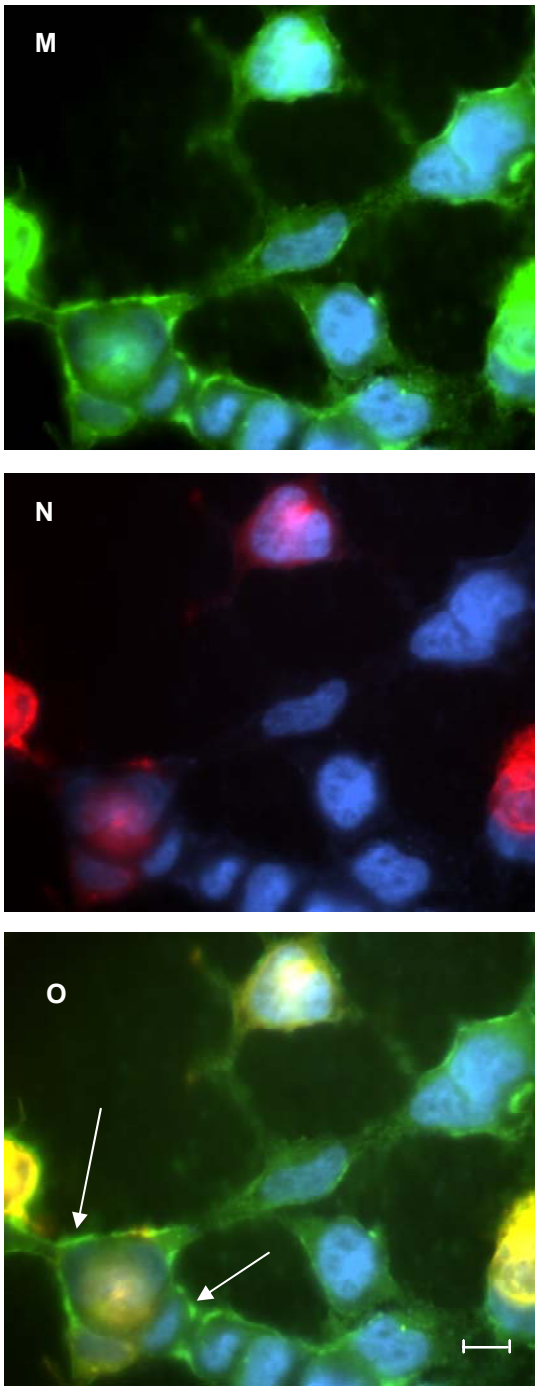


Figure 3.7.5: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 48 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (M) and dsRed-Golgi fluorescence in red (N). An overlay of these images (O) shows colocalisation in yellow. Arrows indicate Vphu-emGFP expression at the plasma membrane.

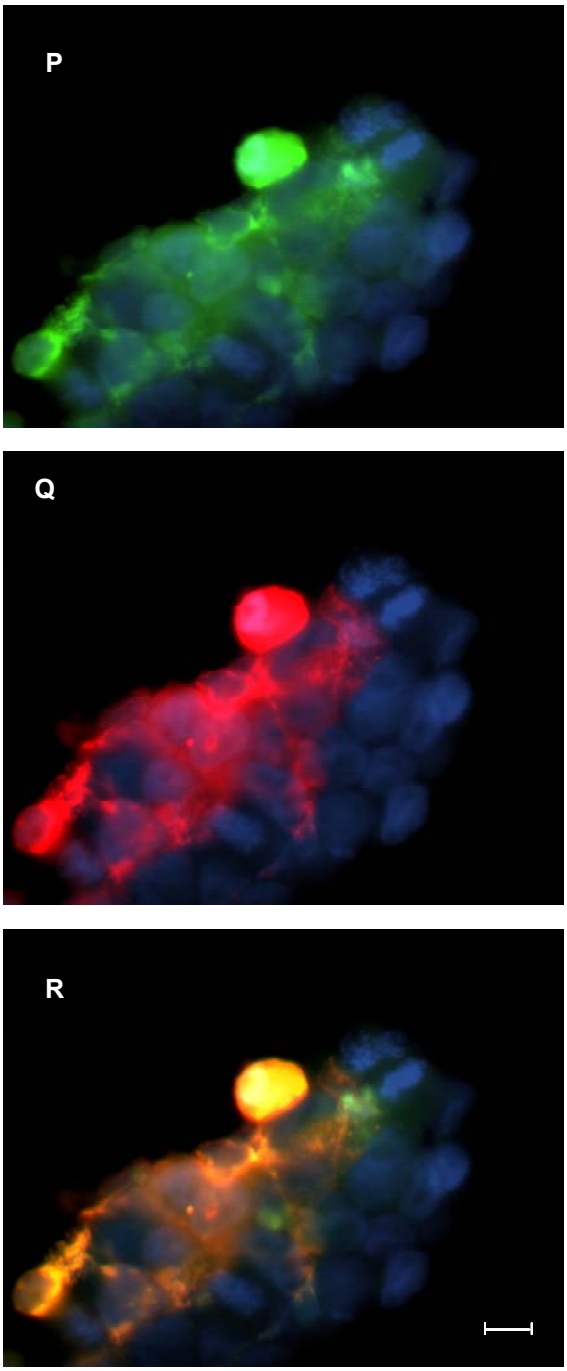


Figure 3.7.6: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 48 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (P) and dsRed-Mem fluorescence in red (Q). An overlay of these images (R) shows colocalisation in yellow.

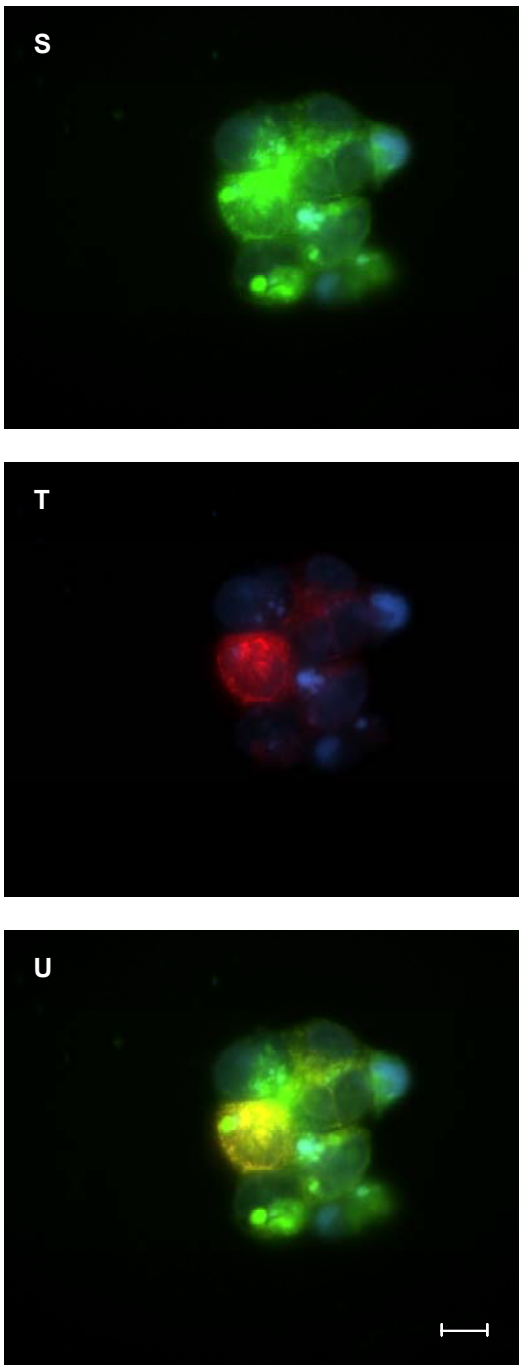


Figure 3.7.7: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 60 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X) . Single colour channels show FV5 Vphu-emGFP fluorescence in green (S) and dsRed-ER fluorescence in red (T). An overlay of these images (U) shows colocalisation in yellow.

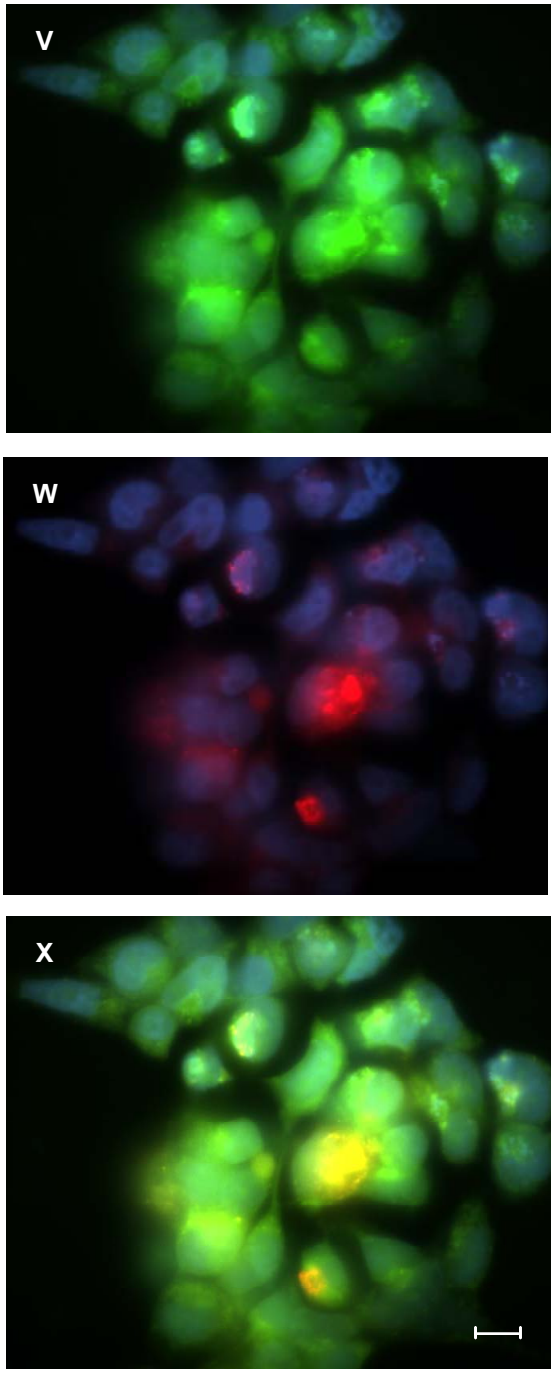


Figure 3.7.8: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 60 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (V) and dsRed-Golgi fluorescence in red (W). An overlay of these images (X) shows colocalisation in yellow.

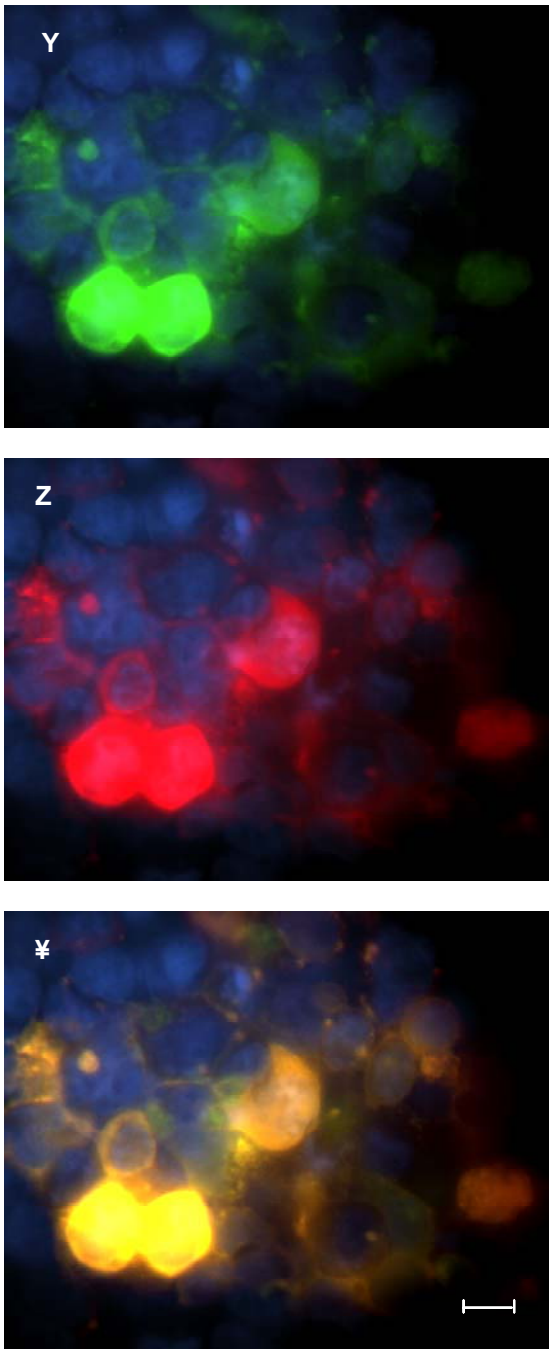


Figure 3.7.9: Subcellular localisation of HIV-1 subtype C FV5 Vphu-emGFP at 60 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (Y) and dsRed-Mem fluorescence in red (Z). An overlay of these images (¥) shows colocalisation in yellow.

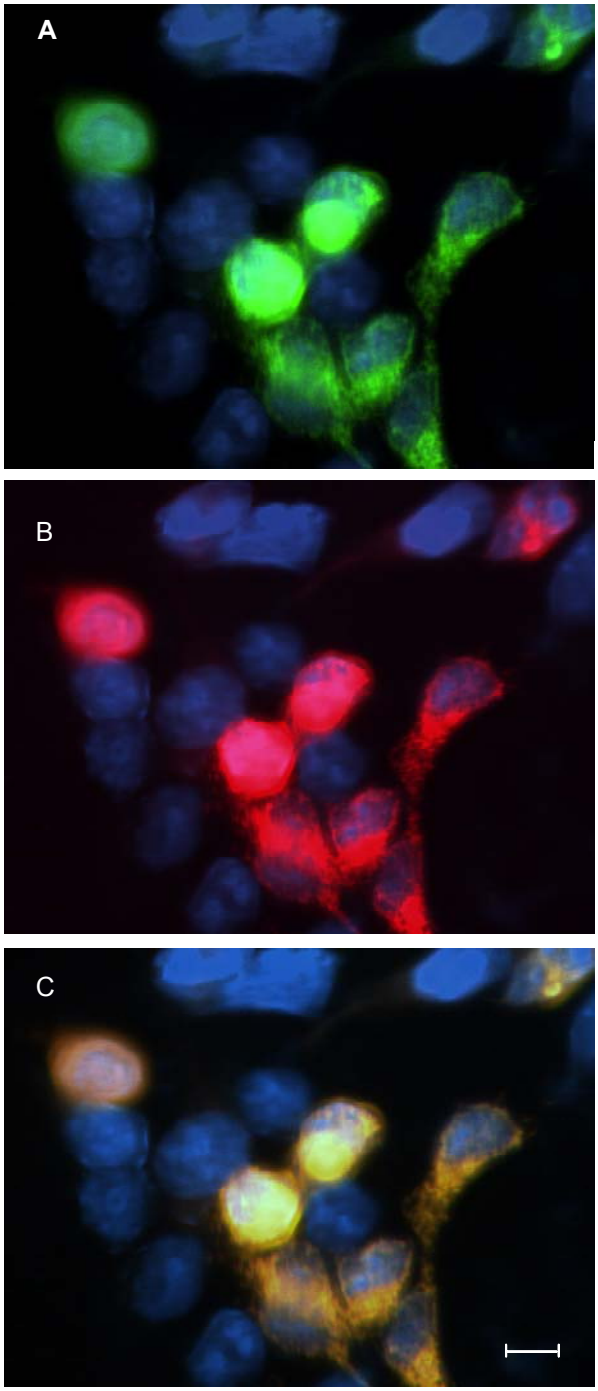


Figure 3.8.1: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 24 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (630X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (A) and dsRed-ER fluorescence in red (B). An overlay of these images (C) shows colocalisation in yellow.

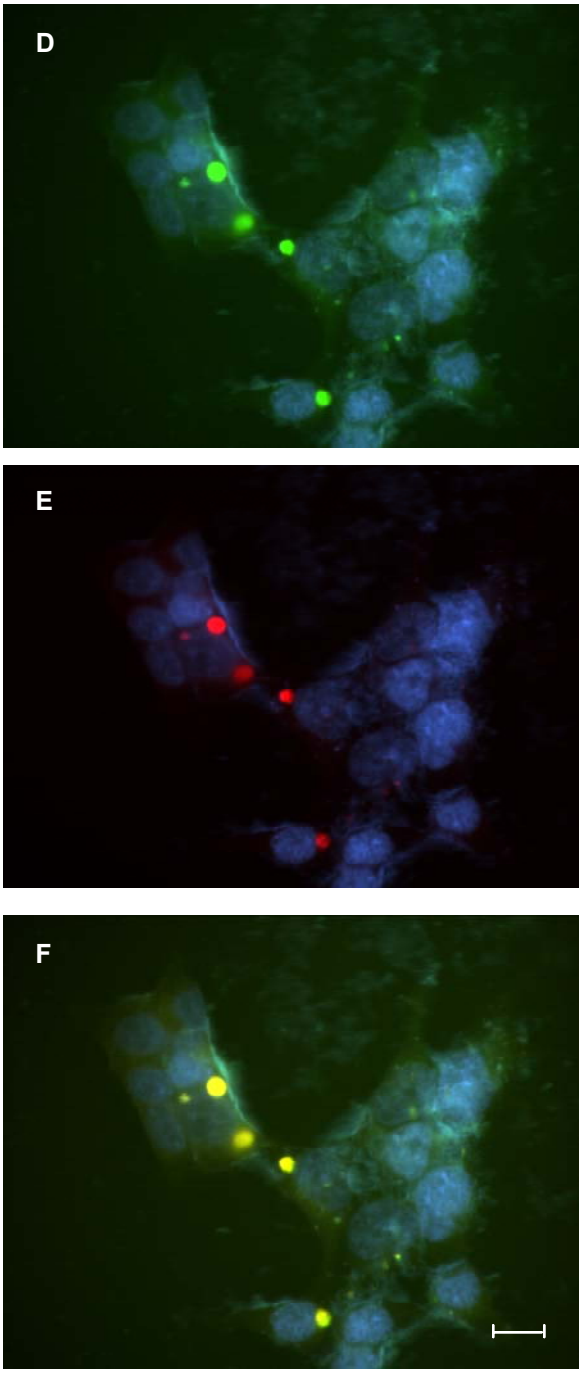


Figure 3.8.2: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 24 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (630X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (D) and dsRed-Golgi fluorescence in red (E). An overlay of these images (F) shows colocalisation in yellow.

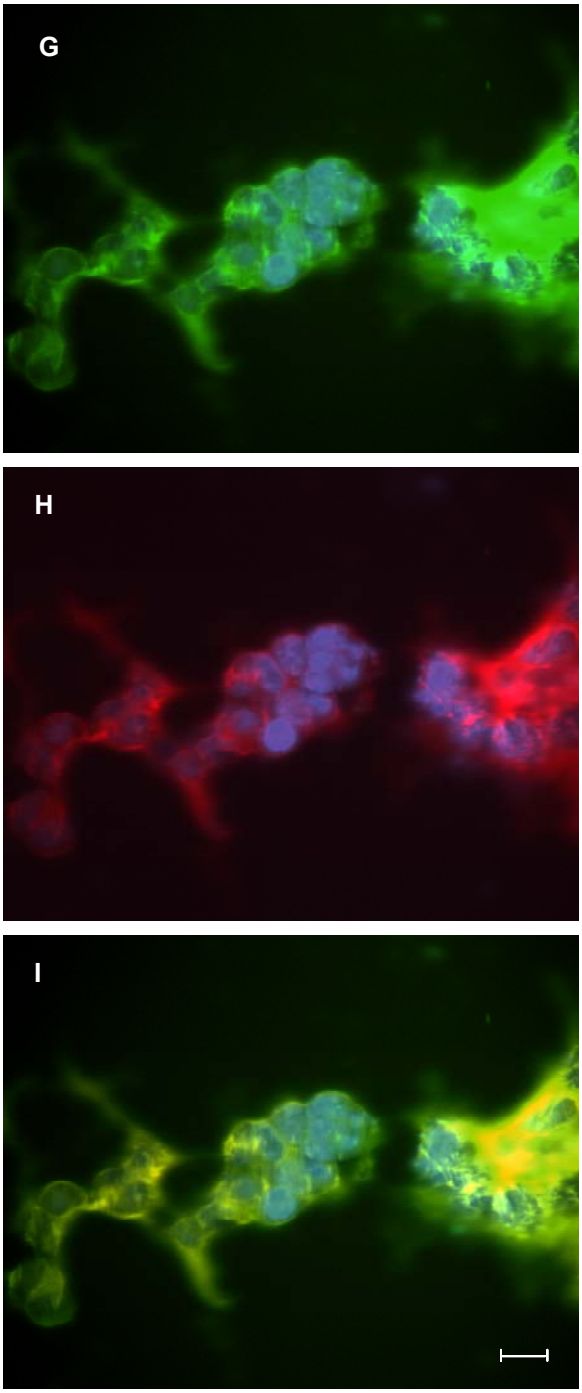


Figure 3.8.3: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 24 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (630X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (G) and dsRed-Mem fluorescence in red (H). An overlay of these images (I) shows colocalisation in yellow.

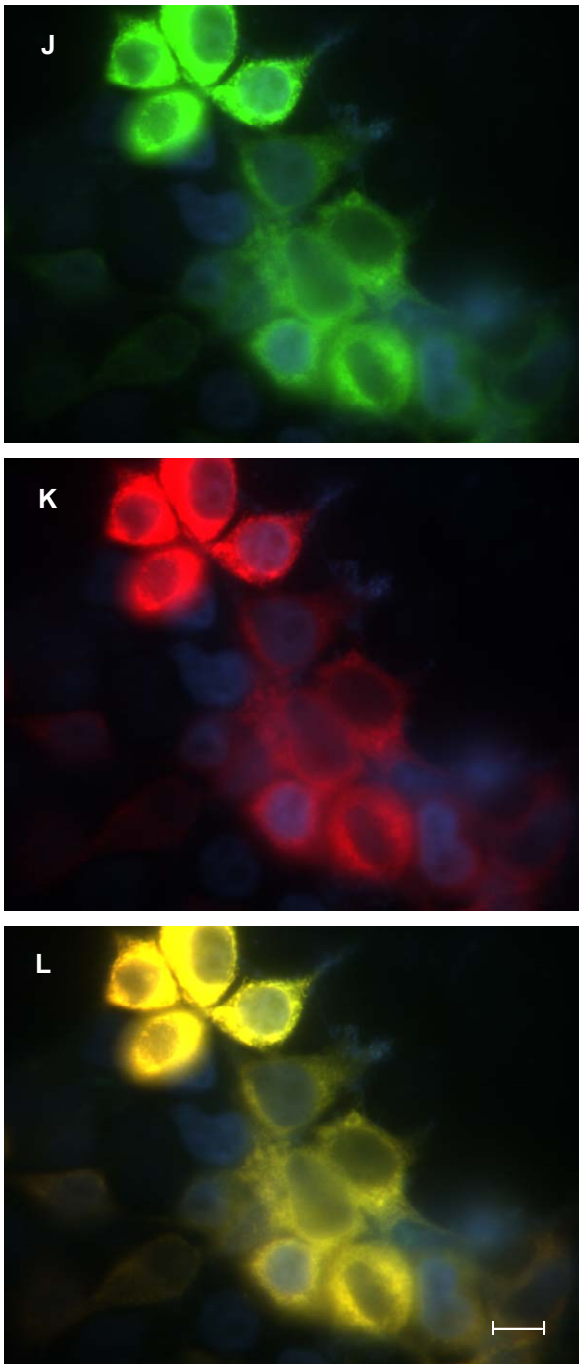


Figure 3.8.4: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 48 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (J) and dsRed-ER fluorescence in red (K). An overlay of these images (L) shows colocalisation in yellow.

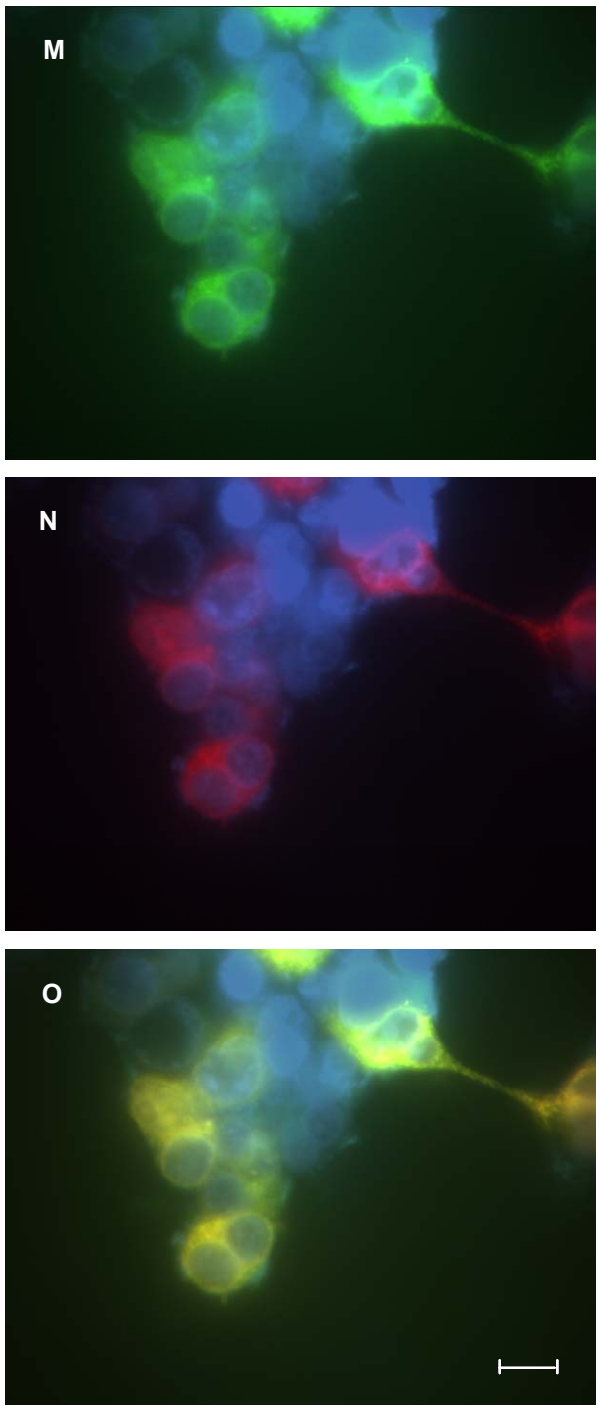


Figure 3.8.5: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 48 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (M) and dsRed-Golgi fluorescence in red (N). An overlay of these images (O) shows colocalisation in yellow.

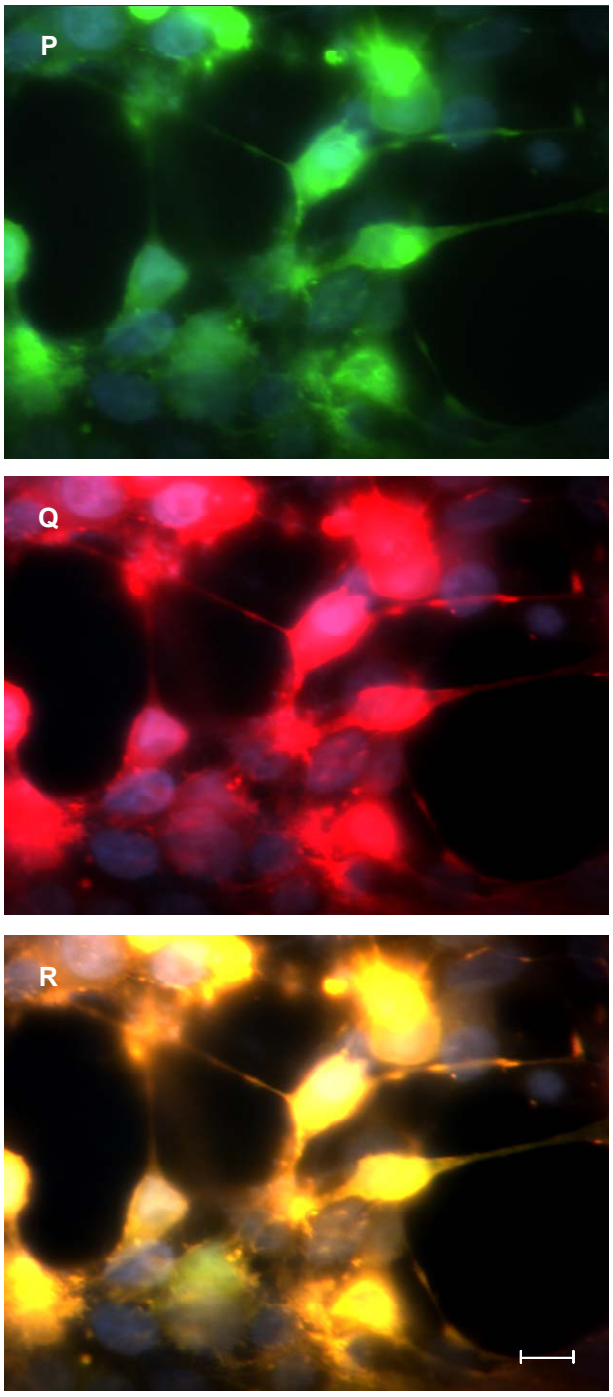


Figure 3.8.6: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 48 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (P) and dsRed-Mem fluorescence in red (Q). An overlay of these images (R) shows colocalisation in yellow.

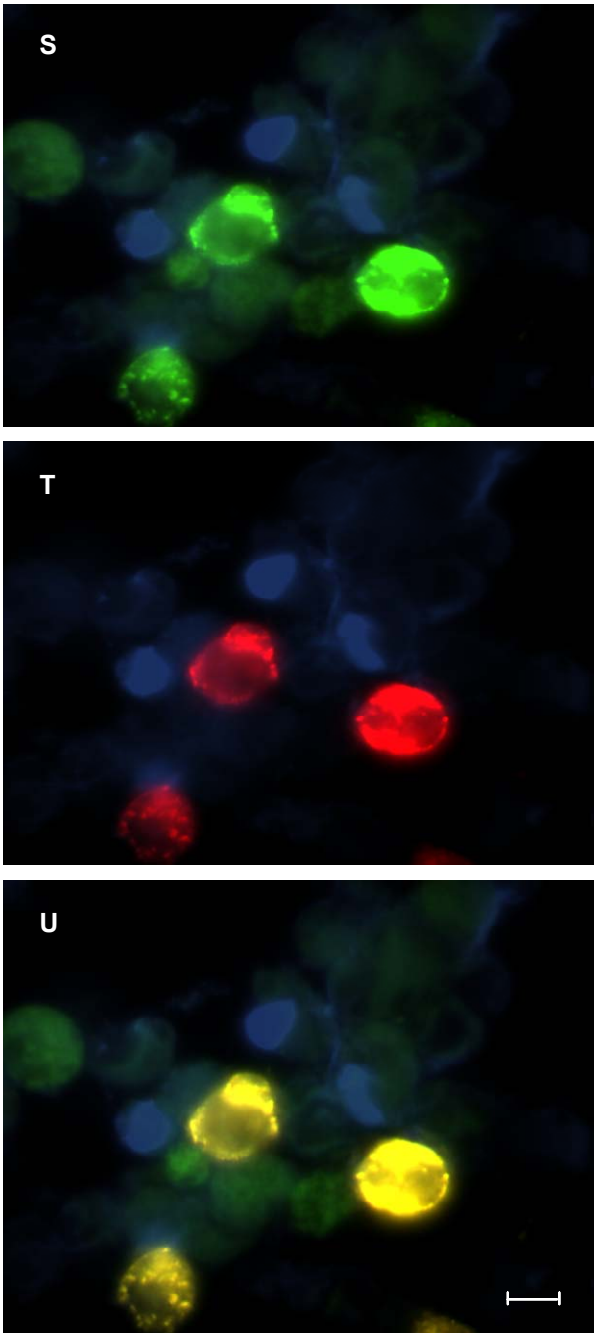


Figure 3.8.7: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 60 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (S) and dsRed-ER fluorescence in red (T). An overlay of these images (U) shows colocalisation in yellow.

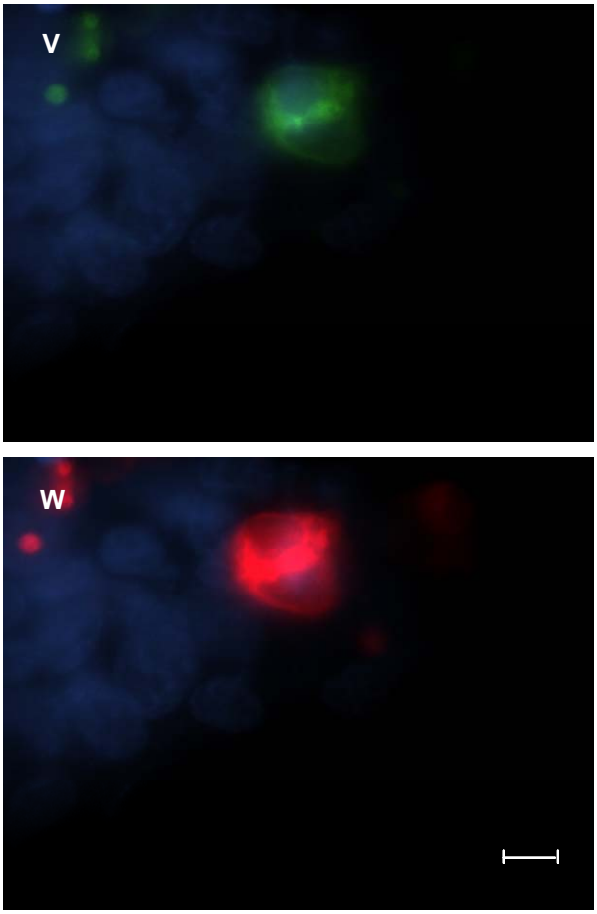


Figure 3.8.8: Subcellular localisation of HIV-1 subtype C FV15 Vphu-emGFP at 60 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (V) and dsRed-Golgi fluorescence in red (W).

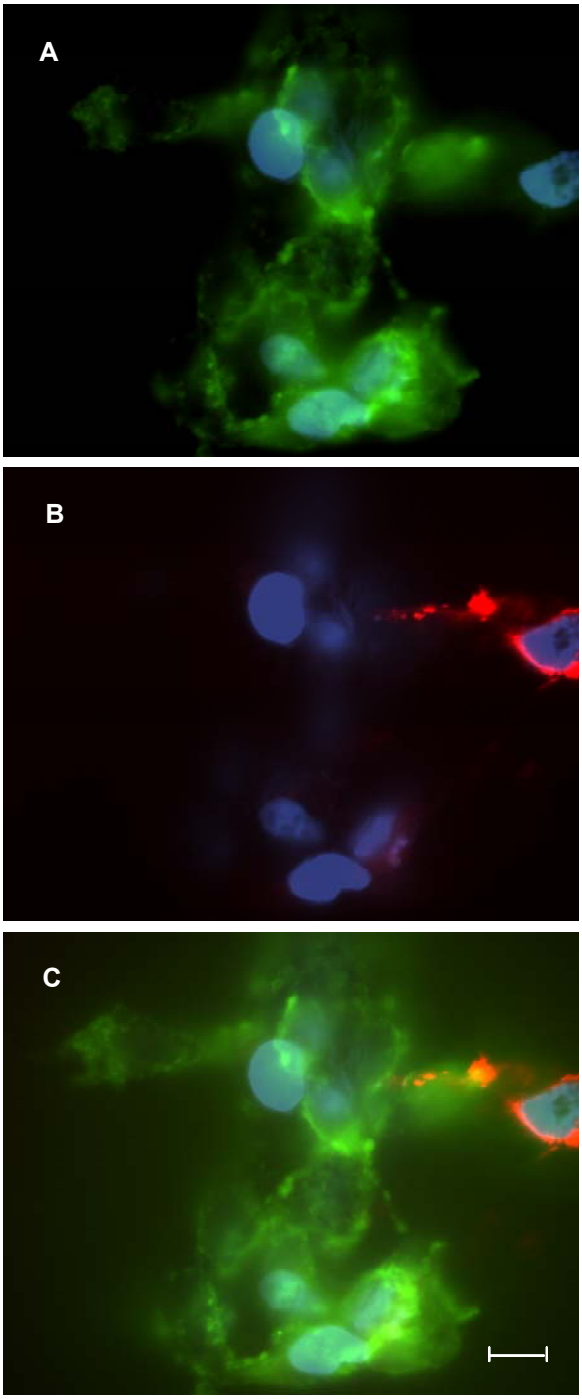


Figure 3.9.1: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 24 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (A) and dsRed-ER fluorescence in red (B). An overlay of these images (C) shows colocalisation in yellow.

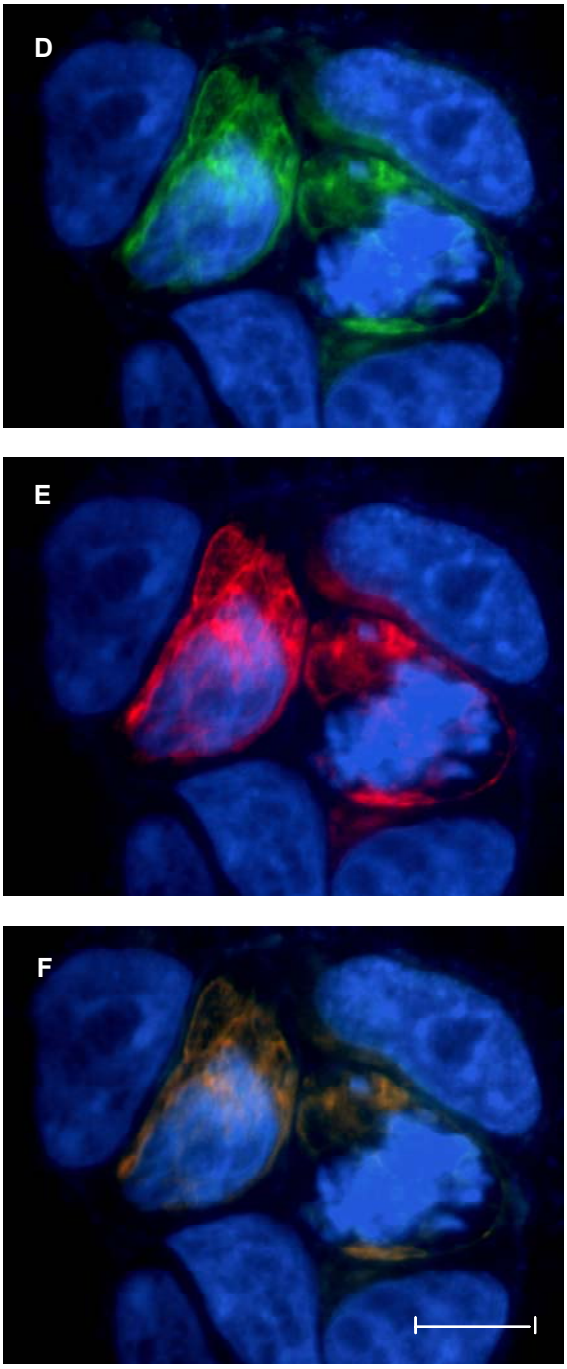


Figure 3.9.2: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 24 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (D) and dsRed-Golgi fluorescence in red (E). An overlay of these images (F) shows colocalisation in yellow.

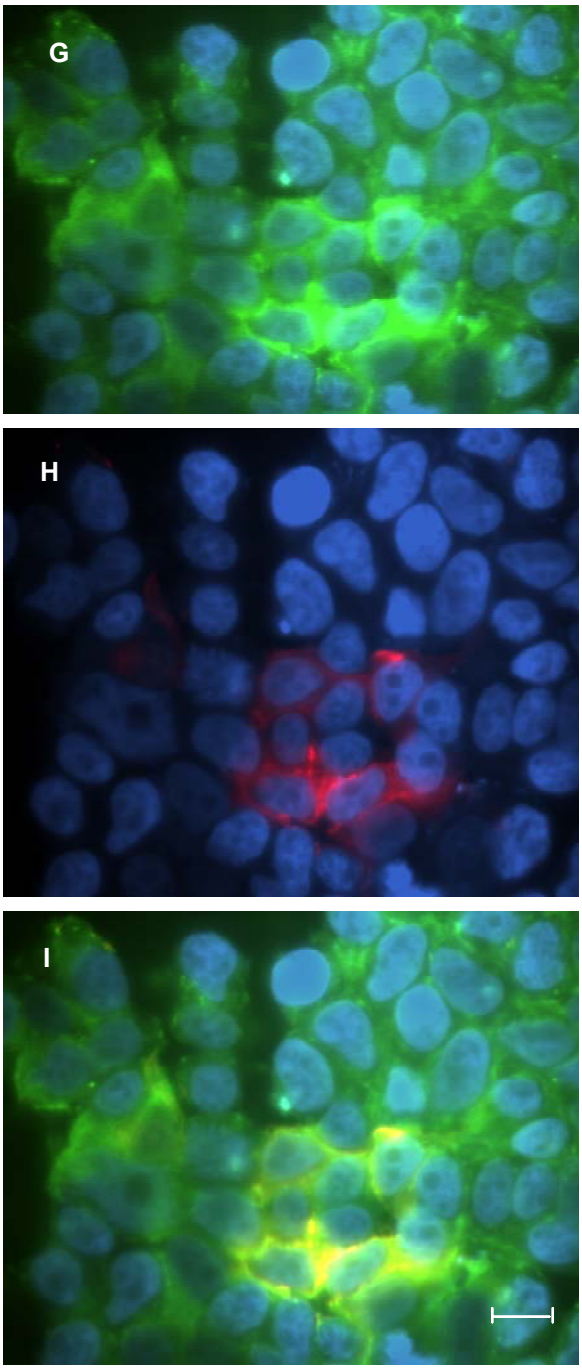


Figure 3.9.3: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 24 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (G) and dsRed-Mem fluorescence in red (H). An overlay of these images (I) shows colocalisation in yellow.

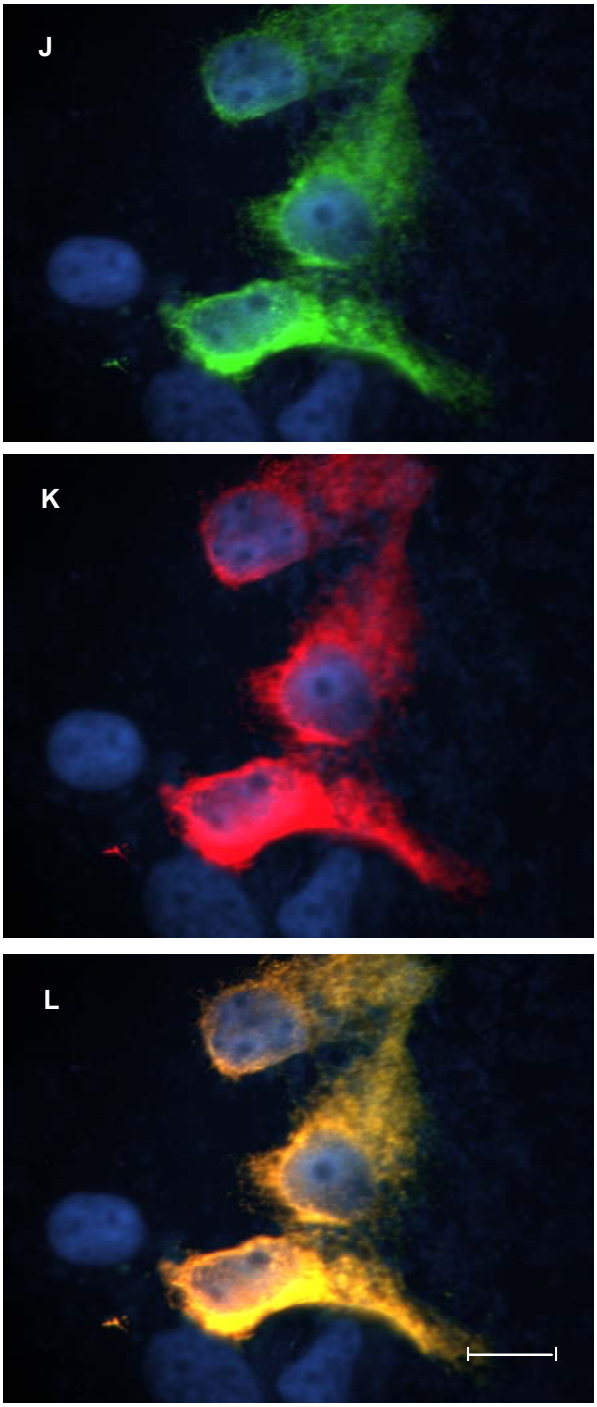


Figure 3.9.4: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 48 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (J) and dsRed-ER fluorescence in red (K). An overlay of these images (L) shows colocalisation in yellow.

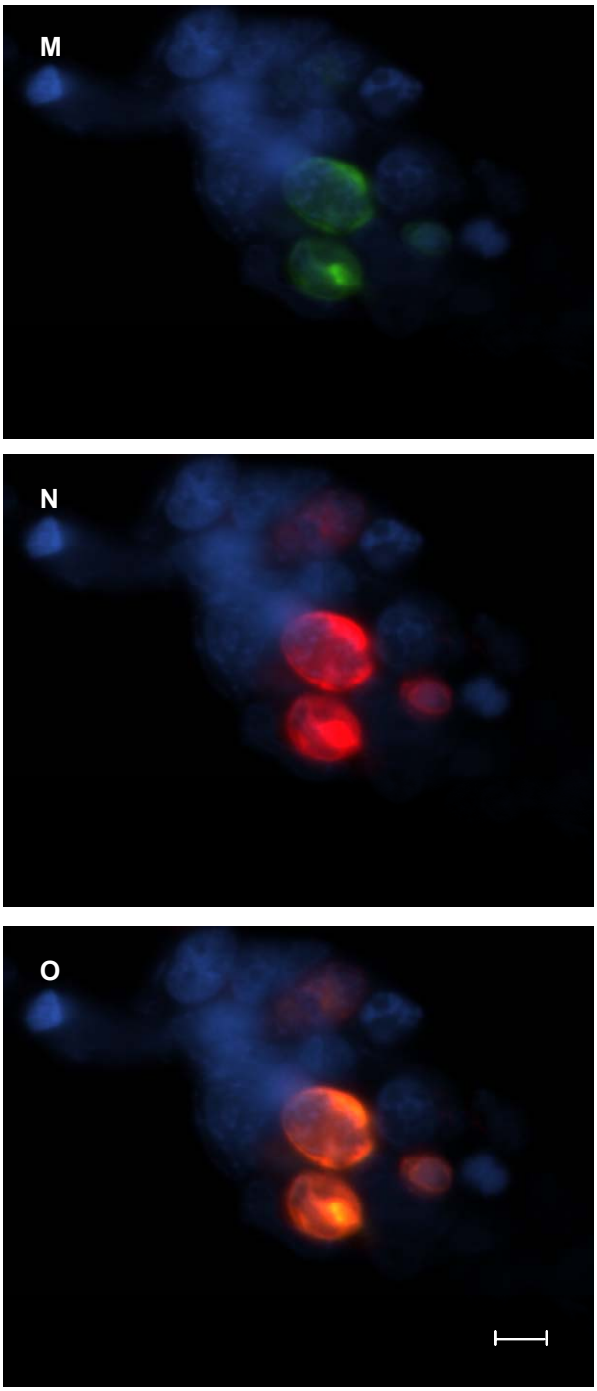


Figure 3.9.5: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 48 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (M) and dsRed-Golgi fluorescence in red (N). An overlay of these images (O) shows colocalisation in yellow.

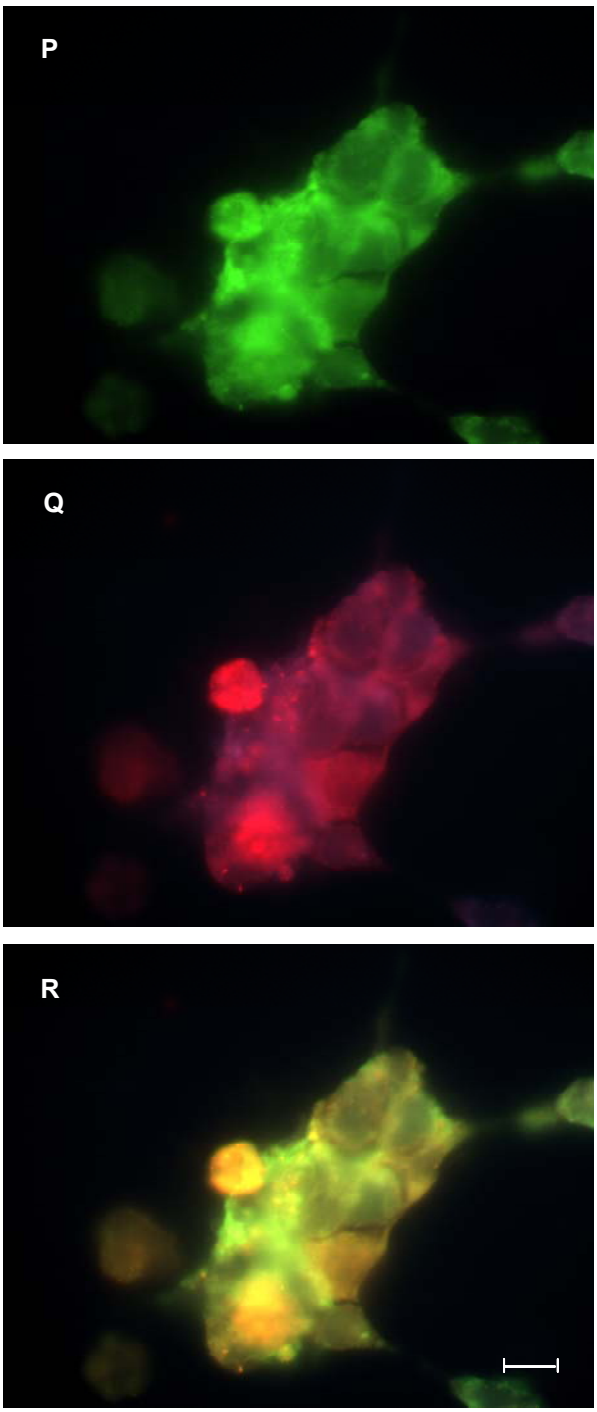


Figure 3.9.6: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 48 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (P) and dsRed-Mem fluorescence in red (Q). An overlay of these images (R) shows colocalisation in yellow.

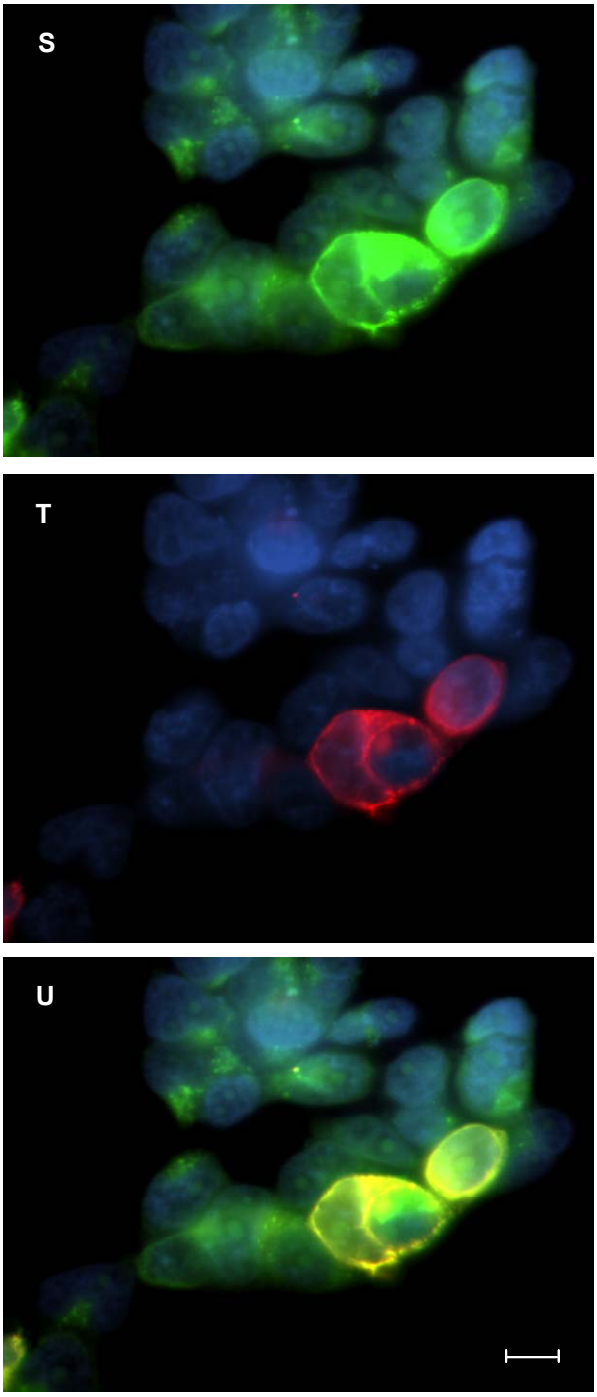


Figure 3.9.7: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 60 hours post co-transfection with pDsRed-ER as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (S) and dsRed-ER fluorescence in red (T). An overlay of these images (U) shows colocalisation in yellow.

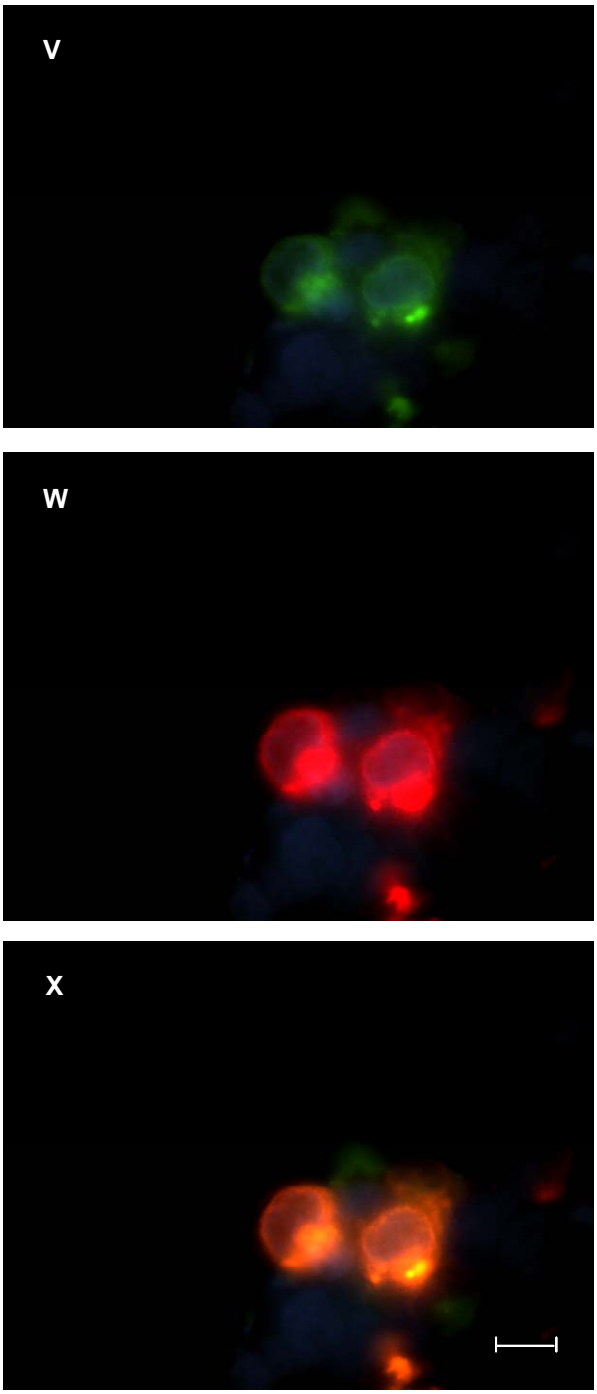


Figure 3.9.8: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 60 hours post co-transfection with pDsRed-Golgi as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (V) and dsRed-Golgi fluorescence in red (W). An overlay of these images (X) shows colocalisation in yellow.

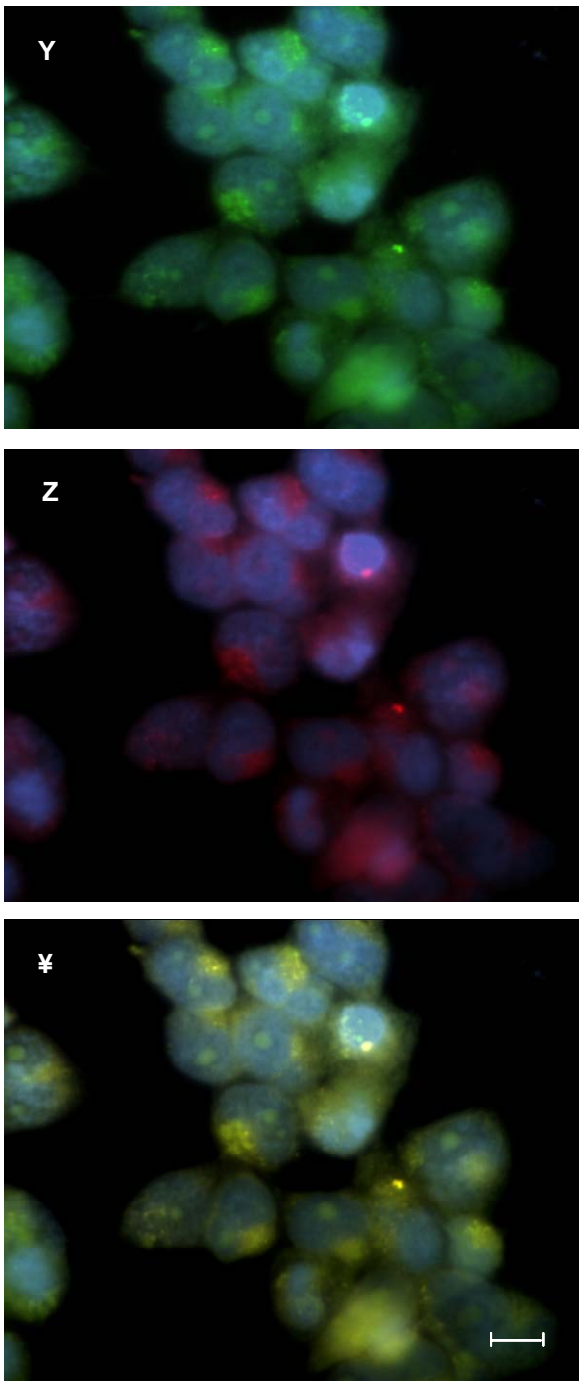


Figure 3.9.9: Subcellular localisation of HIV-1 subtype B Vphu-emGFP at 60 hours post co-transfection with pDsRed-Mem as determined by confocal microscopy. Nuclei are stained blue. Size bars represent 10 microns in measurement (1000X). Single colour channels show FV5 Vphu-emGFP fluorescence in green (Y) and dsRed-Mem fluorescence in red (Z). An overlay of these images (¥) shows colocalisation in yellow.

Chapter 4

Discussion

The HIV-1 subtype B and C Vpu accessory proteins, which are involved in CD4 degradation and virion release, were found to localise to different cellular compartments (the ER/Golgi apparatus and plasma membrane, respectively) when compared at 48 hours (Pacyniak, Gomez et al. 2005). The localisation of the Vpu proteins has an impact on its function in the HIV-1 life cycle. This study confirmed these findings, and in addition showed that subtype B Vpu can localise to the plasma membrane and subtype C Vpu can localise to the ER/Golgi apparatus across the time course. Analysis of subcellular localisation was achieved by cloning representative subtype B and C *vpu* into an emerald-GFP reporter vector and monitoring the movement of these proteins in HEK 293T mammalian cells up to 60 hours.

Two HIV-1 subtype C *vpu* genes were selected for this study because of the distinct N-terminal insertions (McCormick-Davis, Dalton et al. 2000; Scriba, Treurnicht et al. 2001; Bell, Connell et al. 2007). Existing sequence data distinguished 05ZAFV5 as having a six amino acid insertion at the N-terminus and a predicted protein length of 86 amino acids (Bell, Connell et al. 2007). By contrast, 05ZAFV15 has a two amino acid insert at the N-terminus and is 82 amino acids in length. 05ZAFV5 was therefore selected as a representative subtype C isolate, and 05ZAFV15 was selected because it more closely resembled the representative 81 amino acid subtype B Vpu chosen for this study (NL4-3 isolate).

Comparison of the native and codon optimised Vpu sequences cloned into the pcDNA™6.2/C-emGFP-GW/TOPO® vectors showed identical predicted amino acid sequences, except for the presence of a V at position eight in the subtype B Vpu instead of a Q as observed in the wild-type NL4-3 strain (Figure 3.1, see Appendix F for single-letter amino acid code). Valine has a hydrophobic side chain instead of the hydrophilic group of glutamine. This change is not likely to impact on the protein's ability to localise in the membrane since it may only contribute to the already hydrophobic TMD.

Extensive sequence analysis and identification of known functional domains revealed that the NTD, the TMD, and the two α-helices of the C-terminal domain were all predicted to be intact (Figure 3.1). Importantly, the essential tryptophan in the TMD (position 28, in blue), the tyrosine in the hinge region (34-E**Y**RK-37, in red), and the two serines involved in CK-II phosphorylation (56-ED**S**GNE**S**EG-64, in green) were found to be conserved throughout the three isolates. The tryptophan is involved in ion channel gating, while the tyrosine has been shown to be essential for virion release and forms part of the structurally critical salt bridge between the TMD and CD (Cordes, Kukol et al. 2001; Sramala, Lemaitre et al. 2003; Candler, Featherstone et al. 2005).

Besides the distinct subtype C NTD, two other disparate motifs can be singled out. The putative Golgi-retention signal, 79-LR**LL**-82 (Figure 3.1, in purple) is present in

and unique to the subtype C sequences. The subtype B sequence, however, has the sequence 79-HAPW-82 instead. An extra dileucine motif, 4-**LL**AR-7 is present in the longer NTD of 05ZAFV5. A longer NTD is present in about 60% of subtype C Vpu proteins (http://www.hiv.lanl.gov/ 2009) (Appendix G). This extra dileucine or another unidentified motif in the NTD may be involved in signalling the subtype C Vpu to different subcellular compartments.

The potential extra CK-II phosphorylation site, 71-T(X)VD-74 (Figure 3.1, in turquoise), is also present in the subtype C isolates and observed to be TLVD in 05ZAFV5 and TMVD in 05ZAFV15. This sequence is absent in subtype B, which instead harbours a glycine insertion at position 73 within the subtype B sequence, 71-EM**G**VE-75. CK-II is a ubiquitous protein kinase, able to regulate metabolic pathways, signal transduction, as well as transcription, translation and replication (Guerra and Issinger 2008; Olsen and Guerra 2008; Yde, Olsen et al. 2008). It is a tetrameric holoenzyme (Niefind, Guerra et al. 1998) with the alpha and alpha prime subunits having a catalytic function, and the two beta subunits having a regulatory function (Guerra and Issinger 2008). Of significance is the localisation of the enzyme to the ER/Golgi apparatus (Niefind, Guerra et al. 2001). The presence of these different motifs implies a difference in function.

That the function of Vpu in the viral replication cycle is based on its structure is well established for HIV-1 subtype B (Willey, Maldarelli et al. 1992; Schubert, Henklein et

al. 1994; Schubert, Ferrer-Montiel et al. 1996). The presence of Vpu at different cellular levels also informs its function. Its presence is required at the ER/TGN for interaction with CD4 via ßTrCP and the phosphorylation of its two essential serines in the CD (Jabbar 1995; Paul and Jabbar 1997). Vpu also enhances virion release, a function involving the binding of the TMD to tetherin, and its ability to insert into cellular membranes to form ion channels (Strebel, Klimkait et al. 1989; Schubert, Ferrer-Montiel et al. 1996; Paul, Mazumder et al. 1998). Assessing the presence of Vpu at different subcellular compartments may aid in elucidating the link between these functions.

In order to explore differences in subcellular localisation, the selected subtype B and C *vphu* genes were successfully subcloned into the pcDNA™6.2/C-emGFP-GW/TOPO® mammalian expression vector (Invitrogen). Confocal microscopy, as well as SDS-PAGE and Western blot analysis of HEK 293T cells at 24 hours post-transfection with the Vphu-emGFP vectors confirmed the expression of the Vphu-emGFP fusion proteins. The impact of the 27 kDa emGFP on the function of the 16 kDa Vpu (resulting in a 43 kDa fusion protein) is considered to be minimal. Pacyniak *et al*. used an EGFP reporter system to study Vpu subcellular localisation and found that the fusion proteins were functional for CD4 degradation (Pacyniak, Gomez et al. 2005). They also reported Golgi complex localisation of Vpu-EGFP, as well as Gag-EGFP release from transfected cells, which corresponded exactly to the native subtype B Vpu function (Pacyniak, Gomez et al. 2005). Moreover, the EGFP alone

fractionated to the S100 division, while the Vpu-EGFP fractionated to the membrane enriched P100 section after ultracentrifugation (Pacyniak, Gomez et al. 2005). The reporter system used in the current study is based on this EGFP reporter system, and the subtype B construct was found to localise to the Golgi compartment. Accordingly, the emGFP is not expected to effect Vpu expression.

Conversely, Pacyniak *et al*. did not use codon-optimised proteins in their study of Vpu localisation (Pacyniak, Gomez et al. 2005). Vpu is notoriously difficult to express in the absence of viral Tat and Rev, which are involved in the transcription and the translocation of the *vpu* RNA molecule from the nucleus to the cytoplasm (Nguyen, Ilano et al. 2004). Codon-optimisation stabilises the *vphu* mRNA in the nucleus, and thus allow for more efficient export via the chromosome region maintenance protein 1 (CRM1)-independent nuclear export pathway (Nguyen, Ilano et al. 2004). Codon-optimisation in this study led to good expression of the protein, with localisation matching that found by Pacyniak *et al*. (Pacyniak, Gomez et al. 2005). Subtype B expression appears to be more effective on the Western blot (Figure 3.5B) but no assumptions can be made about expression efficiency, as an expression control was not included. This can be included in future work.

Pacyniak *et al.*, reported subtype B Vpu ER/Golgi localisation, and subtype C Vpu localisation at all membranes, including the plasma membrane at 48 hours (Pacyniak, Gomez et al. 2005). The current study confirms the colocalisation of subtype B Vpu at

the ER/Golgi and the accumulation of the subtype C Vpu proteins at the plasma membrane at 48 hours. The average HIV replication cycle is thought to be about two and a half days. This study therefore looked at colocalisation data up to 60 hours post-transfection. Specifically, results from this study show that subtype B Vpu moves from the plasma membrane at 24 hours to the ER/Golgi at 48 hours, and is diffuse at 60 hours, with areas of strong ER localisation. Subtype B Vpu may primarily bind tetherin at the plasma membrane, and redistribute it to the ER/Golgi where it also allows for the degradation of CD4.

The typical subtype C Vpu from isolate 05ZAFV5 behaves differently, moving from the ER/Golgi at 24 hours to the plasma membrane at 48 hours, and is diffuse at 60 hours with strong plasma membrane localisation. This implies that subtype C Vpu primarily acts to degrade CD4 at the ER/Golgi, and then moves to the plasma membrane to enhance virion release via ion channel formation, and/or to antagonise tetherin. The subtype C Vpu from isolate 05ZAFV15 with the shorter NTD has weak colocalisation with the ER/Golgi markers at 24 hours but at 48 hours, strong ER/Golgi and plasma membrane colocalisation is observed. The longer NTD of isolate 05ZAFV5 may be responsible for this difference.

The localisation at 60 hours for subtype B and C Vpu is diffuse and condensed. As a result, it is difficult to interpret this data but it may reflect the localisation of Vpu to a large majority of endomembranous compartments including the ER, the TGN, and

endosomes, as well as the plasma membrane late in the viral life cycle. Overall, this implies that a factor exists that allows subtype B Vpu to be targeted to the plasma membrane at 24 hours and to the ER/Golgi colocalisation at 48 hours, while the subtype C Vpu proteins are primarily at the ER/Golgi region with movement of some of the protein to the plasma membrane at 48 hours.

This factor was originally proposed to lie within the CD by using subtype B and C Vpu chimeras where the CD of each were exchanged (Pacyniak, Gomez et al. 2005). The M_r values of the chimeras differed, indicating a structural difference between the subtype B and C Vpu CDs. Importantly, the subtype C Vpu containing the CD of a subtype B protein allowed for the targeting of the chimeric protein to the Golgi region at 48 hours (Pacyniak, Gomez et al. 2005). Deletion mutants of the subtype B Vpu CD then confirmed that the C-terminal 23 amino acids were sufficient for retention of the protein in the Golgi at 48 hours (Pacyniak, Gomez et al. 2005). This region, the second α-helical domain of the CD, was therefore suspected to harbour a Golgi-retention signal. Remarkably, this region is the most divergent region in terms of sequence between subtype B and C (http://www.hiv.lanl.gov/ 2009), and as mentioned contains two subtype C-unique motifs, the potential CK-II phosphorylation site TMVD, and the dileucine motif, LRLL (Scriba, Treurnicht et al. 2001; Bell, Connell et al. 2007). Subtype C Vpu therefore has extra Golgi-retention signals, which may account for its presence at the ER/Golgi at 24 hours.

Protein signalling and trafficking in eukaryotic cells occurs via an elaborate network of membranous compartments (Cooper 2000). Proteins are synthesized on membrane-bound ribosomes on the RER, and are directed to secretory or endocytic pathways in the cell via the ER and TGN (Lippincott-Schwartz, Roberts et al. 2000). Proteins harbour specific signal codes that are read by specialised ribonucleoprotein complexes (Hobman, Lemon et al. 1997). Proteins directed to the ER usually contain a stretch of hydrophobic residues that is cleaved off by a signal peptidase on the luminal side of the ER (Hobman, Woodward et al. 1995). Signal sequences within the protein are not cleaved, and the position of signal sequences and signal peptidase sequences determines retention in or retrieval from the ER (Hobman, Lemon et al. 1997; Lippincott-Schwartz, Roberts et al. 2000).

Post-translational modifications occur in the ER and later in the Golgi apparatus (Opat, van Vliet et al. 2001). Misfolded proteins are bound by chaperone proteins, and remain at the ER where they are eventually degraded via an ubiquitin-dependent pathway (Nehls, Snapp et al. 2000). The TGN functions as a sorting compartment directing proteins to the plasma membrane or lysosomes (Cooper 2000). Lysosomes contain acid hydrolases that degrade obsolete proteins, RNA, DNA, polysaccharides, and lipids (Cooper 2000). Lysosomes therefore represent a junction between the secretory pathway and the endocytic pathway (Cooper 2000).

The trafficking of membrane proteins to and from diverse cellular compartments usually involves tyrosine, dileucine, and monophenylalanine-based sorting signals (Kirchhausen 1999). CK-II phosphorylation is also important for the retrieval of some proteins. These motifs interact with adaptor complex proteins, AP-1, AP-2, and AP-3 for endocytosis into clathrin-coated vesicles (Kirchhausen 1999; Janvier, Kato et al. 2003). Tyrosine-based motifs generally interact with the μ4 subunit of the AP complexes (Bonifacino and Traub 2003). They are involved in endocytosis of membrane proteins from the plasma membrane (a G at position Y+1), or the targeting membrane proteins to lysosomes (an acidic residue at position Y+1) (Bonifacino and Traub 2003). Dileucine-based motifs also interact with the AP complexes, but with the hemi-complex formed by the small and large subunits (Janvier, Kato et al. 2003). The dileucine is important for efficient transport and expression of proteins at the plasma membrane. An acidic residue four, five, or six bases upstream of the dileucine motif confers more efficient internalisation and the sorting of membrane proteins to cellular sites such as the trans-Golgi network, endosomes, lysosomes, and the plasma membrane via the adaptor proteins (Janvier, Kato et al. 2003). The two subtype C Vpu proteins have a C-terminal dileucine motif with an upstream acidic residue (75-**D**MGP/QLR**LL**-82, Figure 3.1). The Vpu from isolate 05ZAFV5 also has a dileucine within its NTD that is flanked by aspartic acids (1-MV**DLL**ARV**D**-9, Figure 3.1). This may account for the presence of subtype C Vpu at the ER/Golgi at 24 hours post-transfection.

Golgi-retention domains may also be heterogeneous or conformation dependent, and can involve transmembrane, or multimerization domains (Lippincott-Schwartz, Roberts et al. 2000). Glycosyltransferases for example, are retained in the Golgi by oligomerization to prevent their entry into transport vesicles (Sasai, Ikeda et al. 2001; Milland, Russell et al. 2002). Subtype B and C Vpu have several potential Golgi-retention signals. Principally, there is a tyrosine-based membrane-proximal motif (E**Y**RKL), and a CK-II phosphorylation region located between the α-helical cytoplasmic domains (ERADE**S**GNE**S**EGE) (Ruiz, Hill et al. 2008). Vpu is also known to oligomerize into a pentameric bundle (Moore, Zhong et al. 1998), suggesting retention of Vpu in the Golgi. The Vpu proteins in this study have both these potential Golgi-retention motifs, and the subtype C Vpu proteins have an additional CK-II site. This may influence the presence of all three proteins at the ER/Golgi throughout the analysed period, where it performs the important functions of CD4 down-regulation and retention of tetherin at the ER/Golgi.

Ruiz *et al.*, examined the overlapping tyrosine- (**Y**XXΦ) and dileucine-based ([D/E]XXX**L[L/I]**) motifs in the highly conserved membrane-proximal region of subtype C Vpu (E**Y**RK**LL**) at 48 hours (Ruiz, Hill et al. 2008). The subtype C Vpu (from isolate BW16B01) has an E**Y**RK**LL** sequence, as well as the C-terminal TMVD and LR**LL** sequences. It also has a five amino acid insert at the NTD similar to that of 05ZAFV5. Mutating the tyrosine residue to an alanine has no effect on transport to the plasma membrane, or on CD4 surface expression, but causes cytopathic viral stocks (Ruiz,

Hill et al. 2008). Altering the primary leucine to a glycine results in reduced Vpu expression at the plasma membrane, and five-fold more particles in vacuoles at the cell surface than the parental $SHIV_{SCVpu}$ as seen under an electron microscope (Ruiz, Hill et al. 2008). Mutating both the tyrosine and the primary leucine causes intermediate surface localisation of Vpu, less efficient replication, and significantly less effective downregulation of CD4 (Ruiz, Hill et al. 2008).

Thus, the tyrosine residue is involved in increased viral release, the dileucine in Vpu surface expression and retention of virions at the cell surface, while both motifs are important for CD4 degradation at the ER/Golgi (Ruiz, Hill et al. 2008). This membrane-proximal hinge region forms a stabilising salt bridge between the TMD and CD. It is highly conserved across the group M viruses and the tyrosine is conserved in all group M Vpu sequences (http://www.hiv.lanl.gov/ 2009), and has been shown to be essential in enhancing virion release (Paul, Mazumder et al. 1998). The tyrosine is also conserved in the three proteins used in this study (Figure 3.1) suggesting they are functional for increasing virion release. Incredibly, a tyrosine-based signal in HIV-1 Env initiates targeting of virions to the plasma membrane in polarised epithelial cells, and it concentrates viral assembly at one pole of infected lymphocytes (Cervantes-Acosta, Lodge et al. 2001).

Dileucine motifs in the form of [D/E]XXX**L[L/I]** are recognised at the plasma membrane and at intracellular compartments for targeting to lysosomes (Janvier,

Kato et al. 2003). Membrane proximal dileucine motifs also mediate rapid internalisation and targeting of membrane proteins to endosomal and lysosomal compartments (Bonifacino and Traub 2003). Proximity of the Vpu EYRK**LL** motif to the TMD of the protein determines its involvement in endocytosis and/or lysosome targeting (Ruiz, Hill et al. 2008). The primary leucine is critical in signalling while an isoleucine at that position may cause the signal to be less potent. Approximately 80% of subtype C Vpu sequences have a membrane-proximal LL, LI, or LV (McCormick-Davis, Dalton et al. 2000; Ruiz, Hill et al. 2008; http://www.hiv.lanl.gov/ 2009) (Appendix G). In contrast, about 95% of subtype B Vpu proteins have an isoleucine in the crucial primarily leucine position (EYRK**IL**), while only 5.2% have a LL, LI, or LV (Ruiz, Hill et al. 2008; http://www.hiv.lanl.gov/ 2009).

In this study, the subtype C Vpu from isolate 05ZAFV5 has a membrane-proximal LV (E**Y**RK**LV**) while the Vpu from isolate 05ZAFV15 has an unusual membrane-proximal LR sequence (EYR**LR**). The subtype B Vpu used in this study had an isoleucine in the primary position of the membrane proximal dileucine motif (EYRK**IL**). Predictably, it also lacked the additional CK-II phosphorylation site and the second α-helical dileucine (TMVD and LRLL in subtype C). This suggests that subtype B Vpu is not targeted for rapid internalisation from the plasma membrane, and is not taken up into lysosomes. This may account for the presence of subtype B Vpu at the plasma membrane at 24 hours post-transfection. Three subtype C Vpu proteins were used in the Pacyniak *et al*. study. All three proteins had a membrane proximal region with a

LL or LI motif, an extra CK-II site (TMVD) and an extra dileucine (LRLL) (Pacyniak, Gomez et al. 2005). C96-BW16B01 and C96-BW01B22 had a five amino acid insert, while 95IN-21301 had a one amino acid insert at the NTD (Pacyniak, Gomez et al. 2005). These three proteins showed membrane localisation at 48 hours post-transfection (Pacyniak, Gomez et al. 2005), in agreement with this study's results.

Ruiz *et al*. speculate over the reasons subtype C HIV-1 selects for the dileucine when it appears to reduce replication efficiency (Ruiz, Hill et al. 2008). This may give weight to studies showing that subtype C is evolving into a less virulent form (Arien, Abraha et al. 2005). Furthermore, the dileucine may interfere with the overlapping tyrosine-based signal that is essential for enhancing virion release (Ruiz, Hill et al. 2008), and may therefore be important for tetherin interaction. Tetherin causes the retention of fully formed, mature virions at the plasma membrane, inducing their internalisation into endosomes (Neil, Eastman et al. 2006; Neil, Zang et al. 2008; Van Damme, Goff et al. 2008). The TMD of Vpu interacts with tetherin and CAML, thereby impeding their antiviral function (Neil, Zang et al. 2008; Varthakavi, Heimann-Nichols et al. 2008). Mutating the TMD of a subtype B Vpu results in the protein not being able to colocalise with tetherin, whereas a truncated cytoplasmic tail (Vpu1-50) is still able to associate with tetherin (Neil, Zang et al. 2008).

A recent study shows that mutating the conserved and charged arginine and lysine residues within the salt bridge region of a subtype B Vpu causes a decrease in

accumulation of the protein at the ER/TGN and late endosomal compartments, and is correlated to diminished HIV-1 virion release (Dube, Roy et al. 2009). This group also proposed that a second site important for TGN localisation is located in the second α-helical region of the CD (Dube, Roy et al. 2009). This corresponds to all previous research noting the crucial nature of this region. There is a marked amount of Vpu-tetherin colocalisation at the TGN, suggesting that localisation of Vpu to the TGN is vital to the antagonism of tetherin (Dube, Roy et al. 2009). Thus, the membrane-proximal tyrosine, arginine and lysine residues are vital to tetherin antagonism, either by preventing its transport to the plasma membrane, or by sequestering the protein at intracellular compartments (Dube, Roy et al. 2009). These residues are conserved in all the Vpu proteins used in this study. However, the membrane-proximal dileucine motif may interfere with the tyrosine signal in subtype C, causing its presence at the plasma membrane at 48 hours. Subtype B Vpu does not harbour this overlapping dileucine and is able to move to the plasma membrane at 24 hours, and may therefore be more efficient at binding tetherin and transporting it back to the ER/Golgi at 48 hours.

CAML also interacts with Vpu, and CAML-depletion eliminates the need for Vpu-induced viral release (Varthakavi, Heimann-Nichols et al. 2008). There is speculation as to whether it is the mechanistic link between tetherin and Vpu, since both proteins operate within the membrane protein-internalisation pathway (Varthakavi, Heimann-Nichols et al. 2008). CAML and a subtype B NL4-3 Vpu were found to colocalise in

the perinuclear region, with less colocalisation at peripheral vesicular structures (Varthakavi, Heimann-Nichols et al. 2008). Vpu requires functional recycling endosomes to exert its viral release function, but it must exit the pericentriolar endosome to enhance particle release (Varthakavi, Smith et al. 2006). Vpu and CAML may interact along the recycling pathway, as CAML does with EGFR (Tran, Russell et al. 2003). Vpu may prevent CAML from reaching the plasma membrane, or alternatively it may prevent CAML from directing the cycling of another factor involved in virus retention, such as tetherin (Varthakavi, Heimann-Nichols et al. 2008). Antagonism of CAML therefore requires cycling from the plasma membrane to the ER/Golgi, which corresponds to the timing of subtype B movement in this study.

The differences in Vpu localisation are thus likely to have implications for the viral replication cycle and disease pathogenesis. Indeed, HXB2 Vpu is more efficient at down-regulating CD4 surface expression than four subtype C clinical isolates at 48 hours (Hill, Ruiz et al. 2008). A SHIV construct with a subtype C Vpu ($SHIV_{SCVpu}$) has decreased replication kinetics (that is, a delay of two days of p27 production), and a decreased ability to form syncytia in culture compared to the parental strain ($SHIV_{KU-1bMC33}$) (Hill, Ruiz et al. 2008). Inoculation of macaques with $SHIV_{SCVpu}$ also shows a more gradual loss of CD4+ T-cells, and early peak viral loads (in the first three weeks) are 10 fold less than macaques inoculated with the parental strain (Hill, Ruiz et al. 2008). This study is striking in that the subtype of the Vpu protein is the only

difference between the SHIVs used to inoculate the macaques. As shown, the subtype also appears to be responsible for differences in subcellular localisation.

T-cell activation is a major difference between SIV and HIV infections in their respective hosts. Non-pathogenic SIV infection lacks high-level immune activation, T cell turnover, and apoptosis (Apetrei, Robertson et al. 2004). This activation induced cell death (AICD) is present in HIV-1 and SIV_{cpz} where the host fails to decrease the levels TCR-CD3 (Alimonti, Ball et al. 2003). In humans, very few CD8+/CTL and CD4+/T-helper cell responses are directed against Vpu and the antibody response is unknown (http://www.hiv.lanl.gov/content/immunology 2008). The best-defined CD8+ response is against the hinge region motif, EYRKLL that is recognised by HLA*3303 (http://www.hiv.lanl.gov/content/immunology 2008). There are also six recognised epitopes that induce CTL responses, chiefly against the NTD and TMD. There is only one described CD4+/T-helper response, also against the TMD (http://www.hiv.lanl.gov/content/immunology 2008). In light of current data, Vpu can be considered a poorly immunogenic protein, which is remarkable as overcomes specific host factors. Then again, the effects of Vpu on virion release may have downstream effects on T-cell activation and the differences noted between macaques infected with constructs harbouring either a subtype B or C Vpu protein (Ruiz, Hill et al. 2008). HIV-1 Subtype C may be evolving into a less virulent form (Arien, Abraha et al. 2005), and it has been proposed that this slower disease progression may increase transmission rates (Ruiz, Hill et al. 2008), accounting for the prevalence of

subtype C worldwide. The differences in Vpu localisation may also account for this decreased virulence.

In summary, both the subtype B and C Vphu proteins used in this study had ER/Golgi localisation at 24, 48, and 60 hours post-transfection. The sequestering of CD4 molecules at the ER/Golgi prevents its surface expression, thereby preventing its incorporation into budding virions, and the possibility of superinfection (Tanaka, Ueno et al. 2003; Wildum, Schindler et al. 2006). This highlights the importance of capturing CD4 at the ER/Golgi, making more CD4 available for proteasome degradation. The presence of extra dileucine motifs in subtype C, responsible for sorting to intracellular structures, may account for its initial localisation at the ER/Golgi at 24 hours. Subtype C Vphu may therefore accumulate earlier at the ER/Golgi and this may in turn enhance its ability to down-modulate newly synthesised CD4. Alternatively, subtype B Vphu may be localising to the plasma membrane earlier because of tyrosine signalling in its salt bridge region, and the lack of extra Golgi-retention signals in the C-terminal domain. Thus, subtype B Vphu may be able to bind tetherin or CAML at the plasma membrane at 24 hours before redistributing the antiviral proteins to the ER and TGN at 48 hours.

The predominant localization of subtype C Vphu to the plasma membrane at 48 hours may lead to greater ion channel activity, resulting in enhanced virion release by membrane destabilization. However, with its membrane proximal dileucine interfering

with the tyrosine it may not be able to interact with AP complexes for sorting of tetherin/CAML to intracellular compartments. Future work should address whether the dileucine mutation causes more rapid CD4+ T-cell loss, and enhanced interaction with tetherin/CAML. The localisation of subtype C Vphu to the cell membrane at 48 hours may also result in decreased replication kinetics and diminished pathogenesis, in line with the SHIV/Macaque models. In contrast, the subtype B Vphu may have increased CD4 downregulation because of localisation to the ER/Golgi region at 48 hours. This may enhance the CD4-E3-ligase complex formation, and the retention of tetherin at intracellular membranes.

Overall, the results strongly suggest that the replication cycle of subtype C is different from subtype B. Subtype B and C Vpu are known to differ structurally at the carboxy-terminal end. This study emphasizes that the differences in the second α-helical CD may be related to the differences in subcellular localisation. The difference in subcellular localisation may affect CD4 downregulation *in vitro*, and CD4 T cell loss *in vivo*. HIV-1 subtype C also accounts for over 50% of worldwide HIV infection (www.unaids.org 2008). A clue to the marked difference in pathogenesis between these subtypes may therefore reside in the second α-helical domain of the Vpu CD. In conclusion, there is differential timing of translocation of HIV-1 subtype B and C Vpu to the ER/Golgi and plasma membrane compartments. It follows that differential timing of the cycling of tetherin/CAML, and of CD4 degradation, is likely to exist.

A few potential caveats in this study are apparent. Firstly, Vpu was not expressed from its original genomic position, under REV control, but from a codon-optimised gene under the control of a CMV promoter. In turn, the relative contribution of Env and Nef to localisation could not be taken into account. Nef is also involved in CD4 downregulation and has a dileucine motif in its C-terminus that is required for this function (Aiken, Konner et al. 1994). Further, determining whether there are subtype differences in Nef's ability to down-modulate CD4 during infection may be useful in accounting for these subtype specific differences in Vpu function. The interaction between these two proteins and their contribution to CD4 degradation should be determined in future studies. Also, since Vpu is not necessary for infection in HEK 293T cells (Strebel, Klimkait et al. 1988) , it may not be crucial to overcome tetherin or CAML in these cells.

Interestingly, Vpr-GFP studies have failed since Vpr-GFP is prone to site-specific proteolysis resulting in a mixture of intact and truncated forms with impaired function (Caly, Jans et al. 2008). The bioinformatics program, SignalP 3.0, predicts that signal peptidase activity for subtype C Vpu acts between positions 29 and 30 of the TM domain (TIA ^ YI, Figure 3.1) (http://www.cbs.dtu.dk/services/SignalP) (Brendtsen, Nielsen et al. 2004) . This suggests cleavage of the C-terminal from the N-terminal domain, and independent actions. Functional studies will be necessary to show if there is indeed proteolytic activity and whether this interferes with subcellular localisation. Current thinking, however, proposes the C-terminal stabilises the

transmembrane helix in the plasma membrane and remains functional within the cytoplasm (Sramala, Lemaitre et al. 2003).

Future work may also include observing the subcellular localisation of the Vpu at time points earlier than 24 hours. Monitoring Vpu-induced CD4 down-modulation at each time point, possibly using flow cytometry, will also enhance this study. CD4 down modulation is noticeable 16 to 24 hours after infection of Jurkat cells with a NL4-3 construct (Wildum, Schindler et al. 2006). The efficiency of infection of each subtype still needs to be clarified. Monkey data has shown that subtype C virus is less efficient, but HIV-1 subtype C is nonetheless the predominant virus worldwide. Creating chimeric 05ZAFV5/FV15 Vpu proteins containing the FV5 leader sequence may confirm whether the NTD has a role in protein trafficking. Site directed mutagenesis of the other signalling motifs would elucidate their impact on subcellular localisation. Quantitative image analysis could be performed on the confocal images to better define the extent of the colocalisation (Varthakavi, Smith et al. 2006).

Overall, several researchers have established the essential role of Vpu in the HIV-1 viral life cycle and impact on disease pathogenesis. The protein influences CD4 degradation, viral particle budding, and antagonises the antiviral host proteins, tetherin, and CAML. Vpu is also unique to HIV-1 and SIV infecting humans and our recent ancestor, the chimpanzee, respectively. The expression of subtype B or C Vpu in the context of the same backbone has been shown to have a dramatically different

impact on the disease pathogenesis of infected macaques (Ruiz, Hill et al. 2008). As ongoing research uncovers the precise interaction of Vpu-tetherin/CAML, and other as yet unidentified host proteins, novel antiviral treatment strategies will be uncovered. Results from this study highlight the significance of investigating subtype-specific differences in Vpu function, and will lead to research probing the impact of these differences on subtype C-infected individuals.

Appendix A: Ethics Waiver

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat, Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-090330-1revised
29/04/2009

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Ms Catherine Bell**

Project title: **Differential timing of translocation of HIV-1 subtype B and C Vpu to the ER/Golgi and plasma membrane compartments.**

Reason: This is a wholly laboratory study using commercial HEK293T cell lines and viral isolates from a previously approved study (M041002). There are no humans involved.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MED) CHAIR
2009 -04- 29
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

Appendix B: Plasmid Maps

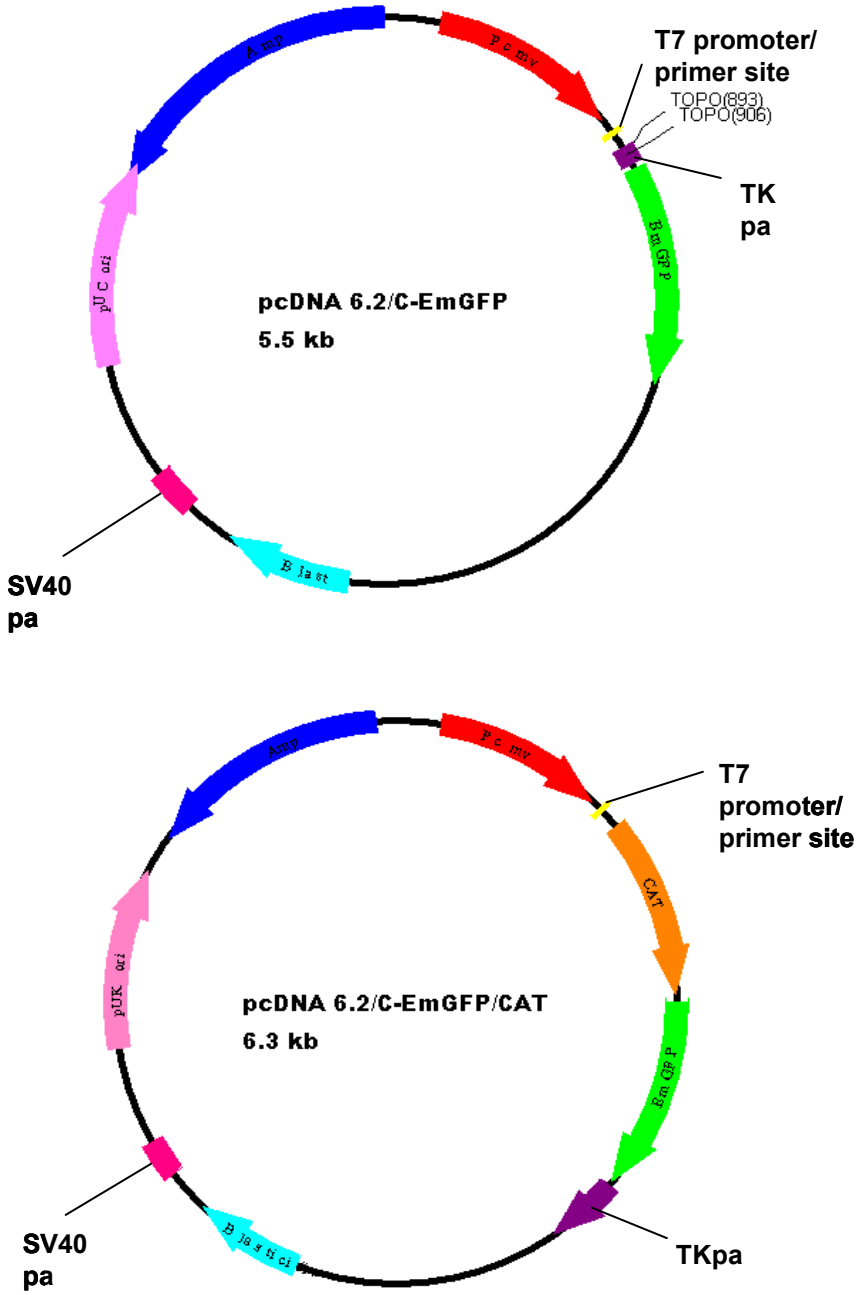


Figure B1: EmGFP reporter plasmid, pcDNA 6.2/C-emGFP and positive control plasmid, pcDNA 6.2/C-emGFP/CAT (www.invitrogen.com).

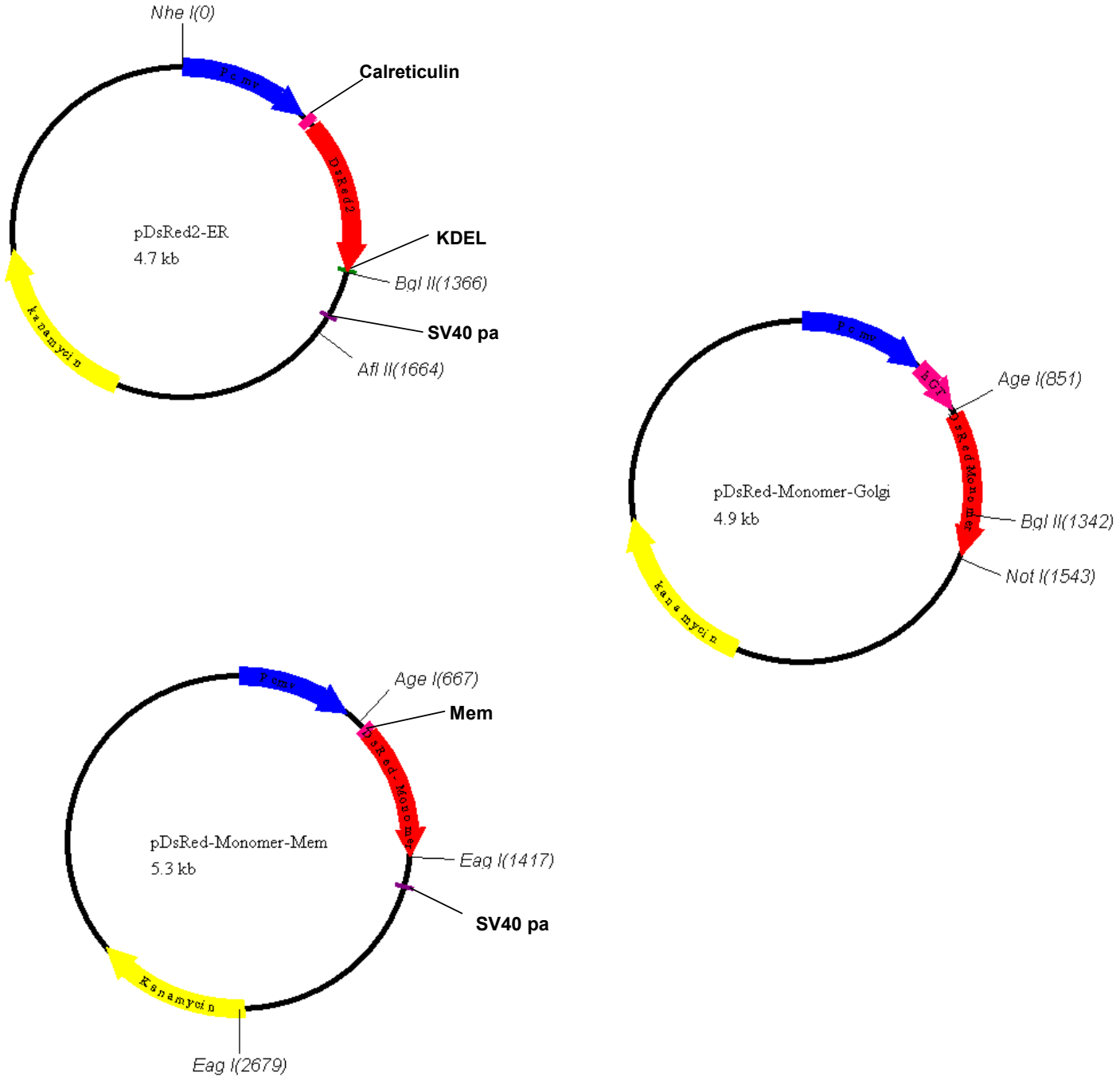


Figure B2: Subcellular localisation plasmids, pDsRed2-ER, pDsRed-Monomer-Golgi, pDsRed-Monomer-Mem (www.clontech.com).

Table B1: Symbol definitions for plasmid maps in Figures B1 and B2.

Symbol	Definition
pCMV	CMV promoter site
T7	T7 promoter/priming site
TOPO	TOPO isomerase cloning sites
EmGFP	Gene encoding the emerald green fluorescent protein
TK pa	TK polyadenylation site
Blast	Blasticidin antibiotic gene
Amp	Ampicillin antibiotic gene
SV40 pa	SV40 polyadenylation signal
pUC ori	pUC origin
CAT	chloramphenicol acetyltransferase gene
DsRED	Gene encoding the fluorescent DsRed monomeric protein
Cal	ER-targeting sequence of calrecticulin gene
hGT	N-terminal 81 amino acids of human ß-1,4-galactosyltransferase
Mem	N-terminal 20 amino acids of neuromodulin (GAP-43) that acts as a palmityolation signal
KDEL	ER-retention sequence, KDEL
Kanamycin	Kanamycin antibiotic resistance gene

Appendix C: Recipes and culture media

C1 Bacterial transformations and cloning reactions

C1.1 Luria Bertani Broth (LB)

10 g tryptone, 5 g NaCl, 5 g yeast extract made up to 1 L with deionised distilled water (ddH_2O). The solution was sterilised by autoclaving for 10 min at 121 °C.

C1.2 Luria Bertani agar (LA)

10 g tryptone, 5 g NaCl, 5 g yeast extract, and 15 g agar powder made up to 1 L with dH_2O.

C1.3 Ampicillin

100 mg/ml: 1 g Amp dissolved in 10 ml 50% ethanol and stored at -20 °C. Add 1 µl/ml to cooled LB or LA.

C1.4 Kanamycin

50 mg/ml: 0.5 g Kanamycin powder dissolved in 10 ml ddH_2O and stored at 4 °C. Add 1 µl/ml to cooled LB or LA.

C1.5 50X TAE

242 g Tris dissolved in 500 ml ddH_2O. To this add 100 ml o.5 M Na_2EDTA (pH 8.0) and 57.1 ml glacial acetic acid. Adjust volume to 1 L with dH_2O. The solution was stored at room temperature.

C1.6 Loading dye

15 ml stock solution: 4.5 ml glycerol, 0.04 g bromophenol blue, in 10.5 ml ddH_2O.

C2 Culture Media

C2.1 HEK 293T cells

Propagation medium: DMEM (Sigma-Aldrich) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco, Invitrogen, NY, USA), 2 mM L-glutamine (Gibco, Invitrogen) and 10 µg/ml penicillin-streptomycin (Gibco, Invitrogen).

Freeze medium: 90 % FCS, and 10% DMSO (Sigma-Aldrich).

C3 SDS-PAGE and Western Blot solutions

C3.1 4X Running gel buffer

36.3 g Tris (FW 121.1) dissolved in 150 ml ddH_2O and adjusted to pH 8.8 with hydrochloric acid (HCL). Made up to 200 ml with ddH_2O and stored at 4 °C for up to three months.

C3.2 4X Stacking gel buffer (0.5 M, Tris-Cl, pH 6.8)

3 g Tris dissolved in 40 ml ddH_2O and adjusted to pH 6.8 with HCL. Made up to 50 ml with ddH_2O and stored at 4 °C for up to three months.

C3.3 5X Tank buffer

144.13 g 196 mM glycine, 10 g 0.1 % SDS, 30.28 g 50 mM Tris made up to 2 L with ddH_2O. Solution stored at room temperature for a month.

C3.4 Transfer buffer

200 ml 20% methanol and 200 ml 5X Tank buffer, dissolved in 500 ml with ddH_2O. Made up to 2 L with ddH_2O and stored at room temperature for a month.

C3.5 2X SDS-PAGE sample buffer

1 ml 1.5 M Tris (pH 6.8), 0.6 ml 20% SDS, 3 ml filter sterilised glycerol, 1.5 ml ß-mecaptoethanol, 0.18 mg bromophenol blue.

C3.6 10% Ammonium persulphate

0.1 g Ammonium persulphate (APS) made up to 1 L with ddH_2O.

C3.7 Tris-buffered saline solution (TBS)

125 mM NaCl, 25 mM Tris pH 8.3.

C3.8 Tween-TBS (T-TBS)

1 ml/L Tween-20 in 1X TBS.

C3.9 Blocking buffer

5 g skim milk powder in 100 ml T-TBS

C3.10 Destain solution (40% methanol, 7% acetic acid)

400 ml methanol, 70 ml ecetic acid, made up to 1 L with ddH_2O and stored at room temperature.

C3.11 Coomassie stain (Coomassie Brilliant Blue R250, 40 % methanol, 7% acetic acid)

0.5 g Coomassie Brilliant Blue R250 dissolved in 800 ml methanol. 140 ml acetic acid added and made up to 2 L with ddH_2O. Stored at room temperature for up to six months.

C4: Confocal microscopy solutions

Dapi counterstain solution

For a 5 mg/ml stock solution, add 10 mg Dapi powder to 2 ml dimethylformamide (DMF). For a 100 ng/ml working solution, add 2 µl Dapi stock solution to 100 ml PBS. Store working solution at 4 °C wrapped in aluminum foil.

Appendix D: Molecular weight markers

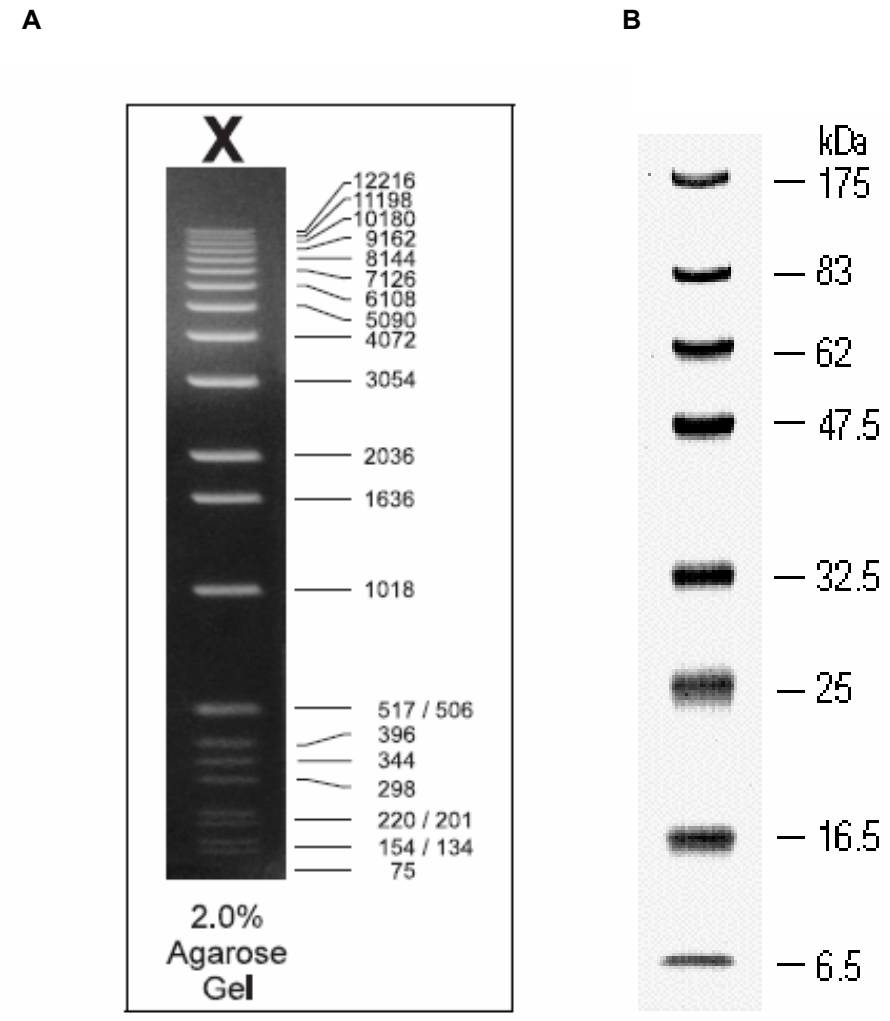


Figure D1: (A) Roche DNA molecular weight marker X (0.07 – 12.2 kpb). Marker used for the size determination of DNA in all agarose gels. (B) NEB Prestained protein marker, broad range (7 – 175 kDa).

Appendix E: Summary of subtype B and C Vphu subncellular localizatio

Table E1: Composite summary of subtype B and C Vphu localisation data as visualised by confocal microscopy.

		24 hours	48 hours	60 hours*
Subtype Vphu	**ER**	Weak	Strong	Strong
	Golgi	Strong	Weak	Intermediate
	Membrane	Strong	Weak	Intermediate
05ZAFV5 Vphu	**ER**	Strong	Strong	Intermediate
	Golgi	Strong	Strong	Intermediate
	Membrane	None	Weak	Strong
05ZAFV15 Vphu	**ER**	Weak	Strong	Strong
	Golgi	Weak	Strong	Intermediate
	Membrane	None	Strong	Intermediate

* Vphu protein appears as large accumulations in relatively shrunken cells. Indicated localisation is observed as the most intense fluorescence.

Appendix F: The single-letter amino acid code

Table F1: The single-letter amino acid code with associated abbreviation.

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

Appendix G: Alignment of all the HIV-1 Subtype C Vpu amino acid sequences available from Los Alamos.

```
                         *        20         *        40         *        60         *        80         *       100
C.ZA.2004. : MVTPSEVGVIAAFILALILAIV-VWTIVYLEYRKIVKQRKIDWLIERIRERAEDSGNESEGDTEELAAMVD--MG--QLRLLDVNGV*---------------- : 82
C.ZM.2002. : MVDYIAIGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDNEELATMVD--MG--QLRLLDVNNL*---------------- : 81
C.ZA.2004. : MVDFIAIGV-GALIVAFIIAIV-VWTIAYLEYRKLLRQKRIDRLIERIRERAEGSGNESEGDTEELSTMVD--MG--NLRLLDVNNL*---------------- : 81
C.BW.1996. : MLEL-IA-KI-DY-RLGG-GALIVALSIAIV-VWIIAYIEYKKLIRQRRINWLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNAL*------- : 86
C.BW.1996. : MLEL-IA-KI-DY-RLGV-GALIVALSIAIV-VWIIAYIEYKKLIRQRKINWLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNAL*------- : 86
C.BW.1996. : MLEL-VA-KV-DY-RLGV-GALIVALSIAIV-VWIIAYIEYKKLIRQRRVNWLIEGIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNAL*------- : 86
C.IN..VB39 : ML-DY-KLGV-GAVIVALILAIV-VWIIVFIEYRKLVAQRKIDWLIKRIRERAEDSGNESEGDTEELSMLVD--MG--HLRLLDVNDL*--------------- : 80
C.IN..VB39 : ML-DY-KLGV-GAVIVALILAIV-VWIIVFIEYRKLVAQRKIDWLIKRIRERAEDSGNESEGDTEELSMLVD--MG--HLRLLDVNDL*--------------- : 80
C.IN..VB49 : MV-DYIQLGV-GALIVAFIIAIV-VWTIAYLEYRKWLRQRKIDRLIERIRERAEDSGNESEGDTEELSAMVD--MG--HLRLLDDNDL*--------------- : 81
C.IN..VB49 : MV-DYIQLGV-GALIVAFIIAIV-VWTIAYLEYRKWLRQRKIDRLIERIRERAEDSGNESEGDTEELSAMVD--MG--HLRLLDDNDL*--------------- : 81
C.IN..VB49 : MV-DYIQLGV-GALIVAFIIAIV-VWTIAYLEYRKWLRQRKIDRLIERIRERAEDSGNESEGDTEELSAMVD--MG--HLRLLDDNDL*--------------- : 81
C.ZA.2004. : MLDL-LA-RV-DY-RLGV-GAFIVALIIAIV-VWIIAYIEYRKWLRQRKINWLIERIRERAEDSGNESEGDTEELSTLVD--ME--HLRLLDVNNG*------- : 86
C.ZA.1998. : MLDL-LA--------IGV-GALIVALIIAIV-VWTIVYIEYRKLLRQKRIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 81
C.ZA.1998. : MLDL-LA--------IGV-GALIVALIIAIV-VWTIVYIEYRKLLRQKRIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 81
C.ZA.1998. : MLNL-LA-RV-DY-RIGV-GALTVALIIAIV-VWTIVYIEYRKLLRQKRIDWLIKRIRERAEDSGNESEGDTEELSTMGD--MG--HLRLLDVHDL*------- : 86
C.ZA.2004. : MLDL-IG-KV-GY-RIGV-GALIVALIIAIV-VWTIVYIEYRKLLRQKRIDWLIKRIRERAEDSGNESEGDNEELSTMVD--MG--HLRLLDVIDL*------- : 86
C.BW.2000. : MLDL-LA-GV-DY-RIGV-GAFLVALSIAIV-VWTIVYIEYRKLLRQKRIDRLIERIRERAEDSGNESEGDIEELSTMVD--MG--HLRLLDVNDV*------- : 86
C.ZA.1998. : MLDL----KV-DY-RLAV-GAFIIALILAII-VWTIAYIKYRKVVRQKKIDWLVKRIKERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*------- : 84
C.ZA.2005. : MLSL-LY-KV-DY-RIGV-GALVIALFLARI-VWTIAYLEYRKLSRQKKIDWLVKRIRERAEDSGNESEGDAEELSTMVD--MG--HLRLLDDNAL*------- : 86
C.YE.2002. : MLDL-LA-KV-DY-RIGI-AAFVVALIIAII-VWTIAYIEYRKLVIQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDINDL*------- : 86
C.FI.1992. : MFDL-TE-RV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.TZ.1998. : MLDL-SA-RV-DY-RLGV-GALLVALIIAIV-VWTIVYIEYKKLVRQRRIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNEL*------- : 86
C.TZ.1997. : MLDL-TA-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDCLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA..99ZA : MLDL-LA-RV-DY-RLGV-GALIVALIIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.KE.1995. : MLDL-IA-RV-DY-RLGV-GALIVALILAIV-VWIIAYLEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVE--VG--HLRLLDVNDL*------- : 86
C.BW.1998. : MVVL-GE-KE-IY-ILGI-GALIVALIIAIV-VWSIAYIEYRKLVRQRRIDQLIKRIGERAEDSGNESDGDTEELSTLVD--MG--HLRLLDDNEL*------- : 86
C.BW.2000. : MLGL-SE-KA-GY-ALGV-GALIVALIIVIV-VWTIVYIEYRKLVRQRKID*LIKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDVNDL*------- : 85
C.ZA.2003. : MLDL-IE-RV-DY-RLGI-GALIVALIIAIV-VWTIAYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--RLRLLDVNDL*------- : 86
C.ZA.2003. : MLGL-PE-RV-DY-RLGV-GALIIALVIAIV-VWTIVYIEYRKLLRQRKINWLAKESGKEQKTVAMRVMGILRSCQQWW--IWG--GLGFWMF-MIY------- : 86
C.ZA.2003. : MLGL-LE-RV-DY-RLGV-GALIIALVIAIV-VWIIAYIEYRKLLRQRKINWLAKRIRERAEDSGNESDGDIEELSTMVD--MG--RLRLLDANDV*------- : 86
C.ZA.2004. : MVDF-TE-RV-DY-RIGV-GALIIALSIAIV-VWIIVYIEYRKLLRQKRIDWLAKRIRERAEDSGNESGGDIEELSTMVD--MG--QLRLLDVHDL*------- : 86
C.TZ.2001. : MVDF-IA-SV-DY-RLGI-GALIIALLIVIV-VWTIVYIEYRKLVRQRKINWLIKRIRERAEDSGNESDGDTEELSEMVD--MG--RLGLLDANDV*------- : 86
C.ZA.2000. : MVNW-AA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLVRQRRIDWLIERIRERAEDSGNESEGDTEELGTMVD--LG--HLRLLDINGV*------- : 86
C.ZA.2000. : MVNW-AA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLVRQRRIDWLIERIRERAEDSGNESEGDTEELGTMVD--LG--HLRLLDINGV*------- : 86
C.ZM.2002. : MINF-AA-RV-DY-RVGV-AAFTIALIIAIV-VWIIVYLE---LVRQRKIDQLIIRIREREEDSGNESEGDIEELSTMVD--MG--QLRLLDGNGL*------- : 83
C.BW.2000. : MVDW-TK-KS-RL-*NSS-SSIYSSSNHSNS-CMDHSIYRI*EIKKTKKNKLVN*KN*RKSRRQWQRE*WGSGGIGNNGG--YG--AS*AFGC**FV------- : 79
C.BW.2000. : MIDW-TE-QV-DY-RIAI-VIIYSSSNHSNS-YMDLSIYRI*EIVKTKKNRLVN*KN*RKGRRQWQ*E*WGS*GTGHHGG--FG--AS*AFGC**FV------- : 78
C.BW.1996. : MIDW-TA-RV-DY-RVAV-V-AFIVALILAIIVWIIAYLEYRKLSKQRKIDCLIKRIRERAEDSGNESDGDQEELATMVD--MG--RLRLLD-TNDL*------ : 86
C.BW.1998. : MSNW-TA-RV-DY-RIAI-A-AFIVALIIAIVVWTIVYIEYRKLSRQRKIDRLIKRIRERAEDSGNESDGDQDELATMVD--MG--HLRLLD-VNDL*------ : 86
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
```

```
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DD-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAI-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HIRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAV-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAIDL*------------ : 82
C.ZM.2003. : ML-EL-DY-KIAV-AALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAIDL*------------ : 82
C.BW.1999. : ML-EL-DY-RIGI-AAFIIALIIAIV-VWTIVYIEYRKLRRQRKIDRLIERIRERAEDSGNESDGDQEELSAMVD--MG--HLRLLDVNDL*------------ : 82
C.AR.2003. : MINL-TD-RV-DY-RIGI-AAFLVALLIAIV-VWIIVYIEYRKLVRQRRIDWLIKRIRERAEDSGNESEGDTEELGTMVD--ME--HLRLLDVNDL*------- : 86
C.BW.2000. : MIDL-SA-RV-DY-RIGV-AAFIIALIIAIV-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLHDNNL*------- : 86
C.TZ.2001. : MLDL-IA-RV-DY-RVGV-VAILIALIIAIV-VWTIVYIEYRKLVKQTKINRLIKRIRERTEDSGNESEGDMEELSTLVD--ME--HLGLLDVNEL*------- : 86
C.ZA.2004. : MISL-LA-RI-DY-RIGI-AALLIALIIAIV-VWTIVYLEYRKLVKQRKIDWLIKRIRERAEDSGNESERDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.TZ.2002. : ML-SL-DY-ILGV-GALGIALIIAIV-VWTIVYIEYRKLVKQRKIDDLIKRIREREEDSGNESDGDVEELSTLVD--MG--QLRLFDANNL*------------ : 82
C.ZA.2003. : MIDF-LA-RV-DY-RIGV-GAFAIALIIAIV-VWTIVYIEYRKLVKQRRIDWLVERIREREEDSGNESDGDIEELSTVVD--MG--RLRLLDVNDL*------- : 86
C.BW.1996. : MIDL-TA-RV-DY-RIAV-AAFTVVLIITII-VWTIVYLEYRKLVKQRKIDRLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNDL**------ : 86
C.BW.1996. : MIDL-TA-RV-DY-RIAV-AAFTVVLIITII-VWTIVYLEYRKLVKQRKIDRLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNDL**------ : 86
C.ZA.2003. : MLDL-AA-RV-DY-RIGI-AAFTVALIIAII-VWTIVYIEYRKLVRQRKIDLLIKRIRERAEDSGNESDGDTEELSTMVD--ME--YIRLFDANDL*------- : 86
C.BW.1996. : MIDL-LA-RV-DY-RIGL-AAFVVALLIAII-VWTIVYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLD-NL*-------- : 84
C.BW.1996. : MIDL-LA-RV-DY-RIGL-AAFVVALLIAII-VWTIVYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLD-NL*-------- : 84
C.BW.1996. : MIDL-LA-RV-DY-RIGL-AAFVVALLIAII-VWTIVYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLD-NL*-------- : 84
C.ZA.2004. : MIDL-SA-RV-DY-RIGI-AAFIIALLIAII-VWTIVYIEYRKLVKQRKVDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--RLRLLDGNV*S------- : 86
C.ZA.2003. : MIDW-VA-RV-DY-RIGI-GAFIVALLIAII-VWTIVYLEYRKLVKQRKLDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLD-DV*-------- : 84
C.ZA.2004. : MIDW-AA-KV-DY-RVGV-VAFIIALLIAII-VWILAYIEYRKLVKQKKIDWLIKRIGEREEDSGNESEGDIEELATMVD--MG--QLRLLDNYV*-------- : 85
C.BW.1998. : MLDL-TE-GV-DY-RIGI-AAFAVALFIAII-VWIIVYLEYRKIVKQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--QLRLL-DVM*-------- : 84
```

```
C.BW.2000. : MLDL-AA-IV-DY-RITI-VAFAIALFIAII-VWTIAYLEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLGHGL*-------- : 85
C.ZA.2004. : MLNL-LA-RV-DY-RIGI-AAFIVALIIAII-VWTIVYIEYRKLVKQRKIDRLIKRIREREEDSGNESEGDIEELATMGD--MG--HLRLLD-GV*-------- : 84
C.BW.2000. : MVDL-LE-KV-DY-RIGI-AAFTVALLIAII-VWIIAYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLDVNDI*------- : 86
C.ZA.2003. : MLDL-AA--I-DY-RIGI-AAFVVALIIAII-VWIIAYIEYRKLVRQRRIDWLIERIRERAEDSGNESEGDNEELATMVD--MG--QLRLLDVNDL*------- : 85
C.ZA.2003. : MLDL-TA-GV-DY-RIGI-VAFAVALIIAII-VWTIVYIEYRKLVRQRKIDWLINRIRERAEDSGNESEGDTEELETMVD--MG--QLRLLDVNVL*------- : 86
C.SO.1989. : MLNL-LA-GV-DY-RIAI-GAFSVALIIAIV-VWTIVYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.SO.1989. : MLNL-LA-GV-DY-RIAI-GAFSVALIIAIV-VWTIVYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.1998. : MLNL-AA-RV-DY-RIGV-GAFIVALIIAII-VWTIVYIEYRKLVRQRKIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.1998. : MLNL-AA-RV-DY-RIGV-GAFIVALIIAII-VWTIVYIEYRKLVRQRKIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.BW.1996. : MLSL-AA-LV-DY-RLGV-GALIVALIIAII-VWTIVYLEYRKLVRQRKINWLVKRIRERAEDSGNESEGDTEELATMVD--LE--HFGLLDIN*L*------- : 85
C.BW.2000. : ML-A--AV-DY-RLGV-GALTVALILVII-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDIEDLAMVVD--MG--QLRLLDVNNL*--------- : 83
C.ZM.1996. : MLNL-EA-RV-DY-RIGV-GALIAALIIAIA-VWIIVYIEYRKLSRQRKIDRLIKRIRERAEDSGNESEGDNEELATMVD--MG--HLRLLDAIDV*------- : 86
C.BW.1998. : MLNL-VA-RV-DY-RLGV-GALIVALIIAIV-VWTLVYIEYRKLVRQRKIDQLVKRIRERAEDSGNESEGDIEELSTMVD--MG--NLRLLDANDL*------- : 86
C.TZ.1997. : MLNL-AA-RI-DY-RLGV-GALIVALIIAII-VWTIAYTEYRKLLRQRKIDRLIKRIKERTEDSGNESEGDVEELATMVD--MG--QLRLLDAIDL*------- : 86
C.ZA.2004. : MLNL-PA-RV-DY-RLGV-GALIIALIITIV-VWTIVWIEYRKWLRQRKIDRLVKRIRERAEDSGNESDGDAEELATMVD--ME--HLRLL*----------- : 81
C.ZA.2000. : MLNL-IA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRKLDWLIKRIIERAEDSGNESEGDVEELSTMVD--ME--HLRLLD--DL*------- : 84
C.ZA.2000. : MLNL-IA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRKLDWLIKRIIERAEDSGNESEGDVEELSTMVD--ME--HLRLLD--DL*------- : 84
C.ZA.1999. : MLNL-NA-RI-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELSTMVD--ME--HLRLLD--DL*------- : 84
C.ZM.2002. : MFDL-SA-RV-DY-RLGV-GALLVALIIAIV-VWTIVYIEYRKILRERKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLD--DL*------- : 84
C.BW.1999. : MFNL-AA-RV-DY-RLGV-GALVIAGIIAIV-VWTIVYIEYRKLSRQRKINEIIKRIRERAEDSGNESDGDMEELSTMVD--LG--HLGLLDANDL*------- : 86
C.ZM.2002. : MYTW-LE-GV-DY-RIGV-AAFIIALIIVIV-VWIIVYIEYRKLVRQTQIDRLIERIRERSEDSGNESDGDIEELATMVD--MG--HIRLLDANDL*------- : 86
C.ZA.2003. : MLNL-LA-GV-DY-RIGV-GAFIIALIIAIV-VWTIVYIEYRKVLRQRKIDRLIERIRERAEDSGNESEGDIEELSTMVD--MG--HLGLLDANGL*------- : 86
C.ZA.2004. : MLNL-LT-KV-DY-RVGV-AALIIALIIAIV-VWTIAYIEYRKLLRQRKIDRLVDRIRERAEDSGNESEGDIEELATTLD--MG--HIRLLDAINL*------- : 86
C.TZ.2001. : MLDL-TA-RV-DY-RIGV-GALIIALIIAII-VWAIVYLEYRKVLRQRKIDRLIQRIRERAEDSGNESEGDIEELATQVD--MDLDHLRLLDANDL*------- : 88
C.ZA.2000. : MLEL-LA-KI-DY-RVGI-AALIIALIMVII-VWTIVYIEYRKIVKQRKIDRLIERIRDRAEDSGNESEGDIEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLNL-LA-RV-DY-RIGV-GALIIALILAII-VWTIVYIEYRKIRKQRKIDNLIERIRERAEDSGNESEGDTEELSTLVD--MG--NLRLLDGNDL*------- : 86
C.ZA.2004. : MWDL-YA-RV-EY-GIGV-GALIIALIIAII-VWTIVYIEYRKILRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZM.2002. : MLDL-FA-GV-DA-RLGI-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 86
C.ZM.2002. : MLDL-FA-GV-DA-RLGI-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 86
C.ZM.2002. : MLDL-FA-GV-DA-RLGI-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 86
C.ZM.2002. : MLNL-FA-GV-NA-KLEI-EALIIALIIAIV-V*TIAYTEYKKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 85
C.ZM.2002. : MLNL-FA-GV-NA-KLEI-EALIIALIIAIV-V*TIAYTEYKKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 85
C.ZM.2002. : MLNL-FA-GV-NA-KLEI-EALIIALIIAIV-V*TIAYTEYKKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 85
C.ZM.2002. : MLNL-FA-GV-NA-KLEI-EALIIALIIAIV-V*TIAYTEYKKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 85
C.ZM.2002. : MLNL-FA-GV-NA-KLEI-EALIIALIIAIV-V*TIAYTEYKKLLRQRKIDKILKRIRERAEDSGNESEGDVEELSTMVD--LE--HLGILDVNNW*------- : 85
C.BW.2000. : M-L--AI-DY-SLGV-GALLVALFIAII---------DRKLVRQAKIDKIIKRIRERAEDSGNESEGDIEELSTMVD--MG--HLRLMDIIN*Y---------- : 74
C.ZA.2004. : M-LE-SI-DY-RLGV-AALLLALIIAII-VWIIAYLEYRKLLRQRRIDKLIKRIRERAEDSGNESEGDIEELSTMVD--VE--HLRLLDVNNL*---------- : 83
C.BW.1996. : MINF-LA-KV-DY-RLGV-GALIVAFIIAIV-VWIIAYIEYRKLLRQRKIDRLIRKIRERAEDSGNESDGDIEELSAMVD--VG--HLRLLDVNN**------- : 85
C.BW.1996. : MINF-LA-KV-DY-RLGV-GALIVAFIIAIV-VWIIAYIEYRKLLRQRKIGRLIRKIRERAEDSGNESDGDIEELSAMVD--VG--HLRLLDVNN**------- : 85
C.ZA.1999. : MLTL-LA-RV-DY-RLGV-GALIVALIIAIV-VWIIAYLEYRKLKRQIKIDRLIRKIRERAEDSGNESDGDIEELSTMVD--MG--HLRLLDVNNG*------- : 86
C.BW.2000. : MLKLATIVDYIL-AA-KV-DY-RVGI-G-ALIAALIITIVVWIIVYREYRKLLRQKKIDRVLEKIRERAEDSGNESEGDLEELSTLVD--VE--HLRLVD-INH : 93
C.ZA.2003. : MNL-LT-RV-DY-RLGI-GALIVGLIIAIV-VWTIVYIEYRKLLRQKKIDWLIKRIGERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDL*---------- : 83
C.ZA.2003. : MLDF-TA-RV-DY-RLGV-GALIIALIIAIV-VWIIAYIEYRKLLRQQNIDRLLKRIREREEDSGNESEGDIEELSTMVD--MG--HLRLLDVNN*-------- : 85
C.BW.1996. : MFSL-IE-KV-DY-RLGV-GALIVAIIIAMI-VWIIAYIEYRKLLRQKKIDRLIERIRERTEDSGNESEGDIEDLSTMVD--MD--HLRLLDINN**------- : 85
C.BW.1996. : MFSL-IE-KV-DY-RLGV-GALIVAIIIAII-VWIIAYIEYRKLLRQKKIDRLIERIRERAEDSGNDSEGDIEDLSTMVD--MD--HLRLLDINN**------- : 85
C.BW.1996. : MFSL-LA-KV-DY-RVGV-GALIVALIIAII-VWIIVYIEYRKLLRQKKIDRLIERIRERAEDSGNDSEGDIEDLSTMVD--MD--HLRLLDINN**------- : 85
C.ZA.2003. : MLDL-TA-GV-DY-RIGV-GAFIVALIIAII-VWIIVYIEYRKLVRHKKLDWLIERIKERAEDSGNESEGDVEELATMVD--ME--HLRLV-VNDL*------- : 85
```

```
C.ZM.2002. : MIDL-AA-KV-DY-RLGV-AALILALIIAII-VWTIAFIEYRKLLKQKKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--LG--HLRL--LDAM*------- : 84
C.BW.1999. : MINL-IA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLKQKKIDWLIKRIRERAEDSGNESEGDTEGLSTMVD--ME--HLRL--LDDL*------- : 84
C.BW.1996. : MVNF-LAAKV-DY-RLGV-GALIVALIIAIV-VWSIVYIEYKKLLKQKNIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRL--LD-L*------- : 84
C.BW.1996. : MVNF-LAAKV-DY-RLGV-GALIVALIIAIV-VWSIVYIEYKKLLKQKKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRL--LD-L*------- : 84
C.BW.1996. : MVNF-LAAKV-DY-RLGV-GALIVALIIAIV-VWSIVYIEYKKMLKQKKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRL--LD-L*------- : 84
C.BW.2000. : MFDL-LA-GV-DY-RLGV-GALIIALIIAIV-VWVIAYIEYKKWLKQKKIDWLIERIRERAEDNGNESEGDTEELATMVD--MG--HLRL--LD-L*------- : 83
C.ZA.2004. : MLNL-LA-RV-DY-RLGV-GALVIALIIAIV-VWTIAYLEYRKLVRQTKINRLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRL--VDDL*------- : 84
C.ZA.2003. : MIDL-LA-KV-DY-RLGV-GALIIALIIAIV-VWTITYIEYRKLVRQRKINWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRL--LDDL*------- : 84
C.IL.1999. : MINF-LA-RV-DY-RLGV-AALIIALIIAIV-VWIIAYIEYRKLVRQRKIDWLIKRIREREEDSGNESDGDTEELATMVD--MG--NLRL--LD-VHNL*---- : 86
C.BW.1996. : MLSL-AA--I-DY-RIGV-GAFVVALIIAII-VWIIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRL--LDDI*------- : 83
C.BW.1996. : MLSL-AA--I-DY-RIGV-GAFVVALIIAII-VWIIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRL--LDDI*------- : 83
C.BW.1996. : MISL-AA-RV-DY-RIGV-GAFIVALIIAII-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRL--LDDN*W------ : 85
C.BW.1996. : MINL-AA-RV-DY-RIGV-GAFIVALIIAII-VWIIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRL--LDDI*------- : 84
C.BW.1996. : MINL-AA-RV-DY-RIGV-GAFIVALILAII-VWIIVYIEYRKLVKQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRL--LDNI*------- : 84
C.ZA.2004. : MLNL-LA-RV-DY-RIGV-GALIIALILAII-VWTLVYLEYRKVVKQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRL--LDAM*------- : 84
C.ZA.2003. : MLNL-LA-RV-DC-RVGV-GALAAALILAII-AWTLVYIEYRKLVKQRKIDWLIERIRERAEDSGNESEGDTEELATMVDMDMG--NLRL--LDDI*------- : 86
C.ZA.2003. : MLSL-LE-RV-DY-RIGV-GALAAALILAII-AWTLVYIEYRKVVKQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRL--LDGIDL*----- : 86
C.TZ.2001. : MIDL-TA-RV-DY-RIGI-GAFIIALIIAIV-VWTIVYIEYRKLVRQKRIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRL--LDAI*W------ : 85
C.ZA.2003. : MIDL-LA-RV-DY-RIGI-GAFIIALIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTLVD--MD--HIRL--LDDI*GD----- : 86
C.ES.2006. : MVDL-LA-RV-DY-RIGV-G?FIVA?IIAIV-VWIIVYIEYRKLVKQRKIDWLIKRIRERAEDSGNESEGDTEELCTMVD--MG--HLGL--LDDI*------- : 82
C.ZM..HIV1 : MIDF-AA-RV-DY-RLGV-GAFIVALIIAII-VWTIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRL--LNDI*------- : 84
C.ZA.2005. : MIDL-YA-GV-DY-RIGV-GALLVALIIAII-VWTIVYIEYRKLVRQRKIDLLIKRIRERAEDSGNESEGDTEELSTMVD--ME--HLRL--LVD-NN*R---- : 86
C.BW.1999. : M-LE-NI-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRL--L*------------ : 78
C.BR.2004. : MTELLEL-LE-KI-DY-RLAV-GAFIVALIIAII-VWTIVYIEYRKLVRQRRIDWLVRRISERAEDSGNESEGDTEELETMGD--MG--HLRL--LGGI*---- : 87
C.BW.2000. : MLDL-AA-RV-DY-RLGV-GALVVALIIAII-VWTIVYIEYRKLVRQRKIDWLIKRIGERAEDSGNESEGDTEELATMVD--MG--HLRL--LDDL*------- : 84
C.KE.1989. : MLDL-LA-RV-DY-KLGV-GALVVALIIAIV-VWTIVYIEYRKLVRQSKIDWLIKRIRERAEDSGNESDGDTEELATMVD--MG--NLRL--LDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FGDL*------- : 84
C.ZM.2003. : MVDL-LA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKLDWLVKRVRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FGDL*------- : 84
C.ZA.2001. : MLNL-LA-KV-DY-RIGV-GAFTVALIIAIV-VWTLVYIEYRKLVKQRKLDWLVKRIRERAEDSGNESEGDIEELATMVD--MG--QLRLLDIDDL*------- : 86
C.TW..TWC2 : ---------------------------------------------------MRVRGIQRNWPQWW--IWG--ILGFCMIIIC------------------ : 27
C.ZA.2004. : MSIL-QA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRQIDRLIRRIRERAEDSGNESEGDTEELATMVD--LG--HLRLLDDNNL*------- : 86
C.ZA.2002. : MLSL-LA-KV-DY-KLGV-GALIIALILAIV-VWIIVYIEYRKLLKQRHIDWLIKRIRERAEDSGNESEGDTEELATMVD--LG--HLRLLDANNL*------- : 86
C.ZA.2004. : MYSL-LE-KV-DY-RLGV-GALIIALIIAIV-VWIIAYIEYRKLLRQRQIDWLIKRIRERAEDSGNESEGDTEELATMVG--MG--HLRLLDDNN**------- : 85
C.ZA.2004. : MLNL-LA-RV-DY-RLGV-GALIIALIIAIA-VWIITYIEYRKLLRQTKINWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2000. : MVDL-LA-GV-DY-RVGV-GALIIALIIAII-VWIWVYIEYRKLLRQRKIDWLIKRLREREEDSGNESEGDTEELATMVD--MG--HLRLLDDNNV*------- : 86
C.ZA.2004. : MVDLVDF-INYRV-DY-RLGV-GALIIALIIAIA-VWIWVYIEYRKLLKQRRIDWLIKGIREREEDSGNESEGDTEELATMVD--MG--YLRLLDDNNL*---- : 90
C.ZA.2000. : MLDL-LE-RV-DY-RLGV-GALIIALIIAIV-VWIWAYIEYRQVLKQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2000. : MSV-----LV-DY-SIAI-GALIIGLIIAII-VWTWAYIEYRKLLKQRKIDCLIKRIRERAEDSGNESDGDTEELATMVD--ME--HLRLLDDNNL*------- : 83
C.ZA.2003. : MSDF-LA-KV-DY-RLGV-GALIIALILAIV-VWIWAYIEYRKLSRQRKIDCLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNN**------- : 85
C.ZA.2003. : MSSLLEK-VLEKV-DY-RLGV-GALIVAVLIAIV-VWIIAYLEYRKLLRQRKIDCLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*---- : 90
```

```
C.ZA.2005. : ----------------------------------------------------MRVRGIQRNWPQWW--IWG--ILGFWMIMVC------------------ : 27
C.ZA.2003. : MLDL-LE-KV-DY-RVGV-GALIIALLIAIV-VWIIAYIEYRKLLRQRQIDRLIKRIREREEDSGNESEGDIEELATMVD--LG--HLRLLDNNNV*------- : 86
C.ZA.2005. : ----------------------------------------------------MRVKGISRNWPQWW--IWG--ILGFWIIMMC------------------ : 27
C.ZA.2003. : MLGL-IE-KV-DY-SLGI-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELAPMVD--MG--HLRLLDNNNV*------- : 86
C.ZA.2003. : MLEL-LA-KV-DY-RLGV-GALIIALIIAIV-VWILAYIEYRKLSRQRKIDRLIKRIRGRAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLEL-LA-NV-DY-RLGV-GALIIALSIVIV-VWTRVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLELMDDNNL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALIIGLIIAIV-VWIIAYLEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--VE--HLRLMDDNNL*------- : 86
C.ZA.2005. : MLNL-LA-KV-DY-RLGV-GALIIALIIAIV-VWIIAYLEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--ME--HLRLLDANNL*------- : 86
C.ZA.2003. : ----------------------------------------------------MRVRGISRNWAQWW--IWG--ILGFWMIIMC---------------- : 27
C.ZA.2003. : MLNL-LE-KV-DY-RLGV-GALIIAIIIAIV-VWIIAYLEYRKLLRQRKIDWLIERIRERAEDSGNESEGDAEELATMVD--MG--HLRLLDDNN**------- : 85
C.ZA.2004. : MLKF-LE-QV-DY-KLGV-GALIIALIIAIV-VWIIAYLEYRKLARQTKIDQLIKRIRERAEDSGNESDGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MLDL---WK-QII-D*E-*E-H***H*S*Q*LCGS*YT*NIENC*GKEK*TG*LKELGKEQKTVAMRVRGTLRNCPQWW--IWG--ILGFWMI-IIC------- : 72
C.ZA.2004. : MFNP--L-DV-DY-RLGV-GALIIAAILAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDGDNL*------- : 85
C.ZA.2003. : MVNL-LA-EV-DY-RLGV-GALIIAAIIAIV-VWIIVYIEYRKLLRQRKINWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLDL-LA-KV-DY-RLGV-GALIIALILTIV-VWIIAYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.1999. : MIDL-LA-RV-DY-RLGV-GALVIALSIAIV-VWILAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.1998. : MIDL-LA-RV-DY-RLGV-GALVIALSIAIV-VWILAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MVDL-LA-EV-DY-RLGV-GALVIALIIAIV-VWIIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATLVD--MG--YLRLLDAIDL*------- : 86
C.ZA.2003. : MLEL-LA-EV-DY-RLGV-GALIIALIIVIV-VWIIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MLDL-TA-VV-DY-KLGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATIVD--MG--HLRLFDDNNL*------- : 86
C.ZA.2000. : MFDL-TA-KV-DA-RLGI-GALIIALIIAIV-VWIIVYIEYRKLLRQRKIDWLIKRIREREEDSGNESEGDTEELATMVD--MG--NLRLLDDNNL*------- : 86
C.ZA.2004. : MLEL-IA-RV-DY-RLGV-GALVIALVIAIV-VWIIVYIEYRKLLRQRKVDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.1998. : MLDF-IA-RV-DY-RLGV-GALILALIIAIV-VWIIVYIEYRKLLRQRKIDRLIKRIRERTEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLNL-LE-KV-DY-RLGV-GALIIAFIIAVV-VWIIAYIEYRKVLRQQRIDWLVRRIRGKGRR*WQ*E*GGYRGIGHNGG--YG--AS*AFGW**FV------- : 81
C.ZA.2005. : MVDL--T-KI-DY-RLGV-GALIIALIITIV-VWIIAYIEYRKLLRQRKIDWLVERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDGNDL*------- : 85
C.ZA.2000. : MLNL-TE-GV-DY-RLGV-GALIIALILAIV-VWIIAYIEYRKLRRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDGNDL*------- : 86
C.ZA.2003. : MLDL-LV-KV-DY-RLGV-GALITAFILAVV-VWIIAYIEYRKLLRQRRIDWLIKRIEERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNV*------- : 86
C.ZA.2005. : ---------------------MDL?IYRI*EISKTKENRLVN*KN*GKRRRQWQ*E*WGHRGIGHNGG--YG--AS*AFG***CV------------------ : 50
C.ZA.2003. : MLDL-MV-GV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELVTMVD--MG--HVRLLDDNNL*------- : 86
C.ZA.2003. : MVDL-IV-RV-DY-RLGV-GALIIALIIAIV-VWILAYIEYRKLLRQRKIDCLIKRIRERAEDSGNESEGDTEELATMVD--MG--YLRLLDDNNL*------- : 86
C.ZA.2000. : MLTL-LE-KV-DY-RIGV-GALIIALILTIV-VWIIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MR--HLRLLDDNN**------- : 85
C.ZA.2003. : MTGL-LE-KV-DY-RLGV-GALIIALIIAIV-VWIIAYLEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MISL-LE-KV-DY-RLGV-GALIIALILTIV-VWIIAYTEYRKLVRQRKIDLLIERIRERAEDSGNESEGDTEELATMVD--MG--QLRLLDDHVL*------- : 86
C.ZA.2000. : MIDL-LA-KV-DY-RLGV-GALIIALILAIV-VWIIAYLEYRKLLRQRKIDWLIERIRERAEGSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.ZA.2004. : MSFL-YA-SV-DY-RLGV-GALIIALILAIV-VWIIVYIEYRKLLRQRKINKLIDRIRDREEDSGNESEGDIEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MWDL-TA-K-*II-D*E-*G-H***HLL*Q*LCGS*HI*NIGSC*DKGK*TD*LKELEKEQKTVAMRVRGMLRNCLRWW--IWG--ILGFWMI-TIS------- : 73
C.ZA.2004. : MLSL-LA-RV-DY-RLGV-AALVIAFIIALV-VWIIAYIEYRKWLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNN**------- : 85
C.ZA.2004. : MVDL-LA-KV-DY-RLGV-GALVVAAIIAIV-VWIIAYIEYRKWLKQKRIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNM*------- : 86
C.ZA.2004. : MLSL-LD-KV-DY-RLGV-AALVIAFILAII-VWIIAYLEYRKLARQRKIDKIIKRIRERAEDSGNESEGDTEELSTLVD--MG--NLRLLDDNNL*------- : 86
C.ZA.2003. : MLSL-ID--I-DY-RLGV-GALVVAFILAIV-VWIIAYLEYRKLVRQRKIDWLIERIRERAEDSGNESEGDAEELATMVD--MG--HLRLLDDNNV*------- : 85
C.ZA.1997. : MFSL-LE-KV-DY-RLGV-GALVIALILTII-VWIIAYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--VG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MFSL-LD-RV-DA-RLGV-GALIVALILAIV-VWVIVYIEYRKLVRQRKIDRLIERIRERAEDSGNESEGDVEELSTMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2000. : ----------------------------------------------------MRVRGIPRNWPQWW--IWG--ILGFWMIIIC-------------- : 27
C.ZA.2003. : MLRL-LD-KV-DY-RLGV-GALIIAFIIAIV-VWIIAYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--ME--HLRLVVDNNL*------- : 86
C.ZA.2003. : MLNL-PE-TV-DY-RLGV-GALIIALIITIV-VWLIVYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRLLDDNNL*------- : 86
C.ZA.2000. : MLNL-IA-RV-DY-RLGV-GALIIALILAIV-VWIIVYIEYKKLLRQRRIDWLIERIRERAEDSGNESEGDTEGLDTMVD--MG--HLRLLDNNNV*------- : 86
C.ZA.2000. : MLNL-IA-RV-DY-RLGV-GALIIALILAIV-VWIIVYIEYKKLLRQRRIDWLIERIRERAEDSGNESEGDTEGLDTMVD--MG--HLRLLDNNNV*------- : 86
```

```
C.ZA.2004. : MSIW-QE-GV-DY-RLGV-GALIIALIIAIV-VWIIVYIEYKKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDHNDV*------- : 86
C.ZA.2004. : MSIF--T-EV-DY-RLGV-GALIVALIIAIV-VWIIAYIEYRKLLRQRKIDCLIKRIRERAEDSGNESDGDTEELATMVD--MG--HLRLLDNNNV*------- : 85
C.ZA.2004. : MSIL-TE-GI-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDVEELATVVD--MG--HLRLLDNNNG*------- : 86
C.ZA.2003. : M-IE-SI-DY-RLGV-GALIIAFIIAIV-VWIIAYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNNQ*---------- : 83
C.ZA.2003. : MLSL-LA-KI-DY-RIGV-GALLIALILAII-VWTLVYIEYRKLSRQRKIDRLIIRIRERAEDSGNESEGDTEELATMVD--MD--HLRLLDNNNL*------- : 86
C.ZA.2003. : MLSL-LE-RV-DY-RIGV-GALIIALILAIV-VWSLAYLEYRKLLRQRKIDRLIRRIRERAEDSGNESEGDTEELATMVD--MG--HLRPLDNNNV*------- : 86
C.ZA.2004. : MISL-LA-EV-DY-RIGV-GALIIALIITII-VWIIVYIEYRKVLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLKL-LA-EV-DY-RLGV-GALIIALIIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--PLRLLDNNN*W------- : 86
C.ZA.2004. : ----------------------------------------------------------MRVRGIPRNWPQWW--IWG--ILGFWMIIMC-------------- : 27
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-GALIIALLIAIV-VWSIVYIEYRKLLRQKQIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HPRLLD--NV*------- : 84
C.ZA.2004. : MFNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWIIVYIEYRKLLRQRKLDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNV*------- : 86
C.ZA.2000. : ----------------------------------------------------------MRVKGILRNWPQWW--IWA--ILGFWMIIMC-------------- : 27
C.ZA.2003. : MLSL-AA-RI-DY-RIGV-GALLIALIIAIV-VWVIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLGDNNL*------- : 86
C.ZA.2004. : MLSL-AA-RV-DY-RIGV-GALLIALIIAIV-VWVIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLGDNNL*------- : 86
C.ZA.2004. : MLSL-LA-RV-GY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--NLRLLDDNNL*------- : 86
C.ZA.2003. : MLSLSLLARSF-LA-GV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--LG--HLRLLDDHNV* : 93
C.ZA.2002. : MLSSM---LSF-LA-RV-DY-RLGV-GALIIALITAIV-AWTLVYIEYRKLVRQGRIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNM* : 90
C.ZA.2005. : ----------------------------------------------------MRVKGIQRNWQQWW--IWG--ILGFWMIIMC-------------------- : 27
C.ZA.2004. : MLNL-LA-RV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDYLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNDNV*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALTVALIIAIV-VWILVYREYRKLVRQRKIDQLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNDV*------- : 86
C.ZA.2003. : MLNL-TA-RV-DY-RLGV-GALTVGLVIAIV-VWIIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--NLRLLD--DL*------- : 84
C.ZA.2003. : MIDL-LA-GV-DY-RLGV-AALVIALIIAIV-VWIIAYIEYRKLVKQTRINQLIKRIRERAEDSGNESEGDNEELATMVD--ME--HLGLLDDNNL*------- : 86
C.ZA.2004. : MLDL-SA-RV-DY-RIGV-GALLIALIIAIV-VWIIVYIEYRKLVRQRKINWLVKRIRERAEDSGNESDGDTEELATMVD--MG--YLRLLDDNNL*------- : 86
C.ZA.2003. : MLDL-TA-RV-DY-RIGV-GALIVALIIAIV-VWIIVYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNGL*------- : 86
C.ZA.2003. : MLDL-TA-RV-DY-RLGV-GALIIALIIAIV-V*IIVYIEYRELVKQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HVRLLDGNDL*------- : 85
C.ZA.2003. : MLNL-LA--------IGV-GALIIALSIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDAEELATMVD--MG--HLRLLDGNN**------- : 80
C.ZA.2003. : MLNL-LA--------IGV-GALIIALIIAIV-VWTIVYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 81
C.ZA.2004. : MLDL-LA--------LGV-AALVIAFIIAII-VWTIVYIEYRKIVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNNV*------- : 81
C.ZA.2003. : MLSL-IA--------IGV-GALIIAFIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDHNV*------- : 81
C.ZA.2000. : MFNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKILKQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDGNDL*------- : 86
C.ZA.2001. : MLDL-LA-RV-DY-RLGV-GALIIALIIAIV-VWIIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATVVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MLDL-IA-RV-DY-RLGV-GALIIALIIAIV-VWIIAYIEYRKLLRQRKIDCLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLDL-IT-RV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLLKQRKIAWLIKRIGERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNIL*------- : 86
C.ZA.2004. : MLDL-IA-RV-DY-RLGV-GAFIIAFILAIV-VWIIAYIEYRKLLGQRKIDWLIKRIRERADDSGNESEGDAEELATMVN--ME--HLGLLDDNDM*------- : 86
C.ZA.2003. : MLGL-IA-RV-DY-RLGV-GALIIALILAIV-VWVLAYIEYRKLLRQQRIDWLIKRIRERTEDSGNESEGDTEELATMVD--ME--HLRLVVGNNL*------- : 86
C.ZA.2003. : MFSL-TE-RV-DY-RLGV-GALIIALILAIA-VWVLAYLEYRKLKRQKRIDWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRLMDGNNL*------- : 86
C.ZA.2003. : MLDL-LA-RV-DY-RLGV-GALLIALILTIV-VWIIAYLEYRKLLRQRRIDWLIKRIRERTEDSGNESEGDTEELATMVD--ME--HLRLLDDNNL*------- : 86
C.ZA.2003. : MVDL-LA-RV-DY-KLGV-GALIIALIIAIV-VWVIAYIEYRKLLRQRRIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLSL-LA-RV-DY-RLGV-GALIVALILAIV-VWVIAYLEYRKLLRQRRIDRLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLNL-LE-RV-DY-RIGV-GAFIIALIIAIV-VWVIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNHM*------- : 86
C.ZA.2003. : MLAL-LE-KV-DY-RLGV-AALIIALIIAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDNIDL*------- : 86
C.ZA.2003. : MSSF-IE-KV-DY-RLGV-AALVIALIIAIV-VWAIAYLEYRKLLKQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDNNDL*------- : 86
C.ZA.2004. : MLDL-IA-KV-DY-RIGV-GALIVAVIIAIV-VWIIVYIEYRKLLRQRKIDCLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MFEL-IA-KV-DY-RLGV-GALIVALIIAIV-VWIIVYIEYRKIVRQRKIDLLIKRIRERAEDSGNESEGDTEELATMVD--VG--QLRLLDANDL*------- : 86
C.ZA.2003. : MLNLNL-LA-KV-DY-RLGV-GALIVALIIAIV-VWIIVYIEYRKLLRQRKIDGLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDANEL*----- : 88
C.ZA.1998. : MLNL-LE-QV-DY-RIGV-GALIIALILAII-VWTIAYLEYRKLVTQKRIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDV*------- : 86
```

```
C.ZA.2003. : MLSL-LE-KV-DY-RLGV-GALIIALIIAII-VWTILYIEYRKLLRQRKIDWLVNRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLVRQRKIDWLVRRIRERAEDSGNESEGDTEELATMVD--MG--NLRLLDDNGV*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWAITYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDDNGL*------- : 86
C.ZA.2003. : MLELLLE-KV-DY-KVGI-AALIIALILAIV-VWTIAYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLVDNDL*------- : 87
C.ZA..99ZA : MFLLA-KV-DY-KIGV-AALIIALILAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDGNDV*--------- : 85
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-GALIIALIIAIV-VWTIVYLEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDKEELATMVD--ME--HLRLLDNIDL*------- : 86
C.ZA.2003. : MVDL-LA-AV-DY-RIGV-GALGIALIIAIV-VWTIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRLLDNNDL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTIVYLEYKKLLRQRRIDWLIERIRERTEDSGNESEGDTEELATIVD--LG--HLRLLDNHDL*------- : 86
C.ZA.2002. : MVHL-LA-RV-DY-RLGV-AALIIALIIAIV-VWIIVYIEYKKLLGQRKIDWLVRRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNDV*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-AALIIALIIAIV-VWTIVYIEYKKLLRQRKLDWLVRRIRERAEDSGNESEGDTEELATMGD--MG--HLRLLDDNNV*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RLGV-GALIIALIIAIA-VWIIVYIEYRKLLRQRKIDWLVKRIREGAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MVNW-LA-EV-DY-RLGV-GALIIALIIAIV-AWSIVYIEYRKVVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDDNN*W------- : 86
C.ZA.1999. : MVHL-PA-EI-DY-RLGV-GALIIALIIAIV-VWIIVYIEYRKLLRQRKIDWLIKGIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDHNV*------- : 86
C.ZA.2003. : MWNFLLA-KV-DY-RLGV-GALIIALIIAIV-VWTITYIEYRKLLRQRKINWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 87
C.ZA.2005. : MWDL-LA-KV-DY-RIGV-GAWLIALIIAII-VWTIVYIEYRKLVRQRKIDQLIKRIRERAEDSGNESEGDTEQLATMVD--ME--HLRLLDNNNL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-GALVTALILAII-VWTITYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLGDNNL*------- : 86
C.ZA.2003. : MLNL-LA-KV-DY-RLGI-GALVIALILAII-VWILIYIEYRKLLRQKKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNNL*------- : 86
C.ZA.2004. : MLKL-LA-RV-DY-RLGV-GALIIALIIAII-VWTIVYIEYRKLVRQRKIDWLVRRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RLGV-GALIIAIIIAII-VWIIAYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNNR*------- : 86
C.ZA.2003. : MVNLLEKVNL-FE-KV-DY-RLGV-GALLIALVIAII-VWTIAYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--LG--HLRLLDVAEL*- : 92
C.ZA.2005. : ----------------------------------------------------MRVRGIQRNWPQWW--IWG--ILGFWIILSC------------------ : 27
C.ZA.2000. : MVNL-LA-RV-DY-RLGV-GALIIALSIAII-VWIIVYIEYRKLLRQKKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--YLRLLDDNNL*------- : 86
C.ZA..99ZA : MLDL-LA-RV-DY-RLEV-GALIIALIIAII-VWIIAYIEYRKVLRQKKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNNL*------- : 86
C.ZA.2003. : MLDL-LA-RV-DY-RLGI-GALIIALIIAIV-VWTIVYIEYRKLVRQKKIDWLIKRIRERAEDSGNESEGDAEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2000. : MLNF-LA-KV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKVLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2004. : MLDF-LA-RV-DY-RLGV-GALIIALIIAIV-IWTITYLEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLEF-LATKV-DY-RLGV-AALIIALIIAIV-VWTIVYIEYRKVLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 87
C.ZA.1998. : MLKL-LT-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.ZA.1998. : MLKL-LT-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.ZA..99ZA : MLNL-LA-EV-DY-RLGV-GALIIALIIAIV-VWTIVYIEYRKLSRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNDL*------- : 86
C.ZA.2000. : MLSL-LA-KV-DY-RLGV-GALIVALIIAIV-VWTITYIEYRKLLRQKQIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLLDVNDV*------- : 86
C.ZA.2003. : MLSL-LA-QV-DY-RIGV-GALIIALIIAII-VWTIAYIEYRKLLRQRQIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.1999. : MLGI-IA-KV-DY-RLGV-AALVIALIIAIV-VWTIAYIEYRKLVKQRQIDRLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MWDL-LG-RV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRQIEWLIKRIRERAEDSGNESEGDTEELATMVD--LG--HLRLLDGIDL*------- : 86
C.ZA.2004. : MLEWLLE-KV-DY-RLGV-GAFIIALIIAIV-VWIIAYIEYRKLLRQQKIDWLIKRIRERAEDSGNESEGDTEELATMVD--ME--HLRFLDNNDL*------- : 87
C.ZA.2004. : MLD-LLA-KI-DY-RVGV-VALVIALIIAIV-VWIIAYIEYRKVLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNDL*------- : 86
C.ZA.2003. : MVD-WLE-KV-DY-KLGV-GALIIALILAIV-VWIIAYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2000. : MLG-LLA-KV-DY-RLGV-GAFIIALIIAIV-VWIIAYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLG-LLA-KV-DY-KLGV-GALIVALIIAIV-VWIIAYIEYRKLIRQREIDQLIKRIRERAEDSGNESEGDIEELATMVD--MG--HVRLLVDNDL*------- : 86
C.ZA.2003. : MLH-LLD-RV-DY-RIAV-GALIIALIIAIV-VWIIAYIEYRKLVKQRKIDNLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.ES.2008. : MLD-LLA-KV-DY-RIAV-VAFLIALILAIV-VWIIAYIEYRKLVKQTRIDQLIKRIRERAEDSGNESEGDTEELATLVD--MG--HLRLLDVNDL?------- : 86
C.ZA.2005. : MWD-LLA-RV-DY-RVGV-GALIIALILAIV-VWIIAYIEYRKLVRQSKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDNNDL*------- : 86
C.TW..TWC3 : ----------------------------------------------------MRVRGILRNYQQWW--IWG--VLGFWMLMIC------------------ : 27
C.ZA.1998. : MFDL-AA-KV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDNNDL*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTITYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDGNDL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYIEYRKLLRQRRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.1997. : MLNL-LV-RV-DY-RLGV-GALIIALIIAIV-VWIIAYIEYRKLLRQKRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
```

```
C.ZA.2003. : MLDL-LA-RV-DY-RLGV-GALVVALIIAIV-VWTIAYIEYRKLVRQRRINWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MVDL-LA-RV-DY-RLGV-GALIIALIIAIV-VWTWAYIEYKKLLRQRKIDWLIKRIRERAEDSGNDSEGDTEELSTLVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLNI-LE-KV-DA-RLGI-GALIIALIIVIV-VWIIVYIEYRKLLRQRKIDRLIERIRERAEDSGNESEGDTEELSTLVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLNL-LE-GV-DY-RLGV-GALIIALILAII-VWTIAYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLNL-LA-RV-DY-RIGV-GALIIALIIAIV-VWTIVYIEYRKLVKQRKIDRLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 86
C.ZA.2000. : MLNL-LE-RV-DY-RLGV-GAFIIALIIAIV-VWTIAYIEYRKVVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLSL-LA-KV-DY-RIGV-GALLIALIIAII-VWTIAYIEYRKVVRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDVNDL*------- : 86
C.ZA.2003. : MLNL-LE-RV-DY-RVGV-GALLIGVIIAII-VWTIVYIEYRKILKQRKIDRLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLKL-LD-RV-DY-RVGV-GAFIIALIIAII-VWTIAYIEYRKLVKQRRIDRLIERIRERAEDSGNESEGDTEELSTMVD--MG--HIRLLDGINL*------- : 86
C.ZA.2004. : MLNL-LE-KV-DY-RIGV-GAFIVALILAII-VWVIAYIEYRKLVKQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVG--MG--RLRLLDVNDL*------- : 86
C.ZA.2003. : MLSL-LD-RV-DY-RLGV-GALVVALILAII-VWIIAYIEYRKLVKQRKIDWLIERIRERAEDSGNESEGDVEELSTMVD--MG--HIRLLDANDL*------- : 86
C.ZA.2000. : MLDL-LA-RV-DY-RVGV-GAFTIALILVII-VWIIVYIEYRKLVKQRRIDWLLERIRERAEDSGNESDGDTEELSTMVE--MG--HIRLLDANDL*------- : 86
C.ZA.2000. : MLDL-LA-RV-DY-RVGV-GAFTIALILVII-VWIIVYIEYRKLVKQRRIDWLLERIRERAEDSGNESDGDTEELSTMVE--MG--HIRLLDANDL*------- : 86
C.ZA.2002. : MLDL-LA-RV-DY-RIGV-GALIVALIIALS-IWIIVYIEYRKLVKQRRINWLVERIRERAEDSGNESEGDTEELSTMVD--MG--HIRLLDGV*--------- : 84
C.ZA.2003. : MLNF-LE-KV-DY-RVAV-GALIVALIIAIV-VWTIVYIEYRKLVKQKRIDWLIKRIRERTEDSGNESEGDTEELSTMVD--MG--HLRLLDNL*--------- : 84
C.ZA.2004. : MLDL-LA-KV-DY-RLGV-GALIIALTIAIV-VWTIAYIEYRKVVKLRRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--ME--HLRLLDNL*--------- : 84
C.ZA.2005. : MLDF-LA-RV-DY-RVGV-GALIIALIIAIV-VWTIVYIEYRKLVRQRRIDWLIERIRERAEDSGNESEGDTEELANMGD--MG--HLRLLDVADL*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RVGV-GAFIVALILAIV-VWTIVYIEYRKLVKQKRTDWLVKRIRERAEDSGNESEGDIEDLSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2002. : MLDL-LA-RV-DY-RVGV-GALTVGVILATV-VWTIVYIEYRKLVKQRKIDWLVERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLEF-FA-RV-DY-RVGV-GALIIALILAIV-VWTIVYIEYKKLVKQRKLDWLVKRIRERAEDSGNESEGDIEELSTMVD--MG--NLRLLDNNDL*------- : 86
C.ZA.2003. : MLDI-LA-KV-DY-RVAV-GAFIVALILAII-VWTIVYIEYRKIVKQRKIDRLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2002. : MLDL-LA-KV-DY-RVGV-GALIVALIIAII-VWTIVYLEYRKLVKQRKIDWLIERIKERAEDSGNESEGDTEELATMVD--MG--YLRLLDVHDL*------- : 86
C.ZA.2004. : MLDL-LA-RV-DY-RIGV-GAFAIGLIIAII-VWTIVYIEYRKLVKQRRIDRLVQRIRERAEDSGNESEGDNEELATMVD--MG--HLRLLDVGDL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-GALTIGLIIAIV-VWTIVYIEYRKLVKQRKIDRLIKRIRERAEDSGNESEGDVEELATMGD--ME--HLRLLDVHDI*------- : 86
C.ZA.2004. : MLDL-LE-RV-DY-RIGV-GAFIVGLIIAIV-IWTIVYIEYRKLVKQRKINRLIERIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDVHDL*------- : 86
C.ZA.2003. : MLDL-LA-RV-DY-RIGV-GAFIIALIIAIV-VWIIVYLEYRKLVKQRKIDWLIERIRERAEDSGNESDEDTEELATMVD--MG--HLRLLDNNDV*------- : 86
C.ZA.2005. : ----------------------------------------------------MRVRGILRNWQPWW--IWG--ILGFWMLMSC------------------ : 27
C.ZA.2003. : MLDL-LE-KV-DY-RVGV-GALIVALILTIV-VWIIAYLEYRKLVGQRRIDLLIKRIRERAEDSGNESEGDIEELSTMVD--MG--NLRLLDVNDL*------- : 86
C.ZA.1998. : MLNL-QT-RI-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDVNEL*------- : 86
C.ZA.1998. : MLNL-QT-RI-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDVNEL*------- : 86
C.US.1998. : MVNL-VA-IV-DY-RLGI-GALIVALIIAII-VWTIAYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDNEELATMVD--MG--QLRLLDVNDL*------- : 86
C.TZ.2001. : MFDI-AA-RV-DY-RLGV-GALIIALIIAIV-VWTIAYLEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDIEELATVVD--VE--HFGLLDVNDL*------- : 86
C.TZ.2001. : MFDL-PA-RV-DY-RLGV-GALLVALIIAIV-VWTIAYIEYRKLLKQKRIDWLVKRIRERAEDSGNESEGEIEELATMVD--VG--QLRLLDVNDL*------- : 86
C.BW.2000. : MLDL-IA-KV-DY-RVGI-GALIVALIIAVV-VWIIAYIEYRKLLKQRKIDWLIKRIRERAEDSGNESEGDIEELATMVD--MG--HLRLLDVHDL*------- : 86
C.FI.1994. : MIDW-TA-KV-DY-RVGV-GALTVGLIIAIV-VWTIVYLEYRKLLRQRKIDWLIKRIRERAQDSGHESEGDTEELETMVD--MG--NLRLLDVNDL*------- : 86
C.ZA.2004. : MLDL-TA-RV-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTGELETMVD--MG--HLRLMDGNDL*------- : 86
C.TZ.2001. : MIDW-KA-RV-DY-RIGV-GAFIVALIIAIV-VWTIVYIEYKKLLRQRKIDWLIKRIRERTEDSGNESDGDIEELSTMVD--ME--HLRLLDGNDL*------- : 86
C.ZA.2004. : MIDW-EA-RI-DY-RIGI-GAFIVALILAIV-VWTIVYLEYRKLVRQRKIDWLVERIRERAEDSGNESEGDTEELAAMVD--MG--HFRLLDGNDL*------- : 86
C.BW.2000. : MVD--LG-RV-DY-RLGV-GALIVALIIAIV-VWIIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HIRLLDGNDL*------- : 85
C.ZM.1996. : MLDL-LA-RV-NY-RVGV-GALIVALLIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.TZ.1998. : MLNL-QA-RI-DY-RLGV-GALIVALSLAIV-VWIIAYLEYRKLVRQRKIDWLIKRIRERAEDSGNESEGVTEELATMVD--MG--HLRLLDNNDL*------- : 86
C.ZA.1998. : MVDL-LA-KV-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLVRQRKID*LIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDNNDL*------- : 85
C.ZA.1998. : ----------------------------------------------------MRVKGILRNCQQWW--TWG--ILGFWIIMTC------------------ : 27
C.ZA.2003. : MVDL-IA-RV-DY-RLAI-GAFTIALIIAIV-VWTIVYLEYRKLRKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDNNEL*------- : 86
C.ZA.2003. : MLDL-LA-RV-DY-RLGI-GAFIIALIIAIV-VWTIAYLEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDNNEL*------- : 86
C.ZA.2003. : MLEL-TA-RV-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDNNEL*------- : 86
C.ZA.2003. : MLNL-----I-DY-RLGV-GAFIVALILAIV-VWIIAYLEYRKLLRQRKIDRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDNNEL*------- : 83
```

```
C.ZA.2004. : MLDL-LV-RV-DY-RLGV-GALIVALLIAIV-VWIIAYLEYRKLLEQRKVDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDNIEL*------- : 86
C.ZA.2003. : MLDL-IA-RV-DY-RLGV-GALIVALIIAIV-VWTITYLEYRKLLRQRKIDWLIKRIRERTEDSGNESEGDIEELSTMVD--MG--HLRLLDINDL*------- : 86
C.BW.2000. : MLEL-TA-RV-DY-RLGV-GALIVAIILAIV-VWIWAYIEYKELLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDGNDL*------- : 86
C.ZA.2004. : MLDL-AA-RV-DY-KLGV-GALIVALIIAIV-VWVIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--QLRLLDVNDL*------- : 86
C.TZ.2001. : MLDL-LA-RI-DY-RVGV-GALIVALILAII-VWIIAYLEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNNL*------- : 86
C.ZA.2004. : MLDL-IA-RV-DY-RLGV-GALIVALIIAIV-AWTIAYLEYRKLLRQKGIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDDNN**------- : 85
C.BW.2000. : MLDL-LT-RV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLLRQRKIDWLIKRIREREEDSGNESEGDTEELAAMVD--MG--HLELLGAHDL*------- : 86
C.TZ.2002. : MLDL-AA-RV-DY-RLGV-GALLVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIREREEDSGNESDGDTEELATMVD--MG--HLRLLDDNNG*------- : 86
C.KE.2000. : MLDL-LA-RV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MLNL-LA-RV-DY-RIGV-GAFTVGLILAII-VWITVYIEYRKLLRQRKIDWLIERIRERTEDSGNESEGDTEELSTMVD--MG--NLRLLDVNDL*------- : 86
C.ZM.2003. : MLDL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESDGDTEELPTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLDL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-KLGV-GALIIALIIVII-VWTIVYIEYRKLLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.ZM.2003. : MLEL-IA-KV-DY-RLGV-GAMIVALIIVII-VWTIVYIEYRKLLRQRKINWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLAVNNW*------- : 86
C.KE.1997. : MLDL-TG-EV-DY-KLGV-GALLVATIIVII-VWTIVYLEYRKLLRQRKIDWLIE?IRERAE?SGNESEGDTEELATMVD--LG--HLRLLDVNNL*------- : 84
C.ZA.1998. : MLDL-----L-DY-RVGV-GALIVALIIAIV-VWTLVYIEYRKVVKQRKLDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--NLRLLDVNDL*------- : 83
C.ZA.1998. : MLDL-----L-DY-RVGV-GALIVALIIAIV-VWTLVYIEYRNVVKQRKLDWLIKRIRERAEDSGNESEGDTEDLATMVD--MG--NLRLLDVNDL*------- : 83
C.KE.2003. : MLDL-TA-RV-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLVRQRKIDRLVKRIRERAEDSGNESEGDTEELAAMVD--MG--NLRLLDVNDL*------- : 86
C.KE.1990. : M?DL-?A-RV-DY-RLGV-GALIIALIIAIV-VWTI?YIEYRKLLRQRKID?LIKRIQ?RAEDSGNESEGDTEELATMVD--M?--HLRLLDVNDL*------- : 80
C.KE.1987. : MLDL-TA-RI-DY-RLGV-GALIVAAIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.KE.1987. : MLDL-TA-RI-DY-RLGV-GALIVAAIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.KE.1987. : MLDL-IA-RI-DY-RLGV-GALIVAAIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNNL*------- : 86
C.KE.1987. : MLDL-IA-RI-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLVRQRKIDWLVKRIKERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.KE.1990. : MLDL-TA-RI-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLIRQRKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.KE.1991. : MLDL--T-RV-DY-RLAV-GALIVVLIIAIV-VWTIVFIEYRKLVRQKKIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 85
C.KE.1995. : MLDL-IA-RV-DY-RLGV-GALIIAGILAIV-VWTIVYIEYRKLVRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
```

```
C.KE.1990. : MIDF-MT-RV-DY-RLGV-GALIVAGIIAIV-VWIIVYIEYRKLLRQRKIDGLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVDDV*------- : 86
C.BW.1999. : MLDL-TA-RV-DY-RLGV-GALIVALIIAIV-VWTIVYLEYRKLQRQRRIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.BW.2000. : MLEL-TA-RV-DY-RLGV-GALIVALIIAII-VWTIASLEYRKLKRQRKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDINDL*------- : 86
C.ZA.2004. : MLDL-IA-RV-DY-RIGV-GALIVAIIIAIV-VWTIVYIEYRRLRRQRKIDRLIKRIREREEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : ML-NLLA-RV-DY-RLGV-GALIVALVIAII-VWIIVYIEYRKLVKQRRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : ML-NLLA-RV-DY-KLGV-GALIVALVITII-VWIIVYIEYRKLVKQRRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZM.2002. : ML-KLLA-RV-DY-RVGV-GAFIVALIIAII-VWTIVYIEYRKLLRQKRIDWLVNRIRERAEDSGNDSDGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.TZ.2001. : ML-NFLA-RV-DY-RLGV-GALIAALIIAII-VWTIAYIEYRKLLKQRKIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2000. : MLNLLEK-VVLA-RV-DY-RLGV-GALIVALILAII-VWTIAYIEYRKLLRQRKIDRLIERIGERAEDSGNESDGDIEELSTMVD--MG--HLRLLDAIDI*-- : 91
C.ZA.2005. : ----------------------------------------------------MRVRGTQRNYQQWW--IWG--ILGFWMLIMC------------------- : 27
C.IL.1999. : MLE-SV-DY-RLGV-GALIVALIIAII-VWIIVYIEYRKLLRQRRIDWLVNRIKERAEDSGNESDGDAEELSTMVD--MG--HLRLLDANDL*----------- : 83
C.ZA.2004. : ML-SLLA-RV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVRQRRIDWLAKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDANDL*------- : 86
C.ZA.2003. : MW-DLSA-RV-DY-RLGV-GALIVALIIAIV-VWSIVYIEYRKLVRQRRIDWLVERIRERAEDSGNESDGDTEELATIVD--MG--HLRLLDANDL*------- : 86
C.ZA.1998. : MVSL-SLFK-GV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLVRQKKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*----- : 88
C.ZA.1998. : MVSL-SLFK-GV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLVRQKKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*----- : 88
C.ZA.1998. : MVSL-SLFK-GV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLVRQKKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*----- : 88
C.ZA.1998. : MVSL-SLVK-GI-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLVRQKKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*----- : 88
C.ZA.2005. : ----------------------------------------------------MRVMGIQRNCPQWW--IWG--ILGFWMLMIC------------------- : 27
C.ZA.2004. : ML-NLYA-KV-DY-RLGV-GALIVALILGLI-VWTIAYIEYRKLVRQRKIDLLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLGLLDANDL*------- : 86
C.ZA.2004. : MI-DLLA-RV-DY-RLGV-GALVVALIIAII-VWIIVYIEYRKLIRQRKIDWLIKRIRERAEDSGNDSDGDTEELSTLVD--MG--NLRLLDVSDL*------- : 86
C.ZA.2003. : MI-DS----I-DY-RLGV-GALIIALIIAII-VWTIAYIEYRKLLKQRKIDWLIKRIRERAEDSGNDSDGDTEELSTMVD--MG--NLRLLDANDL*------- : 83
C.ZA.2003. : ----M-PE-*II-D*E-*EH***H*S*Q*-*CGP*HI*NIESY*DKGE*TG*LKELGKEQKTVAMTVKGTQKNYQQWW--IWG--ILGFWMIMIC--------- : 67
C.ZA.2004. : MI-DVAA-RI-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLLRQKKIDWLVKRIRERAEDSGNDSDGDTEELSTMVD--MG--NLRLLDANDL*------- : 86
C.ZA.2005. : ----------------------------------------------------MRVMGTQRNCQQWW--IWG--ILGFWMLMIC------------------- : 27
C.ZA.1998. : ML-DLTA-RI-DS-RLGI-GALIVALIIAII-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELSTLVD--MG--HLRLLDANDV*------- : 86
C.ZA.1998. : ML-DLTA-RI-DS-RLGI-GALIVALIIAII-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELSTLVD--MG--HLRLLDANDV*------- : 86
C.ZA.1998. : ML-DLTA-RI-DS-RLGI-GALIVALIIAII-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESEGDTEELSTLVD--MG--HLRLLDANDV*------- : 86
C.ZA.2003. : ML-DLSA-RV-DY-RIGV-GALTVALILAII-VWTIVYIEYRKLVRQNRIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDAHDL*------- : 86
C.ZA.2003. : ML-NLSA-RV-DY-RLGI-GALIVALIIAII-VWTIVYIEYRKLVRQSRIDWLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.MW.1993. : ML-ELIA-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVRQRKIDWLVKRIRERAEDSGNESDGDTEELATIVD--MG--HLRLWDANDL*------- : 86
C.ZA.2003. : ML-NLIA-RV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVRQRKIDRLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.BW.1998. : ML-DLIA-RV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVKQRKIDWLIKRIRERVEDSGNESDGDTEELSTLVD--MG--NLRLLDANDL*------- : 86
C.ZA.1998. : ML-NLYA-RV-DY-RVGA-GALIIALIITII-VWTIVYIEIRKIKRQEKIDRLIRRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDANEL*------- : 86
C.ZA.2003. : MI-NLLA-RV-DY-RLGV-GALIVALIITII-VWTIVYLEYRKLRRQRRIDRLIERIRERAEDSGNESEGDTEELSTLVD--MG--HLRLLDNNEL*------- : 86
C.ZA.2005. : ----------------------------------------------------MRVTGIRKNCQQWW--IWG--ILGFWMLMIY------------------- : 27
C.ZA.1998. : MI-NLIA-RV-DY-RIGV-GALIIALIIAII-VWIIVYIEYRKLVRQRKLDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2002. : ML-SLIA-RI-DY-RLGV-GALVVALIITIV-VWIIVYIEYRKLAKQRKIDWLIKRIRERAEDSGNDSDGDTEELATMVD--MG--QLRLLDANDL*------- : 86
C.ZA.2005. : ----------------------------------------------------MKVKGILRNWKQWW--IWG--ILGFWMLMIC------------------- : 27
C.GE.2003. : ML-DF*-QK-*II-D*E-*E-H***H*S*Q**RGP*HI*NIENC*DRKD*TG*LKELRNEQKTVAMGVKGILRNCQRWW--IWG--ILGFWML-MVC------- : 70
C.TZ.2001. : ML-DLTA-RV-DY-RLGV-GALIVALILAII-VWGIVYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLMDAIDL*------- : 86
C.SN.1990. : MV-DLLA-KV-DY-RLGV-GALIVALIIAII-VWIIAYIEYRKLLRQKKLDWLVKRIRERAEDSGNESDGDVEELSTMVD--MG--HLRLLDAIDL*------- : 86
C.SN.1990. : MV-DLLAKS-RL--*IRS-RSIDSSTNHSNNLCGS*HI*NIGNC*DKRN*IG*LRELGKEQKTVAMRVTGMLRNCQPWW--IWG--ILGFWMR-LIY------- : 81
C.KE.2001. : MV-DLLA-KI-DY-RLGV-AALI?AIIIAII-VWIIAYIEYRKLLR?RKIDWLVNRIRERAEDSGNESDGD?EELS?MVD--MG--HLRLLDANDL*------- : 82
C.ZA.2003. : MV-DSLA-RV-DY-RLGV-GALIVAIIIAII-VWTIAYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELATMVD--MG--QLRLFDAIEL*------- : 86
C.BW.1998. : ML-AFLA-RV-DY-RLGV-GAFIIALIIAII-VWTIAYLEYRKLVRQKKIDRLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDAHDL*------- : 86
C.ET.2002. : MF-SFTE-RV-DA-RVGI-GALIVALILAII-VWTIAYIEYRKLLRQRKI?RLIERIRERAEDSGNESEGDTEELSTMVD--MG--TLRLLDVHDL*------- : 85
C.ZA.2003. : ML-NFLA-GV-DY-RIGV-GALIVGLIIAIV-VWIIVYLEYRKLVKQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDAYDL*------- : 86
```

C.ZA.2005. : --MRVRGTQRNWQQWW--IWG--ILGFWMLMIC------------------- : 27
C.IN.1994. : MV-NLLE-RV-DY-RLGV-GALIVALILAII-VWTIAYLEYRKLLRQRKINRLIERIRERVEDSGNESEGDTEELSTLVD--MG--NLRLLDANDL*------- : 86
C.ZA.1998. : MLA-EV-DY-RLGV-GALIIALIIAII-VWIIAYIEYRKLVKQRKINWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*----------- : 83
C.ZA.1998. : MLA-GV-DY-RLGV-GALIIALIIAII-VWIIAYIEYRKLVKQRKINWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*----------- : 83
C.ES.2007. : ML-ELL?-EV-DY-RLGV-GALVVASILTII-VWII?YREYRKLLRQRKIDWLIERIRERAEDSGNESEGD?EELATMVD--VG--HLRLLDVNDL*------- : 83
C.ZA.2004. : MS-GLLE-RI-DY-RLAV-GALIVALILAII-VWTIVYLEYRKLRKQKKIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2005. : ---ML-D*E-*EH***H*S*Q*-LCGP*YI*NIENC*DKRE*TG*LKELGKEQKTVAMRAMGIPRNCQQLL--IWG--ILGFWMLMIC---------------- : 65
C.DK.2001. : ML-DLTA-RV-DY-RLGV-GALVVALILAII-VWTIAYIEYRKLLKQRKIDWLIKRIRERAEDSGNESDGDTEELATMVD--MG--NLRLLDVDDL*------- : 86
C.KE.1998. : MI-ALLA-RV-DY-RLGV-GALIVALIIAII-VWILAYIEYRKLLKQRKIDWLIERIRERAEDSGNESDEDTEELSTMVD--MG--NLRLLDVNDL*------- : 86
C.ZM.2002. : ML-SLLA-KV-DY-KLGV-GALIIALILAIV-VWTLAYIEYRKLLRQKRIDKLIERIREREEDSGNESDGDIEDLSTMVD--MG--NLRLLDANDV*------- : 86
C.ZM.2002. : ML-SLLA-KV-DY-KLGV-GALIIALILAIV-VWTLAYIEYRKLLRQKRIDKLIERIREREEDSGNESDGDIEDLSTMVD--MG--NLRLLDANDV*------- : 86
C.BW.1996. : ML-NLLA-KV-DY-RLGV-GALVIALIIAIV-VWIIAYIEYRKLVRQRKLDELVKRIRERAEDSGNESDGDIEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.BW.1996. : ML-NLLA-KV-DY-RLGV-GALVIALIIAIV-VWIIAYIEYRKLVRQRKLDELVKRIRERAEDSGNESDGDIEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.BW.1996. : ML-NLLA-KV-DY-RLGV-GALVIALIIAIV-VWIIAYIEYRKLVRQRKLDELVKRIRERAEDSGNESDGDIEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.BW.2000. : MI-NLLE-RV----D*E-*E-HWE*H*S***LCGP*YI*NIEN**GRRK*TS*LKELEKEKKTVAMRVMGILRSCQQWW--IWG--ILGFWML-MIC------- : 71
C.ZA.2000. : ML-NLLA-RV-DY-RLGV-GALIVALIIAIV-VWIIAYIEYKKLVRQRRIDKLVERIREREEDSGNESDGDVEELSTMVD--ME--HLRLMDVNNL*------- : 86
C.BW.1996. : ML-YLLE-KV-DY-RLGV-GALIIALIIAII-VWTIAYLEYRKVLRQRRIDRLVERIREREEDSGNESEGDIEELSTMVD--MG--HLRLLDADGL*------- : 86
C.TZ.2002. : MT-YFLE-KV-DY-KLGV-GALIVALVIAII-VWTIVYIEYRKLLRQRRIDRLVKRIRERSEDSGNESDGDVEELSTMVD--MG--HLRLLGVNDV*------- : 86
C.ZA.2004. : ML-GLLE-RV-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLLRQKKIDQLIKRIRERAEDSGNESDGDVEELSSMVD--MG--HLRLLDANGV*------- : 86
C.ZA.2005. : --MRAMGILRNCQRWW--IWG--LLGFWMLLIC------------------- : 27
C.ZA.2005. : --MRVRGILRNCQRWW--IWG--LLGFWMLLIC------------------- : 27
C.ZA.2004. : ML-SLVE-RV-DY-RLGV-GALIVALILAII-VWIIAYIEYRKLVRQKKINWLIERIRERAEDSGNESDGDTEELSTMVE--VG--NLGLLDAN*L*------- : 85
C.ZA.2003. : MV-SLIE-KI-DY-KLGV-GALIVALIIAII-VWTIAYIEYRKLKKQKKIDCLIQRIRERAEDSGNESDGDTEELSTLVD--VG--HLRLLDVNNL*------- : 86
C.FI.1992. : MP-SLIE-KV-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVRQRKINWLIKRIRERAEDSGNESDGDVEELSTMED--MG--HLRLLDVNDL*------- : 86
C.BW.2000. : ML-SLIE-RI-DY-RLGV-GALIVALIIVII-VWTIVYIEYRKLVRQRKLDWLVKRIRERAEDSGNESDGDVEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2003. : MI-SL-DY-RLGV-GALIVAIIIAII-VWIIVYIEYRKLVRQKQLDWLVERIRERAEDSGNESDGDMEELSKMVD--MG--HLRLLDVNDL*------------ : 82
C.ZM.2003. : MI-SLIDY-KIRS-RSIGSSTYHSNNCVDHSIYRI*ESVKTKENKQVN*KN*-GKSRRQWQ*E*WGYRGIVNNGG--SG--ASWAFGC*WFV------------ : 78
C.ZM.2003. : MI-SLIDY-RLGV-GALVVALIIAII-VWTIAYIEYRKVLRQRKINRLIERIRERAEDSGNESDGDTEELSTMVD--LG--HLGLLDVNGL*------------ : 83
C.ZM.2003. : ML-SL-DY-RLGV-GALIVALFIAII-VWIIAYIEYRKLLRQRRINRLIERIRERAEDSGNESDGDIEELSTMVD--ME--HIRL--FDDL*------------ : 80
C.UY.2001. : ML-QFIE-KI-DY-RLAV-GALIVASIIVII-VWSIVYIEYRKLVRQRRIDWLVERIRERAEDSGNESEGDTEELETMVD--MG--HLGLLAVNGL*------- : 86
C.AR.2005. : ML-SLIE-RV-DY-RIV?PE-H***H*S*Q*LCGS*LI*NIGNWSSKEN*TG*LRELGKEQKIAAMRVKGIQRNWKQWW--IWT--ILGFWLL-MFY------- : 76
C.BR.1998. : MLE-SI-DY-RLGV-GALIVAFIIVII-MWIIVYIEYRKLIRQRKIDWLIKRIRERAEDSGNESEGDTEELETMVD--MG--HLRLLDVNDL*----------- : 83
C.BR.1992. : ML-ELIG-RI-DY-RLGV-GALIVALIIVII-VWTIAYIEYRKLVRQRRIDWLVKRIKERAEDSGNESGGDTEELETMVD--MG--HLRLLDGNDL*------- : 86
C.AR.2001. : ---MRAKGIQRNWKQWW--IWG--ILGFCM?MIC------------------ : 26
C.BR.2004. : ML-DLTY-RV-DY-RLGV-GALIIALVLAII-VWIIAYIEYRKLVRQKRIDWLIERIRERAEDSGNESEGDTEELETMVD--MG--NLRLLDVPDL*------- : 86
C.AR.2004. : ML-SLLD-RI-DY-RLGV-AALVIALIIAIV-VWIIAY?EYRKWVRQRRIDWLIERIRERAEDSGNESEGDTEELETMVD--MG--?LRLLDANGL*------- : 84
C.AR.2005. : ML-ELLE-RI-DY-RLAV-AALIVALIIAIV-TWTIVYIEYRKLVRQRRIDWLIERIRERAEDSGNESEGDAEELETMVD--MG--HLRLLDANGV*------- : 86
C.BR.2004. : ML-GLAD-RI-DY-RIGV-GALLIALIIAII-VWTIVYIEYRKLVRQRRINWLIKRIRERAEDSGNESEGDTEELEAMGD--MG--HLRLLDVNGV*------- : 86
C.BR.2004. : MLE-EI-DY-RLGV-GALIVALIIAIV-VWIIAYIEYRKLVRQRRINWLIERIRERAEDSGNESEGDTEELETMVD--MG--NLRLLDVNDV*----------- : 83
C.BW.2000. : MF-SLLE-RI-DY-RLGV-GALLVALIIAIV-VWAIVYIEYRKLVRQRRLDWLVKRIRERAEDSGNESEGDNEELSTVVD--LG--HLRLLDVTDL*------- : 86
C.KE.1991. : ML-NLVA-RI-DY-KLGV-GALLVALSIAII-VWTIVYIEYRKLVKQRRIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.IN..1037 : --MRVRGILRNWQQWW--IWG--ILGFWLLMIC------------------- : 27
C.ZA.2003. : ML-SLLE-RV-DY-KIGV-AALIIAVIIAIV-VWTIAYLEYRKLVRQRKLDWLVKRIRERAEDSGNESEGDMEELATMVD--MG--HLRLLDVNDL*------- : 86
C.FI.1991. : ML-NLLE-KV-DY-RLAV-GALIIALIIAIV-VWTIAYIEYRKLLRQRKINWLVKRIRERAEDSGNESEGDTEELATMVD--MG--HPRLLDVNDL*------- : 86
C.ZA.2004. : ML-DLLE-GV-DY-RLGV-AAFILALIIAIV-VWIIAYIEYRKLLRQRKLDWLVKRIRERAEDSGNESEGDIEELSTMVD--MG--QLRLLDVNN**------- : 85
C.ZA.2005. : ML-NLLE-AV-DY-RVGV-AALIIALILVIV-VWIIAYIEYRKLLRQKRLDWLINRIRERAEDSGNESEGEIEELSTMVD--MG--HLRLLDVNN**------- : 85
C.ZA.2004. : ML-NLLE-KV-DY-RIAV-AALTVALILAIV-VWSLAYREYRKLLKQKKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 86

```
C.ZA.2004. : MI-NFLV-KV-DY-RIGV-GAFVIALIIAIV-VWIIAYIEYRKLVRQRKIDWLIERIRGRAEDSGNESEGDTEELATMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2005. : -------------------------------------------------------MRVRGILRNWKQWW--IWG--ILGFWMLMIC------------------- : 27
C.ZA.2004. : MS-ELLA-QV-DY-RLGV-GALIVALIIAIV-VWIIAYLEYRKLLKQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDAI*LQ------- : 86
C.ZA.2004. : ML-GFLE-KV-DY-RLGV-GALVVAIILVIV-VWTIVFIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDAEELSTVVD--MG--HLRLLDANEL*------- : 86
C.ZM.2003. : ML-SLIA-RV-DY-RLGV-GALIVALIIAII-VWIIAYIEYRKLLRQRRIDWLIKRIRERAEDSGNESEGDVEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2003. : ML-SFLE-RV-DY-RLGV-GALIIALNIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZM.2003. : MI-SLIE-KV-DY-RLGV-GALIVALIIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESDGDTGELSTMVD--ME--HLRLLDVNEL*------- : 86
C.ZA.2004. : ML-SLLE-KV-DY-RIGV-GALIIAFIIAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDAEELSTMVD--MG--QLRLLDVNDL*------- : 86
C.ZA.1998. : ML-SLIA-RV-DY-RLGV-GALIIALILAII-VWIIAYLEYRKLVRQRKIDWLIKRIREREEDSGNESEGDTEELATMVD--MG--NLRLLDGNDL*------- : 86
C.ZA..99ZA : MLEFLLE-RV-DY-RLGV-GALIVALILAIV-VWIIAYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDSEELSTMVD--MG--NLRLLDVNDL*------- : 87
C.FI.1991. : ML-SLLE-RV-DY-KLGV-GALIVAIIIAIV-VWIIAYIEYRKLLRQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--?LRLLDVNDL*------- : 85
C.ZA.1999. : ML-NLLE-KV-DY-RLGV-GALIVALIIAIV-VWIIAYIEYRKLLRQKKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDVAHL*------- : 86
C.ZA.2001. : MS-NLLD-NI-DY-RLGV-GALIVALIIAII-VWTIAYIEYRKLLRQKKIDWLIKRIRERAEDSGNESEGDTEELATMVD--MD--HLRLLDVADL*------- : 86
C.ZA.2000. : ML-SLIA-RV-DY-RLGV-GALIVALIIAII-VWIIAYIEYRKLVRQNKVDWLVKRIRERAEDSGNESEGDTEELATLVD--MG--QLRLLDVADL*------- : 86
C.ZM.2002. : MINL--LEKV-DY-KITV-AAFVIALIIAII-VWTIAYLEYRKLVRQRKLDWLIKRIEERAEDSGNESDGDHEELSTMVD--MG--RLRLLDASDL*------- : 86
C.ZM.2002. : MINL--LEKV-DY-KITV-AAFVIALIIAII-VWTIAYLEYRKLVRQRKLDWLIKRIEERAEDSGNESDGDHEELSTMVD--MG--RLRLLDASDL*------- : 86
C.ZM.2002. : MINL--LEKV-DY-KITV-AAFIIAIILAII-VWTIAYLEYRKLVRQRKIDWLIKRIEERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.BW.2000. : -----------------------------------------------------------MRVMGIKRNCPPWW--IWG--ILGFWMLMIC------------- : 27
C.ZA.2003. : MLSL--IEKV-DY-RIAV-VAFTVALIIVIV-VWTIAYIEYRKLVRQRKIDWLIKRIREGAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLNL--LAKV-DY-RITV-AAFIIALIIAIV-VWTIAYIEYKKLVRQKQIDWLIERIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLSF--LEKV-DY-RIAV-AAFIIALIIAII-VWTIAYIEYRKLVRQRQIDRLIERIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLDANDL*------- : 86
C.BW.2000. : MIEL--IAAV-DY-RIGV-AALIIALIIAIV-VWTIAYIEYRKLLKQKKIDRLIKRIIERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANGL*------- : 86
C.BW.2000. : MLSL--MTRV-DY-RIAV-AAFVIALILAII-VWTIAYLEYRKLVKQKKIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.1998. : MLNF--LEKV-DY-KIGV-AALIIALIIAIV-VWTIVYIEYRQLLRQKRIDWLIERIRERAEDSGNESDGEQEELSTMVD--MG--NLRLLDANGW*------- : 86
C.ZA.1998. : MLSF--LEKV-DY-EIGV-AAFIIALIIAIV-VWIIVYIEYRKLLRQKRIDWLIERIRERAEDSGNESDGEQEELSTMVD--MG--NLRLLDANGW*------- : 86
C.BW.1996. : MVDL--LAKV-DY-KIAV-AAFIIALIIAIV-VWIIVYVEYRKLVKQRKIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.BW.1996. : MVDL--LAKV-DY-KIAV-AAFIIALIIAIV-VWIIVYVEYRKLVKQRKIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.BW.1996. : MVDL--LAKV-DY-RIAV-AAFIIALIIAII-VWTIVYVEYRKLVKQRKIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MVDF--LARV-DY-RVGV-AAIIVALILVIV-VWIIVYIEYRKLSKQRKIDWLIERIRERAEDSGNESDGDQEELSTLVD--MG--HLRLLDVNDL*------- : 86
C.ZA.1998. : MLDLNLLARV-DY-RVGV-AALLIALIIAVV-VWTIAYIEYRKLLRQRRIDKLIERIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAYDL*------- : 88
C.ZA.1998. : MLDLNLLARV-DY-RVGV-AALLIALIIAIV-VWTIAYIEYRKLLRQRRIDKLIERIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAYDL*------- : 88
C.ZA.1998. : MLDLNLLARV-DY-RVGV-AALLIALIIAIV-VWTIAYIEYRKLLRQRRIDKLIERIRERAEDSGNESDGDQEELSTMVD--MG--HLRLLGAYDL*------- : 88
C.TZ.2001. : MVDL---TKI-DY-RLGV-GALIVALILAII-VWTIAYIEYRKLLRQIKIDKLIERIRERAEDSGNESDGDKEELATMED--VG--HLRLLDAYDL*------- : 85
C.ZA.1998. : MVDL--LIEI-DY-RLGV-GALIVALIITII-VWTIAYLEYRKLQRQRKIDRLIERIRERAEDSGNESEGDAEELSTMVD--MG--NLRLLDVNDL*------- : 86
C.ZA.2004. : MLDF--LAQV-DY-RIGV-GALIVALIIAII-VWTIAYLEYRKLKRQRKIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.FI.1993. : MLGL--IAKV-DY-RLGV-GALIVALIIAIV-VWTIAYIEYRKLLRQRRIDWLIERIRERAEDSGNESDGDHEELSTMVD--MG--HLRLLDVNDL*------- : 86
```

```
C.ZA.2003. : MLNL--LARV-DY-RLGV-GALIVALIIAIV-VWTLVYIEYRKLLRQRKIDWLIKRIRERAGDSGNESDGDHEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.IL.1998. : MVDLLDL--LEKV-DY-RLAV-IALIVVAIITIV-VWTIVYIEYRKLLRQRKIDRLIERIRERAEDSGNESEGDAEELSTMVE--MG--NLRLLDVADL*---- : 89
C.IL.1999. : MLEF--LEIV-DY-RITV-GAFIVALIIAIV-VWTIAYIEYRKLSRQRKIDRLIERIRERAEDSGNESDGDADELATMVD--MG--NLRLMDVVDL*------- : 86
C.DJ.1991. : MIDL--LAKV-DY-RLAV-AAFIIAFIIAIV-VWTIAYIEYRKLLRQRKIDRLIERIRERAEDSGNESDGDTDELSTMVD--RG--NLRLLDAVDV*------- : 86
C.FI.1991. : MVDL--LAKV-DY-RLAI-GALIVALIIAIV-VWTIVYIEYRKLLRQRKIDWLVERIRERAEDSGNESEGDTEELSTMVE--MG--NLRLLDVIDL*------- : 86
C.BW.1998. : MLEL--LEKV-DY-KITV-AAFIVALIIAIV-VWTIAYIEYRKLLRQRKIDCLIKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDANDL*------- : 86
C.ZA..99ZA : MVDL--LAQV-DY-RIAV-GAFIVALIIAIV-VWTIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELETMVD--MG--NLRLLDVNDL*------- : 86
C.ZA.2005. : ---------------------------------------------------MRVMGIQRNCQQWW--IWG--ILGFWMLMIC------------------ : 27
C.ET.1986. : MVDL--LAKV-DY-RIVI-VAFIVALIIAIV-VWTIAYIEYRKLLRQRRIDRLIKRTRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDVNDL*------- : 86
C.UG.1990. : MLNL--LAGV-DY-RIGI-GALLIALIIAIV-VWIIVYIEYRKLLKQRRMDRLIKRIRERAEDSGNESDGDTEELSTMVD--MG--NLRLLDINDL*------- : 86
C.ZA.2003. : MLEL-----L-DY-SLGV-GALIVALIIAIV-VWTIVYIEYRKLLRQKKIDQLIKRIRERAEDSGNESDGDTEELATMVD--VG--HLGLLDVADL*------- : 83
C.DJ.1991. : MIDL--PAKV-DY-RLAV-GALIVALIIAIV-VWTIVYIEYRKLLRQRQIDWLIERIRERAEDSGNESDGDTEELATVVG--MG--NLRLLDVVDL*------- : 86
C.IL.1999. : MLDL--LVRV-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLLRVQKIDRLIKRIRERTEDSGNESDGDTEELATMVD--VG--HLRLLDAIDL*RE----- : 88
C.ZA.2004. : MLGL--LAKV-DY-RLGI-GALIVALIIAIV-VWTIAYIEYRKWVRQEKINWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDANDL*------- : 86
C.ZA.2004. : MLDL--LAKV-DY-RVGV-GALIVAIIIAIV-VWTIAYIEYRKWVRQEKINRLIKRIRERAEDSGNESDGDAEELSTMVD--MG--HLRLLDVNDL*------- : 86
C.ZA.2005. : MIDL--LTKV-DY-RLGV-GAFIVALIIAIV-VWTIAYLEYRKWARQKKIDRLIERVRERAEDSGNESDGDTEELAAMGD--MG--HLRLLAVNDL*------- : 86
C.IN.1993. : MID------L-DY-RLGV-GALIVALIIAIV-VWTIVYIEYRKLVRQSKINWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN.1994. : MLD------L-DY-KLGV-GALIVALIIAIV-VWTIVYIEYRKLVKQRKINWLIERIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDINDL*------- : 82
C.MM.1998. : MLD------L-DY-TLAV-GALIVALIIAIV-VWTIVYIEYRRLVNRKNRDWLLKRIRERAEDSGNESEGDTEELSTMVD--MG--R*GFW-C*EL*------- : 79
C.IN..3007 : MLD------L-DY-KLGV-GALIVALIITIV-VWTIVYIEYRRLVNQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN.1993. : MLD------L-DY-KLGV-GALIVALIIVIV-VWTIVYIEYRRLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN.1993. : MLD------L-DY-KLAV-GALIVALIIAIV-VWIIAYIEYRKLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.TW..TWC4 : MLD------L-DY-KLAV-GALIVALIIAIV-VWTIAYIEYRKLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..4051 : MIG------L-DY-KLAV-GALIVALIIAIV-VWTIVYIEYRRLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..2994 : MLE------L-DY-KLGV-GALIVALIIAIV-VWIIVYIEYRRLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN.1998. : MLD------L-DY-RLAV-GALIVALIIAIV-VWIIVYIEYRKLVKQRKIDQLIKRIRERAEDSGNESKGDTEELSTMVD--MG--NLRLLDVNEL*------- : 82
C.IN..4938 : MLD------L-DY-RLAV-GALIVVLIIAIV-VWTIVYIEYRRLVQQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..1038 : MLD------L-DY-KLAV-GALIVALIIVIV-IWTIVYLEYRKLVKQRKIDRLIERIRERAEDSGNESDGDTEELSTLVD--MG--NLRLLDVNDL*------- : 82
C.IN..1106 : MFD------L-DY-KIVV-GAFVVALIIAII-VWTIVYIEYRRLKKQRKIDRLIERIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDANDL*------- : 82
C.IN..4107 : MLN------L-DY-KVVV-GALIAALIIAIV-VWTIAYIEYRKLVEQRKIDRLIERIRERAEDSGNESDGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..1038 : MLN------L-DY-KLAV-AAFILALIIAIV-VWTIVYIEYRKLIKQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--ME--RLRLLDVDDL*------- : 82
C.TW..TWC1 : MID------L-DY-KLGV-GALIVALIIVIV-VWTIVYIEYRKLVRQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 82
C.IN..3007 : MLD------L-DY-KLGV-GALIVALIIVIV-VWTIVYIEYRKLLRQRKIDWLVKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 82
C.IN..1038 : MSD------L-DY-KLAV-VALIVALIIAIV-VWTIAYIEYRKLLKQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN.1998. : MLD------L-DY-KLAV-GAFIVALLIAIV-VWTIVFIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDVNDL*------- : 82
C.IN..1106 : MVD------L-DY-KLTV-GAFVVALIIAIV-VWTIVFIEYRKLLRQRKIDRLIKRIRERAEDSGNESEGDTEELSTLVD--MG--NLRLLDANDL*------- : 82
C.IN.2003. : MLD------L-NY-KLTV-GAFVIALILAIV-VWTIVYIEYRKLVRQRKIDRLIERIRERAEDSGNESGGDTEELPTLVD--MG--NLRLLDVHDL*------- : 82
C.BW.2000. : MLD------L-DY-KVAV-GAFTVALILAIV-VWILVYREYRKLLRQKRIDNLIKRIRERAEDSGNESDGDTEELSTLVD--MG--NLRLL--DDL*------- : 80
C.IN..2994 : MLE------L-DY-KIAV-GALIVALIIAIV-VWTIVYIEYRKLLRQKRIDRLIERIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLAVNNL*------- : 82
C.IN.1993. : ----------------------------------------------------MRVRGILRNYQQWW--IWG--ILGFWMLMIC------------------ : 27
C.IN..4081 : ---------------------------------------------------MRVRGILRNYQQWW--IWG--VLGFWMLMSC------------------ : 27
C.IN..1106 : ---------------------------------------------------MRVRGILRNYQHWW--IWG--ILGFWMLMIY------------------ : 27
C.IN.1999. : MSWN------L-DY-KLAV-GALIIALIIAIV-VWTIVYIEYRRLVRQKKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDVNDL*------ : 83
C.IN.1999. : MSWN------L-DY-KLAV-GALIIALIIAIV-VWTIVYIEYRRLVRQKKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDVNDL*------ : 83
C.IN.1999. : MSWN------L-DY-KLAV-GALIIALIIAIV-VWTIVYIEYRRLVRQKKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDVNDL*------ : 83
C.IN.1999. : MSWN------L-DY-KLAV-GALIIALIIAIV-VWTIVYIEYRRLVRQKKMDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--NLRLLDVNDL*------ : 83
C.IN.1994. : M------L-DL-KLAV-GALIVALIIAIV-VWTIVYIEYRRLVKQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*--------- : 80
```

```
C.ZA.1997. : MRD------L-T--ALGV-GALIVALIIVII-VWTIVYIEYKKLVRQKRIDWLIERIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNDL*------- : 81
C.ZA..99ZA : MLN------L-L--AIGV-GAFIVALIIAIL-VWTIVYIEYRKLVKQRRIDWLVQRIRERAEDSGNESEGDTEELSTMVD--MG--HLRLLDGNDL*------- : 81
C.IN..NARI : MLD------L-AS-ALAI-GALIVAAFIAIV-VWTIVYIEYRKLLRQKRIDQLIKRIRERTEDSGNESEGDVEELST?VD--MG--NLRLLDVNDL*------- : 81
C.MM.1999. : MLE------I-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVKQRKIDWLIERIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNEL*------- : 82
C.CN.1998. : MLG------I-DY-RLGI-GALIVALIIAIV-VWTIVYIEYRRLVRQRKIDRLIKRIRERAEDSGNESEGDTEESSAMVD--MG--NLRLLDVNDL*------- : 82
C.IN.1995. : MVN------L-DY-KLGV-GALIVALIIAIV-VWTIVYIEYRKLVQQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..1106 : MLD------V-DY-RLGV-GALIVALIIAII-VWTIVYIEYRKLVQQRKIDWLIKRIRERAEDSGNESEGDTEELSTMVD--MG--RLRLLDVNDL*------- : 82
C.IN..4958 : ------------------------------------------------------MRVMGTLRNYQQWW--IWG--VLGFWMLMIC------------------- : 27
C.IN..1106 : ------------------------------------------------------MRVRGTRRNYQQWW--IWG--ILGFWMLMIY------------------- : 27
C.BW.2000. : MFALF----EV-DY-RLTI-G-AFIVALFLAIVVWTIAYLEYRKLVRQRRIDQLVKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDV-NDL*----- : 85
C.KE.1998. : ML----AV-DY-RLTV-A-AFIVVLIIAIVVWIIAYIEYRKLLKQRRIDWLVKRIRERAEDSGNESDGDTEE?STMVD--MG--HLRLLDV-NDL*-------- : 81
C.TZ.2002. : MVD-----SI-DY-RLTV-GAFIIAFIIAIV-VWIIAYIEYRKLVRQRKIDQLIKRLGERAEDSGNESDGDIEELSTMVD--MG--NLRLLDANNL*------- : 83
C.TZ.2002. : MVDF----LE-DY-RLGV-GALIVALIIAIV-VWTIAYLEYRKLLRQRRIDQIIKRIRERAEDSGNESDGDTEELATMVD--MG--NLRLLDVNDL*------- : 84
C.ZA.2004. : MVDF-------IV--IGV-GALIVAFIIAII-VWIIAYLEYRKLVKQRKIDWLVKRIGERAEDSGNESDGDTEELSAMVD--MG--YLRLLDANDV*------- : 81
C.ZA.2003. : MLN------L-DY-KIAV-AALIVALIIAII-VWTIVYIEYRKLLRQRKIDWLIKRIRERAEDSGNESEGDAEELSTMVD--MG--HLRLLDDNEL*------- : 82
C.ZA.2003. : MWELLDK-----I-DY-NIAV-AAFIVALIIAIV-VWIIVYIEYRKVVRQRKIDWLIKRIRERAEDSGNESEGDTEELSAMVD--MG--HLRLLDVNDL*---- : 86
C.US.1998. : MLLE------V-DY-RIGV-AALIVALSIAIV-VWTIVYIEYRKLLRQRKIDWLVQRIRERAEDSGNESDGDTEELATMVD--MG--HLRLLDVDEL*------ : 83
C.TZ.2002. : MLD------V-DY-RLGV-AALILALIIAIV-VWTIAYIEYRKLLKQRKIDWLIKRIRERAEDSGNESDGDTEELSTMVD--MG--HLRLLDVNEL*------- : 82
C.BW.2000. : -----------------------------------------------------------MRVKGIQRNWPQWW--IWG--SLGFWMLMFY-------------- : 27
C.ZA.2004. : MIDL-VA-KL-DY-RLGI-AAITVALFIAII-VWTIVYLEYRKLRRQRKIDLLIERIRERAEDSDNESEGDTEELATMAD--MG--*LRLLDVNDL*------- : 85
C.ZA.2003. : ML-DL-DY-RIGV-GALVAALILVII-IWTIVYIEYKKLLKQRKIDWLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLSDVNDL*------------ : 82
C.ZA.2005. : MV-TL-DY-NITI-AAFVVALIIAII-VWTIAYIEYRKLRKQRKIDRLIERIRERAEDSGNESEGDTEELATMVD--MG--QLRLLDVNDL*------------ : 82
C.ZA.2001. : MISL-IE-KI-DY-RLAV-GALIVALIIAIV-VWTIVYLEYRKVVRQRKIDRLIERIRERAEDSGNESEGDTEELATMVD--MG--HLRLLDDNDL*------- : 86
C.BW.2000. : MLDL-T--QI-GY-ELGI-GALIVALIIAIV-VWTIVYIEYRKVLRQRKIDRLIKRIRERAEDSGNESEGDIEELEAIVD--MG--HLRLLGINNL*------- : 85
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.1997. : M--SLLQSCAIIGLVVAIILAIVVWTIVGIEYKKLLKQKRIDRLIRRIR-ERAEDSGNESDGDTDELSKLVE--MG--NYDLGDDNNL*--------------- : 81
C.KE.2001. : M-W??L?ICAIVGLIV??ILAIVVWTI?GIEYKKILKQRKI?RLI?RI?-ERAED????SDGDT?DLAALIE--MG--NYDLGIDNNL?--------------- : 68
C.KE.2001. : M-W??L?ICAIVGLIV??ILAIVVWTI?GIEYKKILKQRKI?RLI?RI?-ERAED????SDGDT?DLAALIE--MG--NYDLGIDNNL?--------------- : 68
             m                   a                                           g
```

Figure F1: An alignment of the 644 HIV-1 subtype C Vpu amino acids sequences available from the Los Alamos sequence database. Sequences are compared to the top line. Dots represent identical residues, and variant residues are shown. The total length of the protein is shown on the far right.

guyenREFERENCES

Ahmad, A., X. A. Yao, et al. (1994). "Surface expression of the HIV-1 envelope proteins in env gene-transfected CD4-positive human T cell clones: characterization and killing by an antibody-dependent cellular cytotoxic mechanism." J Acquir Immune Defic Syndr **7**(8): 789-98.

Aiken, C., N. R. Konner, et al. (1994). "Nef induces CD4 endocytosis:requirement for a critical dileucine motif in the membrane proximal CD4 cytoplasmic domain." Cell **76**: 853-856.

Akari, H., S. Bour, et al. (2001). "The human immunodeficiency virus type 1 accessory protein Vpu induces apoptosis by suppressing the nuclear factor kappaB-dependent expression of antiapoptotic factors." J Exp Med **194**(9): 1299-311.

Aledort, L. M., E. A. Operskalski, et al. (1993). "Low CD4+ counts in a study of transfusion safety." N Engl J Med **328**(6): 441-442.

Alimonti, J. B., T. B. Ball, et al. (2003). "Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS." J Gen Virol **84**(7): 1649-61.

Ameisen, J. C. and A. Capron (1991). "Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis." Immul Today **12**(4): 102-5.

Apetrei, C., D. L. Robertson, et al. (2004). "The history of SIVS and AIDS: epidemiology, phylogeny and biology of isolates from naturally SIV infected non-human primates (NHP) in Africa." Front Biosci **9**: 225-54.

Arien, K. K., A. Abraha, et al. (2005). "The replicative fitness of primary human immunodeficiency virus type 1 (HIV-1) group M, HIV-1 group O, and HIV-2 isolates." J Virol **79**: 8979-90.

Bai, C., P. Sen, et al. (1996). "SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box.." Cell **16**: 263-74.

Ball, S. C., A. Abraha, et al. (2003). "Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C." J Virol **77**: 1021-38.

Barre-Sinoussi, F., J. C. Chermann, et al. (1983). "Isolation of a T-lyphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)." Science **220**(4599): 868-71.

Becker-Herman, S., G. Arie, et al. (2005). "CD74 is a member of the regulated intramembrane proteolysis processed-processed protein family." Mol Biol Cell **16**: 5061-69.

Bell, C. M., B. J. Connell, et al. (2007). "Molecular characterization of the HIV type 1 subtype C accessory genes vif, vpr, and vpu." AIDS Res Hum Retroviruses **23**(2): 322-30.

Besnard-Guerin, C., N. Belaidouni, et al. (2004). "HIV-1 Vpu sequesters beta-transducin repeat-containing protein (betaTrCP) in the cytoplasm and provokes the accumulation of beta-catenin and other SCFbetaTrCP substrates." J Biol Chem **279**(1): 788-95.

Bijlmakers, M. J. and M. Marsh (1999). "Trafficking of an acylated cytosolic protein: newly synthesized p56(lck) travels to the plasma membrane via the exocytic pathway." J Cell Biol **145**(3): 457-68.
Blattner, W., R. C. Gallo, et al. (1988). "HIV causes AIDS." Science **241**(4865): 515-16.
Bonifacino, J. S. and L. M. Traub (2003). "Signals for sorting of transmembrane proteins to endosomes and lysosomes." Annu Rev Biochem **72**: 395-447.
Bour, S., C. Perrin, et al. (2001). "The human immunodeficiency virus type 1 Vpu protein inhibits NF-kappa B activation by interfering with beta TrCP-mediated degradation of Ikappa B." J Biol Chem **276**(19): 15920-8.
Bour, S., C. Perrin, et al. (1999). "Cell surface CD4 inhibits HIV-1 particle release by interfering with Vpu activity." J Biol Chem **274**(47): 33800-6.
Bour, S., U. Schubert, et al. (1995). "The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation." J Virol **69**(3): 1510-20.
Bour, S. and K. Strebel (2003). "The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release." Microbes Infect **5**(11): 1029-39.
Bowers, K., C. Pitcher, et al. (1997). "CD4: a co-receptor in the immune response and HIV infection." Int J Biochem Cell Biol **29**(6): 871-5.
Bram, R. J. and G. R. Crabtree (1994). "Calcium signaling in T cells stimulated by a cyclophilin B-binding protein." Nature **371**: 355-358.
Brendtsen, J. D., H. Nielsen, et al. (2004). "Improved prediction of signal peptides: SignalP 3.0." J Mol Biol **340**: 783-95.
Busch, M. P., J. E. Valinsky, et al. (1994). "Screening of blood donors for idiopathic CD4+ T-lymphocytopenia." Transfusion **34**(3): 192-97.
Callahan, M. A., M. A. Handley, et al. (1998). "Functional interaction of human immunodeficiency virus type 1 Vpu and Gag with a novel member of the tetratricopeptide repeat protein family." J Virol **72**(10): 8461.
Caly, L., D. A. Jans, et al. (2008). "Proteolytic cleavage of HIV-1 GFP-Vpr fusions at novel sites within virions and living cells: concerns for intracellular trafficking studies." J Fluoresc **19**(3): 567-73
Candler, A., M. Featherstone, et al. (2005). "Computational analysis of mutations in the transmembrane region of Vpu from HIV-1." Biochim Biophys Acta **1716**(1): 1-10.
Cervantes-Acosta, G., R. Lodge, et al. (2001). "Influence of human immunodeficiency virus type 1 envelope glycoprotein YXXL endocytosis/polarization signal on viral accessory protein functions." J Hum Virol **4**(5): 249-59.
Chen, M. X., A. E. McPartlin, et al. (1994). "A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus." Embo J **13**(18): 4278-90.
Ciampor, F. (2003). "The ion channels coded by viruses." Acta Microbiol Immunol Hung **50**(4): 433-42.
Clontech Laboratories, I. (2003). "Reef coral fluorescent proteins." Clontechniques **XVIII**(3): 6-7.

Cohen, E. A., E. F. Terwilliger, et al. (1988). "Identification of a protein encoded by the *vpu* gene of HIV-1." Nature **334**(6182): 532-4.

Connor, R. J., H. Mohri, et al. (1993). "Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals." J Virol **10**(4): 1772-1777.

Cooper, G. M. (2000). "The Cell: A Molecular Approach." Sinauer Associates, Inc.(Second Edition).

Cordes, F. S., A. Kukol, et al. (2001). "The structure of the HIV-1 Vpu ion channel: modeling and simulation studies." Biochimica et Biophysica Acta (BBA) - Biomembranes **1512**(2): 291-298.

Cortes, M. J., F. Wong-Staal, et al. (1998). "Cell surface CD4 interferes with the infectivity of HIV-1 particle released from T cells." J Biol Chem **277**: 1770-1779.

Courgnaud, V., M. Salemi, et al. (2002). "Characterization of a Novel Simian Immunodeficiency Virus with a *vpu* Gene from Greater Spot-Nosed Monkeys (Cercopithecus nictitans) Provides New Insights into Simian/Human Immunodeficiency Virus Phylogeny." J. Virol. **76**(16): 8298-8309.

Craig, H. M., M. W. Pandori, et al. (1998). "Interaction of HIV-1 *nef* with the cellular dileucine-sorting pathway is required for CD4 down-regulation and optimal viral infectivity." Proc Natl Acad Sci U S A **95**: 11229-11234.

Daniel, R., R. A. Katz, et al. (1999). "A role for DNA-PK in retroviral DNA integration." Science **284**: 644-647.

Dejucq, N. (2000). "HIV-1 replication in CD4+ T cell lines: the effects of adaptation on co-receptor use, tropism, and accessory gene function." J Leukoc Biol **68**(3): 331-7.

Dube, M., B. B. Roy, et al. (2009). "Suppression of Tetherin-Restricting Activity on HIV-1 Particle Release Correlates with Localization of Vpu in the trans-Golgi Network." J Virol **83**(9):4574-90.

Ewart, G. D., K. Mills, et al. (2002). "Amiloride derivatives block ion channel activity and enhancement of virus-like particle budding caused by HIV-1 protein Vpu." Eur Biophys J **31**(1): 26-35.

Ewart, G. D., N. Nasr, et al. (2004). "Potential New Anti-Human Immunodeficiency Virus Type 1 Compounds Depress Virus Replication in Cultured Human Macrophages." Antimicrob. Agents Chemother. **48**(6): 2325-2330.

Fauci, A. S., D. Mavilio, et al. (2005). "NK cells in HIV infection: paradigm for protection or targets for ambush." Nat Rev Immunol **5**(11): 835-43.

Finkel, T. H., G. Tudor-Williams, et al. (1995). "Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes." Nature Medicine **1**(2): 129-134.

Fischer, W. B. and M. S. Sansom (2002). "Viral ion channels: structure and function." Biochim Biophys Acta **1561**(1): 27-45.

Fliegel, L., K. Burns, et. al. (1989). "Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J Biol Chem **264(36)**: 21522-21528.

Frankel, A. D. and J. A. Young (1998). "HIV-1: fifteen proteins and a RNA." Annu Rev Biochem **67**: 1-25.
Fu, W., B. E. Sanders-Beer, et al. (2009). "Human immunodeficiency virus type 1, human protein interaction database at NCBI." Nucleic Acids Res **37**(Database issue): D417-22.
Garcia, M. L., V. F. King, et al. (1990). "Amiloride analogs inhibit L-type calcium channels and display calcium entry blocker activity." J Biol Chem **265**(7): 3763-71.
Garry, R. F. (1989). "Potential mechanisms for the cytopathic properties of HIV." AIDS **3**(11): 683-694.
Geleziunas, R., S. Bour, et al. (1994). "Cell surface down-modulation of CD4 after infection by HIV-1." Faseb J **8**(9): 593-600.
Geraghty, R. J., K. J. Talbot, et al. (1994). "Cell type-dependence for Vpu function." J Med Primatol **23**(2-3): 146-50.
Gleeson, P. A., R.D. Teasdale et al. (1994). "Targeting of proteins to the Golgi apparatus." Glycoconjugate J. **11**(5): 381-394.
Gonzalez, M. E. and L. Carrasco (1998). "The human immunodeficiency virus type 1 Vpu protein enhances membrane permeability." Biochemistry **37**(39): 13710-9.
Gonzalez, M. E. and L. Carrasco (2001). "Human immunodeficiency virus type 1 VPU protein affects Sindbis virus glycoprotein processing and enhances membrane permeabilization." Virology **279**(1): 201-9.
Gonzalez, M. E. and L. Carrasco (2003). "Viroporins." FEBS Lett **552**(1): 28-34.
Gottlinger, H. G., T. Dorfman, et al. (1993). "Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses." Proc Natl Acad Sci U S A **90**(15): 7381-5.
Gounari, F., I. Aifantis, et al. (2001). "Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development." Nat Immunol **2**(9): 863-9.
Greene, W. C. (1993). "AIDS and the immune system." Sci Am **269**(3): 98-105.
Greene, W. C., Z. Debyser, et al. (2008). "Novel targets for HIV therapy." Antiviral Research **80**: 251-65.
Guerra, B. and O. G. Issinger (2008). "Protein kinase CK2 in human diseases." Curr Med Chem **15**(19): 1870-86.
Handley, M. A., S. Paddock, et al. (2001). "Association of Vpu-binding protein with microtubules and Vpu-dependent redistribution of HIV-1 Gag protein." Virology **291**(2): 198-207.
Hase, T., H. Riezman, et al. (1983). "Import of proteins into mitochondria: nucleotide sequence of the gene for a 70-kd protein of the yeast mitochondrial outer membrane." Embo J **2**(12): 2169-72.
Hill, M. S., A. Ruiz, et al. (2008). "Modulation of the severe CD4(+) T-cell loss caused by a pathogenic simian-human immunodeficiency virus by replacement of the subtype B vpu with the vpu from a subtype C HIV-1 clinical isolate." Virology **371**(1): 86-97.

Ho, D. D., A. U. Neumann, et al. (1995). "Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection." Nature **373**: 123-126.
Hobman, T. C., L. Woodward, et al. (1995). "Targeting of a heterodimeric membrane protein complex to the Golgi: rubella virus E2 glycoprotein contains a transmembrane Golgi retention signal." Mol Biol Cell **6**(1): 7-20.
Hobman, T. C., H. F. Lemon, et al. (1997). "Characterization of an endoplasmic reticulum retention signal in the rubella virus E1 glycoprotein." J Virol **71**(10): 7670-80.
Hout, D. R., E. R. Mulcahy, et al. (2004). "Vpu: a multifunctional protein that enhances the pathogenesis of human immunodeficiency virus type 1." Curr HIV Res **2**(3): 255-70.
Hsu, K., J. Seharaseyon, et al. (2004). "Mutual functional destruction of HIV-1 Vpu and host TASK-1 channel." Mol Cell **14**(2): 259-67.
http://www.hiv.lanl.gov/ (2009). "Los Alamos HIV sequence database."
http://www.hiv.lanl.gov/content/immunology (2008). "HIV Molecular Immunology Database."
Huet, T., R. Cheynier, et al. (1990). "Genetic organization of a chimpanzee lentivirus related to HIV-1." Nature **345**(6273): 356-9.
Hussain, A., C. Wesley, et al. (2008). "Human immunodeficiency virus type 1 Vpu protein interacts with CD74 and modulates major histocompatibility complex class II presentation." J Virol **82**(2): 893-902.
Ishikawa, J., T. Kaisho, et al. (1995). "Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2 that may be involved in pre-B-cell growth." Genomics **26**(3): 527-34.
Jabbar, M. A. (1995). "The human immunodeficiency virus type 1 Vpu protein: roles in virus release and CD4 downregulation." Curr Top Microbiol Immunol **193**: 107-20.
Janvier, K., Y. Kato, et al. (2003). "Recognition of dileucine-based sorting signals from HIV-1 Nef and LIMP-II by the AP-1 gamma-sigma1 and AP-3 delta-sigma3 hemicomplexes." J Cell Biol **163**(6): 1281-90.
Jouvenet, N., S. J. Neil, et al. (2009). "Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin." J Virol **83**(4): 1837-44.
Kerkau, T., I. Bacik, et al. (1997). "The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules." J Exp Med **185**(7): 1295-305.
Kirchhausen, T. (1999). "Adaptors for clathrin-mediated traffic." Annu Rev Cell Dev Biol **15**: 705-32.
Klimkait, T., K. Strebel, et al. (1990). "The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release." J Virol **64**(2): 621-9.
Kupzig, S. e. a. (2003). "BST2/HM1.24 is a raft-associated apical membrane protein with an unusual topology." Traffic **4**: 694-709.
Kyper J, M. J. (1987). "Unusual codon usage of HIV." Nature **327**: 20.

Lakos, Z., B. Somogyi, et al. (1990). "The effect of transmembrane potential on the dynamic behavior of cell membranes." Biochim Biophys Acta **1023**(1): 41-6.
Lama, J. (2003). "The physiological relevance of CD4 receptor down-modulation during HIV infection." Curr HIV Res **1**(2): 167-84.
Lama, J., A. Mangasarian, et al. (1999). "Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner." Curr Biol **9**(12): 622-31.
Laurent-Crawford, A. G., B. Krust, et al. (1991). "The cytopathic effect of HIV is associated with apoptosis." Virology **185**(2): 829-839.
Lee, Y. H., M. D. Schwartz, et al. (1997). "The HIV-1 matrix domain of Gag is required for Vpu responsiveness during particle release." Virology **237**(1): 46-55.
Leitner, T., B. Foley, et al. (2003). "HIV Sequence Compendium 2003." Published by Theoretical Biology and Biophysics Group, Los Alamos National Laboratory.
Lemaitre, V., R. Ali, et al. (2004). "Interaction of amiloride and one of its derivatives with Vpu from HIV-1: a molecular dynamics simulation." FEBS Lett **563**(1-3): 75-81.
Lemaitre, V., D. Willbold, et al. (2006). "Full length Vpu from HIV-1: combining molecular dynamics simulations with NMR spectroscopy." J Biomol Struct Dyn **23**(5): 485-96.
Leng, L., C. N. Metz, et al. (2003). "MIF signal transduction initiated by binding to CD74." J Exp Med **197**: 1467-1476.
Levesque, K., Y. S. Zhao, et al. (2003). "Vpu exerts a positive effect on HIV-1 infectivity by down-modulating CD4 receptor molecules at the surface of HIV-1-producing cells." J Biol Chem **278**(30): 28346-53.
Levy, J. A. (1993). "Pathogenesis of human immunodeficiency virus infection." Microbiol Rev **57**(1): 183-289.
Li Q., V. I. M. (2000). "NF-κB regulation in the immune system." Nat. Rev. Immunol(2): 725-734.
Li, T. J., M. Halloran, et al. (1995). "Persistent infection of macaques with simian-human immunodeficiency viruses." J Virol **69**: 7061-7067.
Lindwasser, O. W., R. Chaudhuri, et al. (2007). "Mechanisms of CD4 downregulation by the Nef and Vpu proteins of primate immunodeficiency viruses." Curr Mol Med **7**(2): 171-84.
Lippincott-Schwartz, J., T. H. Roberts, et al. (2000). "Secretory protein trafficking and organelle dynamics in living cells." Annu Rev Cell Dev Biol **16**: 557-89.
llopis, J. e. a. (1998). Proc Natl Acad Sci U S A **95**: 6803-6808.
Locher, C. P., S. Fujimura, et al. (2003). "Expression patterns of phenotypic markers on lymphocytes from human immunodeficiency virus type 2-infected baboons." AIDS Res Hum Retroviruses **19**(1): 31-40.
Lyerly, H. K., T. J. Matthews, et al. (1987). "Human T-cell lymphotropic virus IIIB glycoprotein (gp120) bound to CD4 determinants on normal lymphocytes and expressed by infected cells serves as target for immune attack." Proc Natl Acad Sci USA **84**(13): 4601-5.

Mackay, G. A., Y. Niu, et al. (2002). "Presence of Intact *vpu* and *nef* genes in nonpathogenic SHIV is essential for acquisition of pathogenicity of this virus by serial passage in macaques." Virology **295**(1): 133-46.

Maldarelli, F., M. Y. Chen, et al. (1993). "Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein." J Virol **67**(8): 5056-61.

Margottin, F., S. Benichou, et al. (1996). "Interaction between the cytoplasmic domains of HIV-1 Vpu and CD4: role of Vpu residues involved in CD4 interaction and in vitro CD4 degradation." Virology **223**(2): 381-6.

Margottin, F., S. P. Bour, et al. (1998). "A novel human WD protein, h-beta TrCp that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif." Mol Cell **1**(4): 565-74.

Matz, M. V. F., A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., Lukyanov, S.A. (1999). "Fluorescent proteins from nonbioluminescent Anthozoa species." Nat Biotechnol **17**: 969-973.

McCormick-Davis, C., S. B. Dalton, et al. (2000). "Comparison of Vpu sequences from diverse geographical isolates of HIV type 1 identifies the presence of highly variable domains, additional invariant amino acids, and a signature sequence motif common to subtype C isolates." AIDS Res Hum Retroviruses **16**(11): 1089-95.

McNatt, M. W., T. Zang, et al. (2009). "Species-specific activity of HIV-1 Vpu and positive selection of tetherin transmembrane domain variants." PLoS Pathog **5**(2): e1000300.

Meusser, B. and T. Sommer (2004). "Vpu-mediated degradation of CD4 reconstituted in yeast reveals mechanistic differences to cellular ER-associated protein degradation." Mol Cell **14**(2): 247-58.

Milland, J., S. M. Russell, et al. (2002). "The cytoplasmic tail of alpha 1,3-galactosyltransferase inhibits Golgi localization of the full-length enzyme." J Biol Chem **277**(12): 10374-8.

Miyagi, E., A. J. Andrew, et al. (2009). "Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion." Proc Natl Acad Sci U S A **106**(8): 2868-73.

Montal, M. (2003). "Structure-function correlates of Vpu, a membrane protein of HIV-1." FEBS Lett **552**(1): 47-53.

Moore, P. B., Q. Zhong, et al. (1998). "Simulation of the HIV-1 Vpu transmembrane domain as a pentameric bundle." FEBS Lett **431**(2): 143-8.

Munro, S. and Pelham, H.R. (1987). "A C-terminal signal prevents secretion of luminal ER proteins." Cell **48(5)**: 899-907.

Nehls, S., E. L. Snapp, et al. (2000). "Dynamics and retention of misfolded proteins in native ER membranes." Nat Cell Biol **2**(5): 288-95.

Neil, S. J., S. W. Eastman, et al. (2006). "HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane." PLoS Pathog **2**(5): e39.

Neil, S. J., V. Sandrin, et al. (2007). "An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein." Cell Host Microbe **2**(3): 193-203.
Neil, S. J., T. Zang, et al. (2008). "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu." Nature **451**(7177): 425-30.
Nguyen, K. L., M. llano, et al. (2004). "Codon optimization of the HIV-1 *vpu* and *vif* genes stabilizes their mRNA and allows for highly efficient Rev-independent expression." Virology **319**(2): 163-75.
Niefind, K., B. Guerra, et al. (1998). "Crystal structure of the catalytic subunit of protein kinase CK2 from Zea mays at 2.1 A resolution." Embo J **17**(9): 2451-62.
Niefind, K., B. Guerra, et al. (2001). "Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme." Embo J **20**(19): 5320-31.
Oliveira, T. d. and S. Cassol (2009). "BioAfrica - HIV bioinformatics in Africa." http://www.bioafrica.net/index.html.
Olsen, B. B. and B. Guerra (2008). "Ability of CK2beta to selectively regulate cellular protein kinases." Mol Cell Biochem **316**(1-2): 115-26.
Opat, A. S., C. van Vliet, et al. (2001). "Trafficking and localisation of resident Golgi glycosylation enzymes." Biochimie **83**(8): 763-73.
Orian, A., H. Gonen, et al. (2000). "SCF(beta)(-TrCP) ubiquitin ligase-mediated processing of NF-kappaB p105 requires phosphorylation of its C-terminus by IkappaB kinase." Embo J **19**(11): 2580-91.
Osmanov, S., W. L. Heyward, et al. (1995). "HIV-1 Genetic Variability: Implications for the development of HIV vaccines." http://data.unaids.org.
Pacyniak, E., M. L. Gomez, et al. (2005). "Identification of a region within the cytoplasmic domain of the subtype B Vpu protein of human immunodeficiency virus type 1 (HIV-1) that is responsible for retention in the Golgi complex and its absence in the Vpu protein from a subtype C HIV-1." AIDS Res Hum Retroviruses **21**(5): 379-94.
Pantaleo, G. and A. S. Fauci (1995). "Apoptosis in HIV infection." Nature Medicine **1**(2): 118-120.
Paul, M. and M. A. Jabbar (1997). "Phosphorylation of both phosphoacceptor sites in the HIV-1 Vpu cytoplasmic domain is essential for Vpu-mediated ER degradation of CD4." Virology **232**(1): 207-16.
Paul, M., S. Mazumder, et al. (1998). "Mutational analysis of the human immunodeficiency virus type 1 Vpu transmembrane domain that promotes the enhanced release of virus-like particles from the plasma membrane of mammalian cells." J Virol **72**(2): 1270-9.
Pelham, H. R. (1996). "The dynamic organisation of the secretory pathway." Cell Struct. Funct. **21**(5): 413-419.
Pinney, J. W., J. E. Dickerson, et al. (2009). "HIV-host interactions: a map of viral perturbation of the host system." Aids **23**(5):549-54
Ploegh, H. L. (1998). "Viral strategies of immune evasion." Science **280**: 248-53.

Premkumar, A., L. Wilson, et al. (2004). "Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride." FEBS Lett **557**(1-3): 99-103.

Ptak, R. G., W. Fu, et al. (2008). "Cataloguing the HIV type 1 human protein interaction network." AIDS Res Hum Retroviruses **24**(12): 1497-502.

Ratajczak, T., A. Carrello, et al. (1993). "The cyclophilin component of the unactivated estrogen receptor contains a tetratricopeptide repeat domain and shares identity with p59 (FKBP59)." J Biol Chem **268**(18): 13187-92.

Ruiz, A., M. S. Hill, et al. (2008). "Requirements of the membrane proximal tyrosine and dileucine-based sorting signals for efficient transport of the subtype C Vpu protein to the plasma membrane and in virus release." Virology **378**(1): 58-68.

Sakai, H., K. Tokunaga, et al. (1995). "Function of human immunodeficiency virus type 1 Vpu protein in various cell types." J Gen Virol **76 (Pt 11)**: 2717-22.

Salim, A. and L. Ratner (2008). "Modulation of beta-catenin and E-cadherin interaction by Vpu increases human immunodeficiency virus type 1 particle release." J Virol **82**(8): 3932-8.

Sasai, K., Y. Ikeda, et al. (2001). "The critical role of the stem region as a functional domain responsible for the oligomerization and Golgi localization of N-acetylglucosaminyltransferase V: The involvement of a domain homophilic interaction." J Biol Chem **276**(1): 759-65.

Schnittman, S. M., S. M. Denning, et al. (1990). "Evidence for susceptibility of intrathymic T-cell precursors and their progeny carrying T-cell antigen receptor phenotypes TCR alpha beta+ and TCR gamma delta+ to human immunodeficiency virus infection: a mechanism for CD4+ (T4) lymphocyte depletion." Proc Natl Acad Sci USA **87**(19): 7727-31.

Schubert, U., A. V. Ferrer-Montiel, et al. (1996). "Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells." FEBS Lett **398**(1): 12-8.

Schubert, U., P. Henklein, et al. (1994). "The human immunodeficiency virus type 1 encoded Vpu protein is phosphorylated by casein kinase-2 (CK-2) at positions Ser52 and Ser56 within a predicted alpha-helix-turn-alpha-helix-motif." J Mol Biol **236**(1): 16-25.

Schubert, U., D. E. Ott, et al. (2000). "Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2." Proc Natl Acad Sci U S A **97**(24): 13057-62.

Schwartz, M. D., R. J. Geraghty, et al. (1996). "HIV-1 particle release mediated by Vpu is distinct from that mediated by p6." Virology **224**(1): 302-9.

Schwartz, O., V. Marechal, et al. (1996). "Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein." Nat Med **2**: 338-42.

Schwartz, S., B. K. Felber, et al. (1990). "Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs." J Virol **64**(11): 5448-56.

Scriba, T. J., F. K. Treurnicht, et al. (2001). "Characterization and phylogenetic analysis of South African HIV-1 subtype C accessory genes." **17**(8): 775-81.
Scriba, T. T., F Zeier, M Engelbrecht, S Janse van Rensburg, E (2001). "Characterization and phylogenetic analysis of South African subtype C accessory genes." AIDS Res Hum Retroviruses **17**(8): 775-81.
Sikorski, R. S., M. S. Boguski, et al. (1990). "A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis." Cell **60**(2): 307-17.
Sousa, A. E., J. Carneiro, et al. (2002). "CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load." J Immunol **169**(6): 3400-6.
Sramala, I., V. Lemaitre, et al. (2003). "Molecular dynamics simulations on the first two helices of Vpu from HIV-1." Biophys J **84**(5): 3276-84.
Stephens, E. B., C. McCormick, et al. (2002). "Deletion of the vpu sequences prior to the env in a simian-human immunodeficiency virus results in enhanced Env precursor synthesis but is less pathogenic for pig-tailed macaques." Virology **293**(2): 252-61.
Stratov, I., A. Chung, et al. (2008). "Robust NK cell-mediated human immunodeficiency virus (HIV)-specific antibody-dependent responses in HIV-infected subjects." J Virol **82**(11): 5450-9.
Strebel, K. (2004). "HIV-1 Vpu: putting a channel to the TASK." Mol Cell **14**(2): 150-2.
Strebel, K., T. Klimkait, et al. (1988). "A novel gene of HIV-1, *vpu*, and its 16-kilodalton product." Science **241**(4870): 1221-3.
Strebel, K., T. Klimkait, et al. (1989). "Molecular and biochemical analyses of human immunodeficiency virus type 1 vpu protein." J Virol **63**(9): 3784-91.
Tanaka, M., T. Ueno, et al. (2003). "Downregulation of CD4 is required for maintenance of viral infectivity of HIV-1." Virology **311**(2): 316-25.
Terwilliger, E. F., E. A. Cohen, et al. (1989). "Functional role of human immunodeficiency virus type 1 vpu." Proc Natl Acad Sci U S A **86**(13): 5163-7.
Tiganos, E., J. Friborg, et al. (1998). "Structural and Functional Analysis of the Membrane-Spanning Domain of the Human Immunodeficiency Virus Type 1 Vpu Protein." Virology **251**(1): 96-107.
Tiganos, E., X. J. Yao, et al. (1997). "Putative alpha-helical structures in the human immunodeficiency virus type 1 Vpu protein and CD4 are involved in binding and degradation of the CD4 molecule." J Virol **71**(6): 4452-60.
Tran, D. D. (2005). "CAML is a p56Lck-interacting protein that is required for thymocyte development." Immunity **23**: 139-52.
Tran, D. D., H. R. Russell, et al. (2003). "CAML is required for efficient EGF receptor recycling." Dev. Cell **5**: 245-256.
Tsien, R. (1998). "The Green Fluorescent Protein." Annu. Rev. Biochem. **67**: 509-44.
Van Damme, N., D. Goff, et al. (2008). "The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein." Cell Host Microbe **3**(4): 245-52.

Van Damme, N. and J. Guatelli (2008). "HIV-1 Vpu inhibits accumulation of the envelope glycoprotein within clathrin-coated, Gag-containing endosomes." Cell Microbiol **10**(5): 1040-57.
Van Harmelen, J., C. Williamson, et al. (2001). "Characterization of full-length HIV type 1 subtype C sequences from South Africa." AIDS Res Hum Retroviruses **17**(16): 1527-31.
Varthakavi, V., E. Heimann-Nichols, et al. (2008). "Identification of calcium-modulating cyclophilin ligand as a human host restriction to HIV-1 release overcome by Vpu." Nat Med **14**(6): 641-7.
Varthakavi, V., R. M. Smith, et al. (2003). "Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production." Proc Natl Acad Sci U S A **100**(25): 15154-9.
Varthakavi, V., R. M. Smith, et al. (2006). "The pericentriolar recycling endosome plays a key role in Vpu-mediated enhancement of HIV-1 particle release." Traffic **7**(3): 298-307.
Watzele, G. and Berger, E.G. (1990). "Near identity of HeLa cell galactosyltransferase with the human placental enzyme."Nucleic Acids Res. **18**(23):7174.
Weiss, A. and D. R. Littman (1994). "Signal transduction by lymphocyte antigen receptors." Cell **76**(2): 263-74.
Wildum, S., M. Schindler, et al. (2006). "Contribution of Vpu, Env, and Nef to CD4 down-modulation and resistance of human immunodeficiency virus type 1-infected T cells to superinfection." J Virol **80**(16): 8047-59.
Willbold, D., S. Hoffmann, et al. (1997). "Secondary structure and tertiary fold of the human immunodeficiency virus protein U (Vpu) cytoplasmic domain in solution." Eur J Biochem **245**(3): 581-8.
Willey, R. L., F. Maldarelli, et al. (1992). "Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4." J Virol **66**(12): 7193-200.
Willey, R. L., F. Maldarelli, et al. (1992). "Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes." J Virol **66**(1): 226-34.
Wilson, L., P. Gage, et al. (2006). "Hexamethylene amiloride blocks E protein ion channels and inhibits coronavirus replication." Virology **353**(2): 294-306.
www3.niaids.nih.gov (2008). "More on how HIV causes AIDS." Accessed April 2009.
www.unaids.org (2008). "Report on the Global HIV/AIDS Epidemic 2008: The Executive Summary." Accessed April 2009.
Yamada, T. and A. Iwamoto (2000). "Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection." Arch Virol **145**(5): 1021-7.
Yamaguchi, N. and Fukuda, M.N. (1995). "Golgi retention mechanism of beta-1,4-galactosyltransferase. Membrane spanning domain-dependent homodimerization and association with alpha- and beta-tubulins." J Biol Chem **270**(20):12170-6.

Yde, C. W., B. B. Olsen, et al. (2008). "The regulatory beta-subunit of protein kinase CK2 regulates cell-cycle progression at the onset of mitosis." Oncogene **27**(37): 4986-97.

Zhang G, G. V., Kain S. (1996). "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells." Biochem Biophys Res Commun **227**: 707-711.