Institute of Molecular Virology
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The transmembrane domain of HIV-1 Vpu is sufficient to confer anti-tetherin activity to SIVcpz and SIVgor Vpus

Dissertation
for the Doctoral Degree in Human Biology (Dr. hum. biol.)
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List of Abbreviations

AIDS acquired immunodeficiency syndrome
Amp ampicillin
APC allophycocyanin
APOBEC3G apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
cART combined antiretroviral therapy
β-gal β-galactosidase
BST-2 bone marrow stromal antigen 2
β-TrCP β-transducin repeat containing protein
°C degree Celsius
CaCl₂ calcium chloride
CBG click beetle green
CCR5 C-C chemokine receptor type 5
CD cluster of differentiation
cpz/CPZ chimpanzee
CO₂ carbon dioxide
CXCR4 C-X-C chemokine receptor type 4
DMEM Dulbecco’s modified eagle medium
DMSO dimethyl sulfoxide
dNTP deoxynucleotide triphosphate
eGFP enhanced green fluorescent protein
EDTA ethylenediaminetetraacetate
ELISA enzyme-linked immunosorbent assay
Env envelope protein
FCS fetal calf serum
FACS fluorescence-activated cell sorting
Fig figure
fw forward
g gram
Gag group specific antigen
gor/GOR gorilla
GP glycoprotein
GPI glycosylphosphatidylinositol
h hour
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HEK293T</td>
<td>human embryonic kidney 293 large T antigen cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HUM</td>
<td>human</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>Luc</td>
<td>luciferase</td>
</tr>
<tr>
<td>m</td>
<td>milli (10^{-3})</td>
</tr>
<tr>
<td>(\mu)</td>
<td>micro (10^{-6})</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>n</td>
<td>nano (10^{-9})</td>
</tr>
<tr>
<td>Nef</td>
<td>negative factor</td>
</tr>
<tr>
<td>NF-(\kappa)B</td>
<td>nuclear factor 'kappa-light-chain-enhancer' of activated B cells</td>
</tr>
<tr>
<td>NTB-A</td>
<td>natural killer, T and B cell antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light unit</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>rv</td>
<td>reverse</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>SIVcpzPts</td>
<td>SIV from Eastern chimpanzees (Pan troglodytes schweinfurthii)</td>
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SIVcpzPtt</td>
<td>SIV from Eastern chimpanzees (<em>Pan troglodytes troglodytes</em>)</td>
</tr>
<tr>
<td>SIVgor</td>
<td>SIV from Western lowland gorillas (<em>Gorilla gorilla gorilla</em>)</td>
</tr>
<tr>
<td>SOE PCR</td>
<td>splicing by overlap extension PCR</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>Tris</td>
<td>trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>TRIM5α</td>
<td>tripartite motif-containing protein 5 α</td>
</tr>
<tr>
<td>Vpr</td>
<td>viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>viral protein U</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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### Amino acids

<table>
<thead>
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<th>Amino Acid</th>
<th>Symbol</th>
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<tbody>
<tr>
<td>A</td>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>C</td>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>D</td>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>E</td>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>φ</td>
<td></td>
<td>amino acid with bulky hydrophobic side chain</td>
</tr>
<tr>
<td>G</td>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>H</td>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>I</td>
<td>Ile</td>
<td>isoleucine</td>
</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>L</td>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>M</td>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>N</td>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>P</td>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>Q</td>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>S</td>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>T</td>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
<td>valine</td>
</tr>
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List of Abbreviations

W  Trp  tryptophan
Y  Tyr  tyrosine
1 Introduction

1.1 The AIDS pandemic

AIDS, the acquired immunodeficiency syndrome, was first described in the United States in 1981 as a general immunodeficiency characterized by an increase of otherwise rare opportunistic infections in homosexual men (Gottlieb et al., 1981; Masur et al., 1981). In 1983, the group of Luc Montagnier was the first to isolate and identify the causative agent, the human immunodeficiency virus (HIV) (Barré-Sinoussi et al., 1983). In the following years it became clear that two immunodeficiency viruses are found in humans: HIV-1 and HIV-2 (Clavel et al., 1986; Gallo et al., 1983; Popovic et al., 1984). HIV-1 comprises four phylogenetically separable groups: M, N, O and P. Group M (‘major’) is largely responsible for the AIDS pandemic with currently more than 35 million infected individuals world-wide (UNAIDS, 2013). HIV-1 M is subdivided into nine subtypes (A-D, F-H, J and K) based on its genomic sequence diversity (Thomson et al., 2002). Group O (‘outlier’) infects about 100,000 people and is mainly restricted to West Africa, especially Cameroon (Leys et al., 1990; Peeters et al., 1997; Vallari et al., 2010; Vessière et al., 2010). Groups N (‘non-M non-O’ or ‘new’) and P are very rare with only a few reported cases (Delaugerre et al., 2011; Plantier et al., 2009; Roques et al., 2004; Simon et al., 1998; Vallari et al., 2010, 2011). HIV-2 is phylogenetically highly diverse and subdivided into nine groups (Ayouba et al., 2013). It mainly circulates in Western African countries with a prevalence of 1-10 % but its overall prevalence is declining (Hamel et al., 2007; Langley et al., 1996; van der Loeff, Maarten F Schim et al., 2006; Poulsen et al., 1993; Wilkins et al., 1993).

In general, infection with HIV-1 leads to a decline of CD4+ T cells and is associated with increased susceptibility to opportunistic infections that finally lead to death within several years after infection (Gottlieb et al., 1981; Masur et al., 1981). Up to now, HIV has killed more than 36 million people around the world and each year about 2.3 million individuals are newly infected (UNAIDS, 2013). Although long-term suppression of HIV by combined antiretroviral therapy (cART) has substantially reduced AIDS-related fatalities, effective treatment is still expensive and often accompanied by severe side effects (Reid et al., 2013). Furthermore, cART does not allow curing HIV infection and no effective AIDS vaccine is currently available (Burton et al., 2012; Menéndez-Arias, 2013).

1.2 Evolution of HIV-1

The breakthrough in deciphering the origins of the AIDS pandemic came in 1999, when Gao et al. showed that a simian immunodeficiency virus (SIV) from the Central chimpanzee (Pan
troglodytes troglodytes, Ptt) is closely related to HIV-1 (Gao et al., 1999). Since then, SIVs have been detected in more than forty African non-human primate species of which most are infected with a species-specific virus strain (Pandrea et al., 2008). Nowadays it is known that three of these species have transmitted their viruses to humans giving rise to the different groups of HIV-1 and HIV-2 (Sharp and Hahn, 2011). These transmissions most likely occurred via blood exposure during hunting or bush meat preparation (Peeters et al., 2002).

Figure 1. Distribution and infection status of chimpanzee and gorilla species in Central Africa and the evolution of the four HIV-1 groups. The habitats of different chimpanzee and gorilla (sub)species in Central Africa are indicated. Colored areas: chimpanzee habitats; dashed areas: gorilla habitats. Red star: not infected with SIV; filled green spot: infected with SIV that was transmitted to humans; unfilled green spot: infected with SIV but no transmission to humans (modified from Li et al., 2012). SIVcpzPtt was transmitted from Central chimpanzees (P. t. troglodytes) (light blue) to humans giving rise to HIV-1 groups M (pandemic) and N (17 reported cases) and to Western lowland gorillas (G. g. gorilla) (light brown) giving rise to SIVgor. This virus was subsequently transmitted to humans resulting in the emergence of groups O (epidemic) and P (two reported cases). In contrast, SIVcpzPts from Eastern chimpanzees (P. t. schweinfurthii) (yellow/green), a virus closely related to SIVcpzPtt, has not been found in humans yet.

The direct precursors of HIV-1 are SIVs infecting chimpanzees and gorillas (Gao et al., 1999; Van Heuverswyn et al., 2006; Huet et al., 1990; Peeters et al., 1989). They are thought to have crossed the species barrier on at least four separate occasions in the early and mid-20th century (figure 1) (Korber et al., 2000; Lemey et al., 2004; Sauter et al., 2011; Wertheim
et al., 2009; Worobey et al., 2008). Transmission of SIVcpzPtt from Central chimpanzees (*Pan troglodytes troglodytes*) to humans resulted in the emergence of the pandemic group M and the rare group N (Gao et al., 1999; Keele et al., 2006; Simon et al., 1998). SIVcpzPtt was also transmitted to Western lowland gorillas (*Gorilla gorilla gorilla*) giving rise to SIVgor (Van Heuverswyn et al., 2006; Takehisa et al., 2009). This virus was subsequently transmitted to humans resulting in the emergence of HIV-1 groups O and P (figure 1) (Leys et al., 1990; Plantier et al., 2009).

Apart from the Central chimpanzee there is a second SIV infected chimpanzee subspecies, the Eastern chimpanzee (*Pan troglodytes schweinfurthii*), that lives in close proximity to the Central chimpanzee (figure 1) (Gagneux et al., 1999). Interestingly, SIVcpzPts infecting the Eastern chimpanzee or progenies of this virus has never been detected in humans, suggesting that this virus has not successfully crossed the species barrier. Of note, the absence of a SIVcpzPts zoonosis cannot be explained by an insufficient primate reservoir in Eastern chimpanzees. Li et al. and others showed that SIV infection is common and widespread among Eastern chimpanzee communities (Keele et al., 2006, 2009; Li et al., 2012). In some communities, the prevalence rates were similar to or even exceeded those previously observed for Central chimpanzees in Cameroon and Gabon (Li et al., 2012). Thus, Li et al. hypothesized that greater adaptive hurdles may have prevented the successful transmission of SIVcpzPts to humans.

### 1.3 The host restriction factor tetherin

One important hurdle for successful cross-species transmission is the interferon (IFN)-inducible cellular restriction factor tetherin. Tetherin, also known as bone marrow stromal antigen 2 (BST-2), HM1.24 or CD317, has been identified by Neil et al. and Van Damme et al. in 2008 and has been shown to restrict HIV release from infected cells (Van Damme et al., 2008; Ishikawa et al., 1995; Neil et al., 2008; Ohtomo et al., 1999). It is a type II integral membrane glycoprotein of 30-36 kDa with an unusual topology (figure 2a) (Kupzig et al., 2003). Tetherin bears two membrane anchors: an N-terminal transmembrane domain (TMD) and a C-terminal glycosylphosphatidylinositol (GPI) anchor. These two anchors are separated by an extracellular coiled-coil domain, which is important for the formation of disulfide-linked dimers (Andrew et al., 2009; Hinz et al., 2010; Kupzig et al., 2003). Moreover, it comprises a small N-terminal cytoplasmic tail containing a clathrin-dependent endocytosis motif (Masuyama et al., 2009; Rollason et al., 2007). This motif is important for the cycling of tetherin molecules between lipid rafts at the cell surface and the trans-Golgi network (TGN) (Rollason et al., 2007).
As its name suggests, tetherin physically tethers newly formed enveloped virions to the membrane of infected cells with one anchor present in the plasma membrane of the cell and the second inserted into the viral membrane, thereby inhibiting virus release (figure 2a) (Lehmann et al., 2011; Perez-Caballero et al., 2009; Venkatesh et al., 2013). Tethered virions are subsequently endocytosed and degraded (Neil et al., 2006). Since this mechanism does not involve a specific viral target but the lipid envelope, tetherin restricts the release of a wide range of enveloped viruses, including members of the families of filo-, arena-, paramyxovirus-, alpha-, gammaherpesvirus-, rhabdo- and flaviviruses (Amet et al., 2014; Jones et al., 2013; Le Tortorec et al., 2011). Apart from its ability to inhibit virus release, tetherin was recently identified as a pattern recognition receptor (Cocka et al., 2012; Galão et al., 2012). Upon binding of virions at the cell surface, tetherin activates the canonical NF-κB pathway thereby inducing the expression of pro-inflammatory cytokines (Galão et al., 2012; Tokarev et al., 2013).

![Figure 2. Tetherin restricts virus release and is antagonized by the lentiviral proteins Nef or Vpu. (a) Inhibition of virus release by the host restriction factor tetherin.](image)

The IFN-inducible membrane protein physically tethers newly formed virus particles to the cell surface with one membrane anchor being present in the viral membrane and the other in the cellular membrane. Retained virions remain tethered at the cell surface or are endocytosed and subsequently degraded. (b) Species-specific counteraction of tetherin by SIVcpz and SIVgor Nef and HIV-1 M and N Vpu. SIVcpz and SIVgor Nefs target a five amino acid stretch (DIWKK or AILKK, respectively) in the cytoplasmic tail of ape (CPZ/GOR) tetherin and remove it from the cell surface. On the contrary, this five amino acid stretch is deleted in the human (HUM) orthologue of tetherin. After transmission to humans, the Vpu proteins of HIV-1 groups M and N evolved the ability to counteract the human orthologue. This down-modulation requires a direct interaction of their transmembrane domains and the transmembrane domain of tetherin.
1.4 Tetherin counteraction by SIVcpz, SIVgor and HIV-1

The lentiviral genomes of SIVcpz, SIVgor and HIV-1 contain three structural genes (gag, pol, env) and two regulatory genes (tat, rev) that are essential for replication both, in vivo and in vitro. Additionally, they encode four accessory proteins (Vif, Vpr, Vpu, Nef) that equip the virus with tools to counteract and evade the immune response of the host and enable efficient replication in vivo. Two of these accessory proteins of HIV-1 and its precursors SIVcpz and SIVgor have been shown to counteract tetherin: the negative factor (Nef) and the viral protein U (Vpu) (figure 2b) (Van Damme et al., 2008; Neil et al., 2008; Sauter et al., 2009).

1.4.1 Nef-mediated antagonism

Most SIVs, including the direct precursors of HIV-1, SIVcpz and SIVgor, use their Nef proteins to counteract tetherin in their respective host species (figure 2b) (Lim et al., 2010; Sauter et al., 2009; Yang et al., 2010). Nef is a myristoylated multifunctional protein that localizes throughout the endosomal system but is mainly found at the cell surface and in a juxta-nuclear region of the cell (Arhel and Kirchhoff, 2009; Craig et al., 2000; Tokarev and Guatelli, 2011). Nef also down-modulates CD4, the primary receptor of HIV and SIV, and a variety of cell surface receptors with immunological functions such as MHC-I and MHC-II, CD28 and CD8 (Garcia and Miller, 1991; Schwartz et al., 1996; Stove et al., 2005; Stumptner-Cuvelette et al., 2001; Swigut et al., 2001).

Tetherin antagonism by SIVcpz and SIVgor Nef requires a specific five amino acid stretch (DIWKK/AILKK) in the cytoplasmic tail of chimpanzee or gorilla tetherin (figure 2b) (Rong et al., 2009; Sauter et al., 2009). This counteraction is adaptor protein 2 (AP-2) dependent and most likely involves enhanced internalization of the restriction factor from the cell surface thereby removing it from the sites of viral budding (Serra-Moreno et al., 2013; Zhang et al., 2011).

In contrast to ape and monkey tetherins, the human orthologue is characterized by a deletion of the five amino acids that are targeted by Nef (figure 2b) (Jia et al., 2009; Sauter et al., 2009). Thus, this deletion renders human tetherin resistant to counteraction by SIV Nef and constitutes a barrier for successful transmission and spread of SIV in the human population (Sauter et al., 2009).

1.4.2 Vpu-mediated antagonism

In case of HIV-1 groups M and N, the accessory protein Vpu has evolved to counteract human tetherin after cross-species transmission (figure 2b) (Sauter et al., 2009, 2012; Yang
et al., 2011). Vpu is a 16 kDa type I transmembrane protein encoded by the genomes of HIV-1 and a few closely related SIV strains (Bailes et al., 2003; Cohen et al., 1988; Courgnaud et al., 2002; Kirchhoff, 2009; Strebel et al., 1988). It consists of a short N-terminal domain, a single \( \alpha \)-helical transmembrane domain (TMD), two cytosolic \( \alpha \) helices separated by a short flexible connector loop and a C-terminal tail (figure 2b, figure 3) (Henklein et al.; Park and Opella, 2005; Park et al., 2003; Schubert et al., 1994; Wray et al., 1995, 1999).

HIV-1 M Vpu is a multi-functional protein which does not only counteract tetherin but also degrades CD4 to prevent superinfection of the infected cell and to increase virus release (table 1) (Van Damme et al., 2008; Kimura et al., 1994; Lama et al., 1999; Wildum et al., 2006; Willey et al., 1992). In addition, Vpu reduces cell surface expression of the NK-, T- and B-cell antigen (NTB-A), the lipid antigen presenting surface molecule CD1d and the natural killer cell activating ligand PVR (Matusali et al., 2012; Moll et al., 2010; Shah et al., 2010). These functions allow evasion from NK cell recognition of HIV-1 infected cells and inhibition of lipid antigen presentation to NK-T cells. Recently, counteraction of tetherin by HIV-1 M Vpu was also shown to contribute to the evasion of antibody-dependent cellular cytotoxic responses by NK cells (Alvarez et al., 2014; Arias et al., 2014). Apart from that, Vpu has been shown to inhibit the activation of NF-\( \kappa \)B by interfering with \( \beta \)-TrCP-mediated degradation of I\( \kappa \)B (Bour et al., 2001).

In contrast to Vpus from pandemic HIV-1 group M, those from rare group HIV-1 N strains show only weak anti-tetherin activity (table 1). Moreover, they are unable to reduce CD4 surface expression and are poorly active against CD1d and NTB-A, respectively (Sauter et al., 2009, 2012). Interestingly, HIV-1 N is still adapting to the human host and we have shown that the Vpu of a recently isolated strain representing the first infection with group N outside Cameroon is as active in counteracting tetherin as group M Vpus but lacks the CD4 degradation function (Delaugerre et al., 2011; Sauter et al., 2012). In comparison, the Vpu proteins of SIVcpz and SIVgor as well as HIV-1 groups O and P efficiently down-

<table>
<thead>
<tr>
<th>HIV-1 Vpus</th>
<th>SIV Vpus</th>
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<tbody>
<tr>
<td>M</td>
<td>N</td>
</tr>
<tr>
<td>Tetherin</td>
<td>+++</td>
</tr>
<tr>
<td>CD4</td>
<td>+++</td>
</tr>
</tbody>
</table>
modulate CD4 but are inactive against ape or human tetherin (table 1) (Sauter et al., 2009, 2011).

Counteraction of human tetherin by Vpu involves direct interaction between the transmembrane domains of both proteins (figure 2b, figure 3) (Iwabu et al., 2009; Kobayashi et al., 2011; McNatt et al., 2013; Pang et al., 2013; Rong et al., 2009; Sauter et al., 2012; Skasko et al., 2012; Vigan and Neil, 2010). Moreover, the cytoplasmic part of HIV-1 M Vpus contains a highly conserved β-TrCP binding motif (DSGXXS) that includes a pair of serine residues. These serines residues are phosphorylated by casein kinase II and interact with the adaptor protein β-TrCP (figure 3). β-TrCP recruits an E3 ubiquitin ligase complex that finally leads to the ubiquitination of tetherin and its subsequent degradation (Douglas et al., 2009; Friborg et al., 1995; Mangeat et al., 2009; Margottin et al., 1998; Mitchell et al., 2009; Schubert et al., 1994). Interestingly, most of the weakly active group N Vpus harbor mutations in this motif (Sauter et al., 2012).

It has also been reported that certain tyrosine- (YXXφ; φ: amino acid with bulky hydrophobic side chain) and di-leucine-based ([D/E]XXXL[L/I/V/M]) sorting motifs in the cytoplasmic domain of Vpu are critical for effective tetherin antagonism by Vpus (figure 3) (Dubé et al., 2009; Petit et al., 2011; Ruiz et al., 2008; Vigan and Neil, 2010). These motifs are implicated in endocytosis as well as the targeting of transmembrane proteins to lysosomes or lysosome-related organelles (Bonifacino and Traub, 2003; Kirchhausen, 1999). However, the relative contribution of these sorting motifs to Vpu-mediated counteraction of tetherin is still under debate (Arias et al., 2011, 2012).

Interestingly, the Vpus of the direct precursor of HIV-1 M and N, SIVcpzPtt, harbor most of these motifs in their cytoplasmic parts (table 2). In contrast, the Vpu of SIVcpzPts, that has not been detected in humans, and SIVgor, whose descendants have not evolved Vpu-
mediated tetherin counteraction after zoonotic transmission, lack most of these motifs (table 2) (Li et al., 2012).

**Table 2. Motifs in SIVcpz and SIVgor Vpus involved in tetherin counteraction.**
-: motif not present; ✓: motif present

<table>
<thead>
<tr>
<th>Virus</th>
<th>Clone</th>
<th>Tetherin interaction</th>
<th>Trafficking</th>
<th>β-TrCP binding</th>
<th>Trafficking</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVcpzPtt</td>
<td>MB897</td>
<td>AXXXXAXXAXXXW</td>
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<td></td>
<td>EK505</td>
<td>AXXXXAXXAXXXW</td>
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<td>✓</td>
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<td>-</td>
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<td></td>
<td>ANT</td>
<td>AXXXXAXXAXXXW</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SIVgor</td>
<td>CP2139</td>
<td>XYYXXφDSGXSS[D/E][XXX][L/I/V]</td>
<td>-</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>BQ664</td>
<td>XYYXXφDSGXSS[D/E][XXX][L/I/V]</td>
<td>-</td>
<td>-</td>
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</table>

### 1.5 Scientific aim

The acquisition of effective Vpu-mediated anti-tetherin activity distinguishes pandemic HIV-1 group M strains from non-pandemic group N, O and P viruses and may have been a prerequisite for their global spread (Sauter et al., 2009). Sequence analyses revealed that the motifs in the cytoplasmic tail of Vpu reported to be required for effective antagonism of human tetherin are often conserved in SIVcpzPtt Vpus from Central chimpanzees but usually absent in SIVcpzPts Vpus from Eastern chimpanzees and SIVgor Vpus from Western lowland gorillas (table 2) (Li et al., 2012). The aim of my thesis was to clarify whether the SIVcpzPtt Vpu only requires adaptive changes in the transmembrane domain whereas SIVcpzPts or SIVgor Vpus require adaptation in both TMD and cytoplasmic part to acquire activity against the human tetherin orthologue. This might explain (1) why SIVcpzPts has never been detected in humans yet and (2) why the descendants of SIVcpzPtt but not SIVgor evolved Vpu-mediated tetherin antagonism and caused the AIDS pandemic (Keele et al., 2006; Li et al., 2012; Sauter et al., 2009, 2011).
2 Material and methods

2.1 Material

2.1.1 Cell culture and bacteria

2.1.1.1 Cell culture

HEK293T is a human renal epithelial cell line that was transformed with adenovirus type 5 and expresses the SV40 (simian virus 40) large T antigen (Graham et al., 1977).

HeLa is a cell line with an epithelial phenotype derived from cervical cancer cells taken from Henrietta Lacks (Masters, 2002).

TZM-bl is a HeLa cell line derivative that expresses high amounts of human CD4, CCR5 and CXCR4. It contains firefly luciferase and β-galactosidase genes under the control of the HIV-1 long terminal repeat (LTR) promoter (Charneau et al., 1994).

Dulbecco’s modified eagle medium (DMEM) Gibco/LifeTechnologies; Darmstadt
Ethylendiamintetraacetate (EDTA)-trypsine Gibco/LifeTechnologies; Darmstadt
Fetal calf serum (FCS) Gibco/LifeTechnologies; Darmstadt
L-glutamine Gibco/LifeTechnologies; Darmstadt
Lipofectamine LTX Reagent Invitrogen/LifeTechnologies; Darmstadt
OPTI-MEM Invitrogen/LifeTechnologies; Darmstadt
Phosphate-Buffered Saline (PBS) Invitrogen/LifeTechnologies; Darmstadt
Penicillin/Streptomycin Gibco/LifeTechnologies; Darmstadt

Calcium-phosphate

2 M CaCl$_2$ was prepared and sterilized by filtration.

HBS (10x)

8.18 % NaCl (w/v), 5.94 % HEPES (w/v) and 0.2 % Na$_2$HPO$_4$ (w/v) in distilled water. The pH was adjusted to 7.1 and sterilized by filtration.

Cell lines were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 350 µg/ml L-glutamine, 120 µg/ml streptomycin sulfate and 120 µg/ml penicillin. Cells were split when they were 90% confluent.
Material and methods

2.1.1.2 Bacteria

*Escherichia coli* (E. coli) XL-Blue:

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1
lacF’ proAB lacIqZM15 Tn10 (Tetr) Amy Camr
(Bullock et al., 1987)

One Shot TOP10 Chemically Competent *E. coli*:

F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZAΔM15
ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697
galE15 galK16 rpsL(StrR) endA1 λ- (Invitrogen/
Gibco, Karlsruhe) (Xu and Li, 2008).

Ampicillin (Amp) ratiopharm; Ulm
Bacto-agar BD; Heidelberg
Bacto-tryptone BD; Heidelberg
Bacto-yeast extract BD; Heidelberg
Dimethyl sulfoxide (DMSO) Fluka; Neu-Ulm
Glucose Roth; Karlsruhe
Kanamycin (Kan) Invitrogen/LifeTechnologies; Darmstadt
SOC medium Invitrogen/LifeTechnologies; Darmstadt

Luria Bertani (LB) medium

10 g/l bacto-tryptone, 5 g/l yeast extract, 8 g/l NaCl, 1 g/l glucose, aqua dist.

LB Amp Agar

15 g/l agar, 100 mg/l ampicillin in LB medium

LB Kan Agar

15 g/l agar, 100 µg/l kanamycin in LB medium

2.1.2 Oligonucleotides

Oligonucleotides were obtained from biomers.net GmbH (Ulm).

<table>
<thead>
<tr>
<th>Name</th>
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<th>Sequence 5’-&gt;3’</th>
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## Material and methods

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</tr>
</tbody>
</table>
Material and methods

2.1.3 Plasmids

<p>| | | | | |</p>
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<th></th>
<th></th>
<th></th>
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<td>SK77</td>
<td>rv</td>
<td>GCCGATCCGACATGTGAAAAG</td>
<td>p_CBG-N</td>
<td>BQ664 vpu</td>
</tr>
</tbody>
</table>

**pBR_NL4-3 Δvpu**  
Vpu deleted mutant of a CCR5-tropic (92TH014.12) HIV-1 NL4-3 proviral derivative (Papkalla et al., 2002).

**p_CBG-N**  
Construct encoding the N-terminal fragment of click beetle green (CBG) in frame with β-catenin at its N-terminus was kindly provided by Piwnica-Worms (Villalobos et al., 2010). β-catenin was replaced by the respective Vpu alleles via SalI and BamHI. Contains kanamycin resistance gene for selection in bacteria.

**p_CBG-C**  
Construct encoding the C-terminal fragment of CBG in frame with β-TrCP1 at its C-terminus, was provided by Piwnica-Worms (Villalobos et al., 2010). Contains ampicillin resistance gene for selection in bacteria.

**pcDNA3.1(+)**  
CMV-promoter based expression vector (Invitrogen/Life Technologies; Darmstadt). Contains ampicillin resistance gene for selection in bacteria.

**pCG_IRES_eGFP**  
CMV-promoter based expression vector in which the vpus were cloned via the restriction sites MluI and XbaI. It promotes eGFP expression via an IRES (Münch et al., 2007; Tanaka and Herr, 1990). Contains ampicillin resistance gene for selection in bacteria.

**pCG_ΔIRES_eGFP**  
pCG_IRES_eGFP where the IRES eGFP cassette was removed via BamHI digestion.

2.1.4 Restriction enzymes

- **BamHI**  
  NEB enzymes; Frankfurt
- **MluI**  
  NEB enzymes; Frankfurt
- **SalI**  
  NEB enzymes; Frankfurt
- **XbaI**  
  NEB enzymes; Frankfurt

2.1.5 Vpu alleles and accession numbers

The accession number refers to the HIV sequence database Los Alamos (http://www.hiv.lanl.gov).
### Material and methods

<table>
<thead>
<tr>
<th>Clone</th>
<th>Virus</th>
<th>Species/subspecies/origin</th>
<th>Vpu size (bp)</th>
<th>Source</th>
<th>Accession number</th>
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<tbody>
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<td>NL4-3</td>
<td>HIV-1 M</td>
<td>human</td>
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<td>Infectious molecular clone/human T cell line</td>
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<td>MB897</td>
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</table>

All wild type vpu alleles and the respective chimeric alleles were already available or were cloned for this study as an untagged and a C-terminally AU-1-tagged version.

#### 2.1.6 Polymerase chain reaction (PCR)

- dNTPs Invitrogen/Life Technologies; Darmstadt
- HiPerSolv Chromanorm water VWR; France
- Phire Hot Start DNA polymerase Kit Fisher Scientific GmbH; Schwerte

#### 2.1.7 Cloning and DNA purification

- Agarose Roth; Karlsruhe
- DNA Ligation Kit Ver.2.1 Takara Bio Inc. Takara Bio INC; Japan
- Ethanol Sigma-Aldrich; Steinheim
- Ethidiumbromide Sigma-Aldrich; Steinheim
- HiPerSolv Chromanorm water VWR; France
- Isopropanol Merck; Darmstadt
- Miniprep Kit Qiagen; Hilden
- Molecular weight size marker “1 kb ladder” Invitrogen/Life Technologies; Darmstadt
- Restriction endonucleases BioLabs; Frankfurt
- TAE buffer 5Prime GmbH; Hamburg
- UltraClean DNA Purification Kit MoBio Laboratories Inc.; USA
- Wizard™ Plus Midiprep Kit Promega; USA
Material and methods

2.1.8 Western blot

Antibodies

- $\alpha$-AU-1 (MMS-130P) Covance; USA
- $\alpha$-eGFP (290–50) Abcam; UK
- $\alpha$-β-actin (8227–50) Abcam; UK
- $\alpha$-mouse IRDye Odyssey (926–32210) Li-COR; USA
- $\alpha$-rabbit IRDye Odyssey (926–32221) Li-COR; USA

Antibody buffer

- 0.2% Tween-20 (v/v) in PBS with 1% milk powder (w/v)

$\beta$-mercaptoethanol (β-ME) Sigma-Aldrich; Steinheim

Blocking buffer

- 0.2% Tween-20 (v/v) in PBS with 5% milk powder (w/v)

Immobilon-FL Transfer Membrane Merck Millipore; USA
Milk powder Roth; Karlsruhe
M-PER Mammalian Protein Extraction Thermo Scientific; USA

Reagent

NuPAGE Antioxidant Invitrogen/LifeTechnologies; Darmstadt
NuPAGE MES SDS Running Buffer Invitrogen/LifeTechnologies; Darmstadt
NuPAGE Novex Bis-tris gels Invitrogen/LifeTechnologies; Darmstadt
PBS Invitrogen/LifeTechnologies; Darmstadt
Precision PlusProtein Standard Bio-Rad; USA
Protein Loading Buffer Invitrogen/LifeTechnologies; Darmstadt
Sodiumdodecylsulfate (SDS) Sigma-Aldrich; Steinheim

Transfer buffer

- 47.9 mM Tris, 38.6 mM glycine, 1.3 mM SDS, 20% methanol (v/v) in distilled water; adjusted pH to 8.3

Tween-20 Roth; Karlsruhe
Whatman paper Whatman; UK

Wash buffer

- 0.2% Tween-20 (v/v) in PBS

2.1.9 Fluorescence-activated cell sorting (FACS)

Antibodies

- $\alpha$-CD4-APC (MHCD0405) Invitrogen/LifeTechnologies; Darmstadt
Material and methods

α-tetherin (16-3179-82) eBioscience; USA

α-mouse-APC (A865) Invitrogen/LifeTechnologies; Darmstadt

FACS buffer

1% FCS (v/v) in PBS

Paraformaldehyde (PFA) Merck; Darmstadt

PBS Invitrogen/LifeTechnologies; Darmstadt

2.1.10 β-galactosidase assay

Gal-screen substrate Applied Biosystems; USA

2.1.11 Enzyme-linked immunosorbent assay (ELISA)

Antibodies

mouse anti-p24 monoclonal (MAK183) EXBIO; Czech Republic

rabbit anti-p24 polyclonal Eurogentec; Belgium

goat anti-rabbit IgG, Fc-HRP Dianova; Hamburg

Antibody diluent

0.05 % Tween-20 (v/v) in 10 % FCS (v/v) in PBS

Blocking solution

10 % FCS (v/v) in PBS

BSA Diluent/Blocking Solution (10 %) KPL Vertrieb Fa. Medac; Wedel

Lysis solution

10 % Triton X-100 in distilled water

PBS Invitrogen/LifeTechnologies; Darmstadt

Sample diluent

0.05 % Tween-20 (v/v) in 0.05 % (v/v) Triton X-100 PBS

Stop solution

0.5 M H2SO4

3,3’,5,5’-tetramethylbenzidine (TMB) KPL; USA

Peroxidase Substrate

Triton X-100 Sigma-Aldrich; Steinheim

Tween-20 Roth; Karlsruhe

Wash buffer

0.05 % Tween-20 (v/v)
2.1.12 Confocal immunofluorescence microscopy

Antibodies

- $\alpha$-AU-1 (PRB-130P) Covance; USA
- Alexa Fluor 488 anti-sheep Invitrogen/LifeTechnologies; Darmstadt
- Alexa Fluor 568 anti-rabbit Invitrogen/LifeTechnologies; Darmstadt
- Alexa Fluor 647 anti-mouse Invitrogen/LifeTechnologies; Darmstadt
- $\alpha$-tetherin (16-3179-82) eBioscience; USA
- $\alpha$-TGN46 (AHP500GT) AbD Serotec; UK

Antibody diluent

- PBS with 0.1 % (v/v) Tween-20 and 1 % (v/v) BSA

BSA Diluent/Blocking Solution (10 %)

- KPL Vertrieb Fa. Medac; Wedel

PBS

- Invitrogen/LifeTechnologies; Darmstadt

Permabilization buffer

- PBS with 0.1 % (v/v) Saponin

PFA

- Merck; Darmstadt

Saponin

- Santa Cruz; USA

Tween-20

- Roth; Karlsruhe

2.1.13 Click-beetle assay (CBA)

D-luciferin regis; USA

Modified Eagle’s balanced salt solution (MEBSS) buffer

- 144 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO$_4$, 0.8 mM Na$_2$HPO$_4$, 1.2 mM CaCl$_2$, glucose 5.6 mM, and HEPES 4 mM [pH 7.4]

2.1.14 Consumables

- 5 ml Polystyrene round-bottom tubes 12x75mm BD; Heidelberg
- Cell culture flasks Sarstedt; Nümbrecht
- Cell culture plates Greiner Bio-one; Frickenhausen
- Filter tip (10, 200, 1000 µl) Greiner Bio-one; Frickenhausen
- µ-Slides 8 well ibiTreat ibidi GmbH; Martinsried
- Nuncl immuno plates, maxi sorb surface Thermo Scientific; USA
- Nunclon-delta white microwell plates Thermo Scientific; USA
- Reaction tubes (1.5/2 ml) Sarstedt; Nümbrecht
- Serological pipettes (2, 5, 10, 25 ml) Sarstedt; Nümbrecht
- Tubes (15, 50 ml) Sarstedt; Nümbrecht
2.2 Methods

2.2.1 Cloning

2.2.1.1 PCR
PCR was performed using the Phire Hot Start DNA Polymerase Kit according to the manufacturer’s protocol in a PTC-100 Programmable Thermal Controller (Veriti, Applied Biosystems).

2.2.1.2 Restriction digestion
Digestion was performed using NEB enzymes according to the manufacturer’s instructions.

2.2.1.3 Isolation of DNA from agarose gel
DNA fragments were separated by gel electrophoresis (agarose gel (1 % (w/v))) and visualized on a UV screen (366 nm) (Syngene) and the bands were cut. Afterwards, the DNA was purified using the UltraClean 15 DNA purification Kit according to the manufacturer’s instructions.

2.2.1.4 Ligation
Vector and insert DNA were mixed in a ratio of 1:4 and ligated using the DNA Ligation Kit Ver.2.1 according to the manufacturer’s instructions.

2.2.1.5 Transformation
DNA was incubated with 15 µl of *E. coli* XL2-Blue cells (ligated DNA) or 12 µl OneShot TOP10 Chemically Competent *E. coli* (retransformation of plasmid DNA) on ice for 20 min. After the cells were heat-shocked for 30 s at 42 °C they were incubated on ice for 2.5 min. 200 µl SOC medium was added and the transformed cells were incubated at 37 °C on a shaker for 40 min before being plated on LB agar plates containing the appropriate antibiotics.

2.2.1.6 Mini preparation
Plasmid DNA for cloning and sequencing was prepared with the Miniprep Kit according to the manufacturer’s protocol. The DNA concentration and quality was determined using the Nano Drop spectrophotometer (peqlab, Erlangen).

2.2.1.7 Sequencing
6 µl of Mini-prep DNA or 3 µl of Midi-prep DNA (filled up to 15 µl with HiPerSolv Chromanorm water) and 15 µl Primer (10 pmol/µl) per sample were sent to Eurofins MWG GmbH for sequencing.
Material and methods

2.2.1.8 Midi preparation
Plasmid DNA for transfection was prepared by using Wizard Plus Midiprep Kit. The DNA concentration and quality was determined using the Nano Drop spectrophotometer (peqlab, Erlangen).

2.2.1.9 Transfection
One day before transfection, 0.2x10^6 HEK293T or HeLa cells were seeded in 6-well plates. For microscopic analysis 40,000 HeLa cells were seeded in ibidi 8-well slides (1 cm²). For the click-beetle assay, 25,000 HEK293T cells were seeded in 96-well plates (white plates). At a confluency of 60-70 % cells were used for transfection.

2.2.1.10 Calcium-phosphate transfection
Appropriate amount of DNA and 13 µl 2 M CaCl₂ were mixed and filled up to 100 µl with aqua dist.. 100 µl 2x HBS was added dropwise, the mixture was pipetted ten times and added dropwise to the cells after changing the cell culture medium. Cells were incubated for 8-16 h before medium was replaced by fresh medium. 48 h post-transfection cells were used for analysis.

2.2.1.11 Lipofectamine LTX transfection
Lipofectamine LTX reagent was used for transfection of HeLa cells according to the manufacturer’s instructions.

2.2.2 Western blot
To monitor Vpu expression, HeLa cells were transfected with 5 µg of the pCG Vpu-AU-1 expression constructs. Two days post-transfection cells were harvested, lysed in M-PER buffer containing 1 % SDS and a protease inhibitor cocktail and cell lysates were separated in 10 % SDS-PAA gels in a Tris-Tricine buffer system. After gel electrophoresis, proteins were transferred onto PVDF membranes and probed with anti-AU-1 antibody. For internal controls, blots were incubated with antibodies specific for eGFP and actin. Subsequently, blots were probed with anti-mouse or anti-rabbit IRDye Odyssey antibodies and proteins detected using a LI-COR Odyssey scanner.

2.2.3 FACS
To determine the effect of Vpu on tetherin and CD4 cell surface expression, HEK293T were transfected in 6-well plates by the calcium phosphate method and HeLa cells using Lipofectamine LTX reagent with 1 µg of a tetherin (for HEK293T cells) or CD4 (for 293T and HeLa cells) expression vector and 5 µg of pCG constructs expressing eGFP alone (as
control) or together with Vpu. Two days post-transfection, tetherin or CD4 expression was examined by FACS analysis with the BD FACS CantoII (BD Biosciences). An allophycocyanin (APC)-conjugated anti-human CD4 antibody was used for staining of surface CD4. For staining of surface tetherin an unconjugated anti-tetherin antibody and an APC-conjugated secondary anti-mouse antibody were used. The fluorescence of stained cells was detected by two-color flow cytometry and Vpu-mediated tetherin or CD4 down-modulation was calculated (BD FACSDiva software). For exogenously expressed proteins, the mean fluorescence intensity (MFI) obtained for cells transfected with the control construct expressing only eGFP was compared to the MFI obtained for cells co-expressing Vpu and eGFP to determine the efficiency of tetherin or CD4 down-regulation. For endogenously expressed proteins, the MFI of untransfected cells (eGFP negative) was compared to the MFI of transfected cells (eGFP positive). The transfection of the control construct expressing only eGFP served as reference.

2.2.4 Virus release assays

To determine the capability of Vpu to antagonize tetherin, HEK293T or HeLa cells were seeded in 6-well plates and transfected with 4 µg of NL4-3 ∆vpu IRES eGFP, 1 µg Vpu expression plasmid and (for HEK293T cells) different amounts of a tetherin expression vector (6.25, 12.5, 25, 50, 125 and 250 ng). Two days post-transfection, supernatants were harvested and analyzed for infectious virus release by a 96-well infection assay on TZM-bl indicator cells (2.2.4.1) and the release of p24 antigen by a homemade ELISA (2.2.5.2)

2.2.4.1 β-galactosidase assay

TZM-bl cells were seeded in 96-well plates (6000 cells/well). On the following day, cells were infected. Three days post-infection, the supernatant was removed and 40 µl of 1:2/1:4 diluted Gal-screen substrate in PBS was added to each well. After 30 min incubation at room temperature, 35 µl of the cell lysates were transferred into a 96-well Nunclon-delta white microwell plate and light emission was monitored with an Orion Microplate Luminometer (Berthold Detection systems). The enzyme activity was measured as relative light units/second (RLU/s) using the Software Simplicity 4.02 (Berthold detection systems).

2.2.4.2 ELISA

Nunc immuno maxi sorb surface plates were incubated with p24 antibody at room temperature overnight. Plates were washed and blocked with blocking buffer for 2 h. Virus stocks and cells were lysed with Triton X-100 (1%/10 %) and transferred to the 96-well plates. After incubation overnight, plates were washed and incubated with a rabbit anti-HIV-
1 p24 antibody for 1 h before washed and incubated with a goat anti-rabbit antibody conjugated with horseradish peroxidase followed by the addition of TMB peroxidase substrate. The reaction was stopped with 0.5 M H$_2$SO$_4$ after 20-30 min. The plates were measured at 450 nm and 650 nm with the Thermomax microplate reader (Molecular devices).

2.2.5 Confocal immunofluorescence microscopy

HeLa cells were transfected using Lipofectamine LTX Reagent with pCG Vpu-AU-1 constructs and stained 24 h post-transfection. Briefly, cells were fixed with 4% PFA, blocked with BSA and surface tetherin was stained with an anti-tetherin antibody and a secondary antibody conjugated to Alexa Fluor 647. Cells were then permeabilized with 0.5% saponin. After blocking with BSA, Vpu, tetherin and TGN46 were stained with an anti-AU-1, anti-tetherin and anti-TGN46 antibody, respectively. Secondary antibodies conjugated to Alexa Fluor 568, Alexa Fluor 647 and Alexa Fluor 488 were used for detection. A confocal microscope (LSM 710, Zeiss) with the corresponding software (Zeiss Zen Software, 2010) was used for analysis.

2.2.6 CBA

The click beetle luciferase complementation assay allows the real-time analysis of protein-protein interactions in living cells (Villalobus et al., 2010). pCBGC_β-TrCP1 and pβ-catenin_CBG-N constructs encoding the N- or C-terminal fragment of click beetle green (CBG) were kindly provided by Piwnica-Worms. *SalI* and *BamHI* restriction sites were added to the *vpu* alleles by PCR and standard cloning techniques were used to insert the *vpu* alleles into the pCBG-N vector replacing the β-catenin gene. The luciferase assay was essentially performed as described before (Villalobos et al., 2010). Briefly, HEK293T cells in white 96-well plates with clear bottom were transfected with equal amounts of the click beetle green constructs. 48 h after transfection the cells were washed once and then incubated in MEBSS buffer containing 1% heat-inactivated fetal bovine serum and 150 µg/ml D-luciferin. Photon flux was quantified at room temperature with a SAFAS Xenius spectrofluorimeter for 1 min or an Orion microplate luminometer for 10 s.

2.2.7 Sequence analysis and statistics

Vpu sequences were obtained from the HIV Sequence Database (www.hiv.lanl.gov). Vpu amino acid sequences were aligned using multiple sequence alignment with hierarchical clustering (http://multalin.toulouse.inra.fr/multalin).
Material and methods

The activities of \textit{vpu} alleles were compared using a two-tailed Student’s \textit{t} test. The PRISM package version 4.0 (Abacus Concepts, Berkeley, CA) was used for all calculations.
3 Results

SIVcpzPtt and SIVgor from the Central chimpanzee and the Western lowland gorilla have successfully crossed the species barrier to humans giving rise to HIV-1 groups M, N, O and P (figure 1). In contrast, SIVcpzPts from the Eastern chimpanzee or progeny thereof has never been detected in humans. Of the two transmitted viruses only SIVcpzPtt evolved effective Vpu-mediated anti-human tetherin activity after transmission (figure 2b). To clarify whether SIVcpzPtt Vpu requires less adaptive changes to evolve activity against human tetherin than SIVcpzPts and SIVgor due to predispositions in its cytoplasmic part, I generated a set of chimeric Vpu proteins. These chimeras contain the N-terminus of an HIV-1 M Vpu (NL4-3) and the cytoplasmic part of six representative Vpu proteins of primary isolates of SIVcpzPtt (MB897, EK505), SIVcpzPts (TAN3, ANT) and SIVgor (CP2139, BQ664) (table 2). The transmembrane domain of the NL4-3 Vpu directly interacts with the transmembrane domain of human tetherin and this interaction is known to be essential for efficient counteraction (Rong et al., 2009; Skasko et al., 2012; Vigan and Neil, 2011). Thus, the generated Vpu chimeras only differ in the presence or absence of functional motifs in the cytoplasmic part. These motifs have been reported to be involved in correct localization, degradation or rerouting of tetherin and to be required for effective counteraction of tetherin (figure 3) (Van Damme et al., 2008; Douglas et al., 2009; Kueck and Neil, 2012; Mangeat et al., 2009; Mitchell et al., 2009; Ruiz et al., 2008). The expression of the six chimeric and the corresponding parental Vpu proteins was confirmed by Western blot analysis of cell lysates of HEK293T and HeLa cells that were transfected with the respective expression construct (figure 4).

![Figure 4. Chimeric Vpu proteins are expressed in HEK293T and HeLa cells.](image)

Expression of wild type Vpu proteins and chimeras between the TMD of HIV-1 NL4-3 Vpu and the cytoplasmic part of SIVcpz or SIVgor Vpus. Cells were transfected with expression plasmids encoding the indicated AU-1-tagged Vpus and eGFP. Mock transfected cells were used as negative control. Actin and eGFP expression levels were analyzed to control for loading and transfection efficiency, respectively.
Results

All Vpus were expressed in transfected HEK293T and HeLa cells although the detection levels of the chimeras as well as their respective wild types varied (figure 4). It is known that the functional activity of Vpu proteins as well as their detection level by immunofluorescence microscopy do not always correlate with their in vitro expression levels detected by Western blot because portions aggregate and/or remain associated with the insoluble membrane fraction (Sauter et al., 2012).

3.1 Tetherin down-modulation is independent of reported trafficking motifs in the cytoplasmic part of Vpu

First, I examined the ability of the wild type and chimeric Vpu proteins to reduce cell surface expression of tetherin. Removing tetherin from the surface and hence from its site of action is thought to circumvent the inhibition of virion release, virus sensing and activation of NF-κB. I co-transfected HEK293T cells with a construct co-expressing Vpu and eGFP and a plasmid expressing human tetherin. Cells were stained for surface tetherin and analyzed by flow cytometry. In agreement with previous studies, HIV-1 M NL4-3 Vpu significantly reduced the levels of cell surface tetherin, whereas all wild type SIVcpz and SIVgor Vpus were largely inactive (figures 5a and 5b) (Sauter et al., 2009). The TMD of the NL4-3 Vpu, however, significantly increased the capability of the SIVcpz\textit{Pts} MB897 and SIVgor BQ664 Vpus to reduce the surface levels of tetherin (NL-MB, NL-BQ). In contrast, the exchange of the TMD had only a minor if any effect on the ability to down-modulate surface tetherin of the EK505, TAN3, ANT and CP2139 Vpus (NL-EK, NL-TA, NL-AN, NL-CP).

Exogenous over-expression of tetherin in HEK293T results in substantially higher expression levels of this restriction factor compared to those found in cells that endogenously produce tetherin (figure 5d). To exclude that weak activities of some Vpus were missed due to high tetherin levels, I repeated the experiment using HeLa cells which endogenously express tetherin (figures 5a and 5c). The results showed that all chimeric Vpus were able to efficiently reduce the expression of endogenously expressed tetherin at the cell surface of HeLa cells (figures 5a and 5c). Surprisingly, the chimera between the TMD of NL4-3 and the cytoplasmic part of SIVcpz\textit{Pts} ANT Vpu was most active (NL-AN), although the cytoplasmic part of this SIVcpz\textit{Pts} Vpu does not contain the β-TrCP binding motif or the tyrosine- or di-leucine-based sorting motifs (figure 2).
3.2 Chimeric Vpus relocalize tetherin to the trans-Golgi network

To investigate the fate of tetherin and its subcellular localization in more detail, I performed confocal immunofluorescence microscopy. As Vpu-mediated down-modulation of tetherin
was more pronounced in HeLa than in HEK293T cells, I transfected HeLa cells with expression constructs for AU-1-tagged Vpus and stained the cells for tetherin, Vpu and the TGN marker TGN46.
In the absence of Vpu, tetherin co-localized with the TGN marker but was also present at the cell surface (figure 6a, empty vector). As expected, the expression of tetherin at the surface of transfected HeLa cells was markedly reduced in the presence of HIV-1 M NL4-3 Vpu. Co-localization studies showed, that NL4-3 Vpu as well as tetherin localized to the TGN (figure 6b). In contrast, none of the tested wild type Vpus of SIVcpzPtt, SIVcpzPts and SIVgor removed tetherin from the cell surface (MB897, ANT, BQ664). In agreement with my previous findings, however, all chimeric Vpus (NL_MB, NL_AN, NL_BQ). relocalized tetherin from the cell surface to the TGN.

3.3 Chimeric Vpus enhance virus release despite the absence of previously described sorting motifs

The removal of tetherin from the cell surface and therefore from the viral budding site is usually associated with an increase of virus release (Van Damme et al., 2008; Mitchell et al., 2009). To determine the potential of the chimeric Vpus to enhance virion release, I co-transfected HEK293T cells with a vpu defective HIV-1 NL4-3 proviral construct, the Vpu and eGFP co-expressing constructs and a tetherin expression plasmid. Importantly, the amount of tetherin was carefully titrated to detect possible weak anti-tetherin activities (figure 7a). Virus release was measured using a TZM-bl reporter cell assay and the results were confirmed by p24 ELISA.

As shown in figures 7a and 7b, all parental wild type SIV Vpus were inactive in promoting the release of infectious virus from transiently transfected HEK293T cells compared to the empty vector control. In contrast, all chimeric Vpus enhanced infectious virus release. Unexpectedly, the chimeric Vpus with the cytoplasmic part of SIVcpzPtt Vpus that harbored the β-TrCP binding motif as well as the tyrosine- and di-leucine-based sorting motifs showed the weakest activity (NL_MB, NL_EK). On the contrary, the chimeras between the NL4-3 TMD and the cytoplasmic part of SIVgor BQ664 that lacks most of the reported motifs was the most active (NL_BQ). Of note, enhancement of infectious virus yield correlated with the release of p24 showing that the enhancement of infectious virus yield was not due to an effect on infectivity of the viral particles (figures 7c and 7d).
Figure 7. Chimeric Vpu proteins enhance infectious virus yield in HEK293T and HeLa cells. (a)-(c) Enhancement of virus release by chimeric Vpus in HEK293T cells. Infectious virus yield from HEK293T cells co-transfected with the proviral HIV-1 NL4-3 Δvpu construct, the indicated vpu allele and decreasing amounts of a vector expressing human tetherin. (b) Infectious virus release and (c) p24 release (p24(supernatant)/p24(supernatant+cells)) in the presence of 50 ng co-transfected tetherin expression plasmid. The release in the absence of tetherin of each respective Vpu was set to 100%. Shown are average values (±SEM) derived from three independent experiments each involving triplicate infections of TZM-bl indicator cells. (d) Correlation analysis of infectious virus yield and p24 release from HEK293T cells. (e), (f) Enhancement of virus release by chimeric Vpus in HeLa cells. Infectious virus yield from HeLa cells co-transfected with the proviral HIV-1 NL4-3 Δvpu construct and the indicated vpu allele. (e) Infectious virus release and (f) p24 release (p24(supernatant)/p24(supernatant+cells)). The release in the presence of NL4-3 Vpu was set to 100%. Shown are average values (±SEM) derived from two independent experiments each involving triplicate infections of TZM-bl indicator cells. (g) Correlation analysis of infectious virus yield and p24 release from HeLa cells.
To confirm these results in the presence of endogenous tetherin, I transfected HeLa cells with the \textit{vpu}-defective HIV-1 NL4-3 proviral construct and the Vpu and eGFP co-expressing constructs and quantified virus release. As shown in figures 7e-7g, most chimeric Vpus enhanced infectious virus yield and p24 release from HeLa cells with efficiencies similar to those observed in HEK293T cells. Thus, most Vpus that promoted infectious virus release in transiently transfected HEK293T cells were also active in HeLa cells (figures 7b-7g). Surprisingly, the chimeras between the transmembrane domain of NL4-3 Vpu and the cytoplasmic tails of SIVcpz \textit{Pts} or SIVgor Vpus were the most active ones in both experiments although they do not contain any (SIVcpz\textit{Pts}) or only one (SIVgor) of the motifs previously reported to be involved in tetherin counteraction.

**3.4 Gain of anti-tetherin activity may come at the cost of CD4 degradation activity**

Besides counteraction of tetherin, another well-established and conserved function of Vpu is the ability to down-modulate the viral CD4 entry receptor (Kimura et al., 1994; Willey et al., 1992). Down-modulation of CD4 protects infected cells from superinfection and enables efficient release of fully infectious progeny viruses (Lama et al., 1999; Levesque et al., 2003; Wildum et al., 2006).

To analyze Vpu-mediated down-modulation of CD4, I co-transfected HEK293T and HeLa cells with the Vpu co-expressing eGFP and the CD4 expression constructs. Cells were stained for CD4 surface expression and analyzed by FACS. As shown in figures 8b and 8c, all wild type HIV-1, SIVcpz and SIVgor Vpu proteins strongly reduced CD4 cell surface expression. Surprisingly, however, several Vpu chimeras (NL-MB, NL-EK and NL-BQ) were only poorly active against CD4. To exclude differences between transiently and endogenously expressed proteins, I used TZM-bl cells stably expressing CD4 to confirm the results. The effects on endogenously expressed CD4 were similar although less pronounced (figure 8d).
Figure 8. Some chimeric Vpus are less active in down-modulating CD4 than their wild type Vpu. (a) Primary FACS data. HEK293T and HeLa cells were co-transfected with the indicated Vpu construct co-expressing eGFP and a plasmid expressing CD4. TZM-bl cells were only transfected with the indicated Vpu construct co-expressing eGFP. Cells were stained for CD4 surface expression (APC). The range of eGFP expression and the MFIs used to calculate down-modulation are indicated. (b) Vpu-mediated reduction of CD4 surface expression in HEK293T, (c) HeLa and (d) TZM-bl cells. Shown are the levels of CD4 cell surface expression relative to those measured in cells transfected with the empty vector (100%). Data represent average values (±SEM) derived from three to five independent experiments.

3.5 Exchange of the TMD does not affect ability of Vpu to bind β-TrCP

It has been reported that effective tetherin counteraction by HIV-1 M Vpus requires a functional β-TrCP binding motif with the consensus sequence DSGXXS (Van Damme et al., 2008). This very same motif is also believed to be critical for Vpu-mediated CD4 down-modulation (Margottin et al., 1998; Paul and Jabbar, 1997). Phosphorylation of both serines
Results

by casein kinase II enables recruitment of the adapter protein β-TrCP and subsequently the E3 ubiquitin ligase complex. The E3 ligase finally ubiquitinates CD4 or tetherin thereby inducing the degradation of these target proteins (Douglas et al., 2009; Margottin et al., 1998; Schubert et al., 1998).

I established a click beetle luciferase fragment complementation assay to examine whether the wild type Vpus are capable of interacting with β-TrCP and to test whether the exchange of the TMD affects binding. To this end, I fused the N-terminal fragment of the click beetle green luciferase (N-CBG-Luc) to the C-terminus of Vpu, and the C-terminal fragment of this luciferase (C-CBG-Luc) to the N-terminus of β-TrCP (figure 9, right panel). HEK293T cells were co-transfected with both constructs. If the expressed proteins interact, the luciferase fragments assemble to a functional enzyme. Thus, this assay allows the real-time analysis of protein-protein interactions in living cells (Villalobos et al., 2010).

As expected, NL4-3 Vpu interacted with β-TrCP (figure 9). In agreement with published data, mutations in the DSGXXS motif (S52A/S56A or G53D) significantly reduced the interaction with β-TrCP (Sauter et al., 2012). The wild type MB897 Vpu and its chimera that harbor the consensus DSGXXS motif efficiently bound β-TrCP. In comparison, the ANT and BQ664 wild type Vpus containing alterations in the β-TrCP binding site were less efficient in β-TrCP binding. Most importantly, exchanges of the TMD had no significant effect on the interaction of Vpu with β-TrCP. Thus, altered β-TrCP binding was not the reason for the gain of anti-tetherin activity or the loss of the ability to down-modulate CD4.

![Figure 9. Exchange of the TMD does not alter β-TrCP binding ability.](image)

**Figure 9. Exchange of the TMD does not alter β-TrCP binding ability.** HEK293T cells were co-transfected with constructs expressing the fusion proteins C-CBG-Luciferase_β-TrCP and the indicated Vpu_N-CBG-Luciferase. Interaction of β-TrCP and Vpu leads to the assembly of the click beetle green luciferase protein. Luciferase activity was determined in living cells by addition of D-luciferin and quantification of bioluminescence. Values represent averages (±SEM) derived from five independent experiments.
4 Discussion

SIVcpzPtt and SIVgor from the Central chimpanzee and the Western lowland gorilla have successfully crossed the species barrier to humans giving rise to HIV-1 groups M, N, O and P. In contrast, SIVcpzPts from the Eastern chimpanzee that lives in close proximity or progenies of this virus has thus far never been detected in humans. Of the two transmitted viruses only SIVcpzPtt evolved effective Vpu-mediated anti-human tetherin activity after transmission and resulted in the emergence of pandemic HIV-1 group M strains. Sequence analyses suggested that Vpus of the SIVcpzPtt only had to evolve an interaction site with human tetherin in their transmembrane domains, whereas SIVcpzPts and SIVgor Vpus lack several motifs in their cytoplasmic part that were reported to be required for effective Vpu-mediated tetherin antagonism (table 1).

The present thesis shows that the Vpu proteins of six primary isolates of SIVcpzPtt, SIVcpzPts and SIVgor all became antagonists of human tetherin when their transmembrane domain was exchanged by that of the HIV-1 group M NL4-3 Vpu. Thus, enabling binding of tetherin by replacing the transmembrane domain of SIV Vpus to that of an HIV-1 M Vpu was sufficient to confer anti-tetherin activity to all SIVcpz and SIVgor Vpus analyzed (table 3). Efficient counteraction was independent of the previously reported motifs in the cytoplasmic part of Vpu, namely the tyrosine and di-leucine trafficking motifs and the di-serine β-TrCP binding motif (table 3). This is in agreement with a study by Lim et al. who reported that a chimera between the TMD of an HIV-1 M Vpu (Q23-17) and the cytoplasmic part of a SIVcpzPtt Vpu (US) that lacks the tyrosine motif was as active as the wild type HIV-1 M Vpu in human tetherin counteraction (Lim et al., 2010). My experiments revealed, that chimeric Vpus lacking the tyrosine trafficking motif and the di-serine motif (NL_BQ) or even all three motifs (NL_AN) were as active as the control HIV-1 M Vpu in tetherin down-modulation and enhancing virus release (table 3). Of note, one of the chimeric Vpus that contained all functional motifs was the least active one (NL_MB). Thus, the requirement of the previously reported motifs seems to be highly allele specific.
Table 3. Previously described motifs involved in tetherin and CD4 counteraction in the cytoplasmic part of SIVcpz and SIVgor Vpus and anti-tetherin activities of the chimeric Vpus.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Clone</th>
<th>Tyrosine</th>
<th>Di-serine</th>
<th>Di-leucine</th>
<th>Tetherin ↓</th>
<th>Release ↑</th>
<th>CD4 ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIVcpzPtt</td>
<td>MB897</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EK505</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SIVcpzPts</td>
<td>TAN3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td></td>
<td>ANT</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SIVgor</td>
<td>CP2139</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>BQ664</td>
<td>-</td>
<td>-</td>
<td>✓</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

The membrane proximal tyrosine-based trafficking motif is highly conserved in HIV-1 group M and its involvement in tetherin antagonism has first been studied in the context of subtype C (Ruiz et al., 2008). Ruiz et al. showed that changing the tyrosine to an alanine decreases virus release and concluded that the YXXφ motif is essential for efficient release. In a subsequent study, however, Dubé et al. analyzed the closely related subtype B Vpu and did not observe an effect when mutating the tyrosine in this putative motif (Dubé et al., 2009). Thus, the tyrosine-based motif seems neither to be involved in tetherin counteraction nor in Vpu trafficking in subtype B although it is highly conserved. Interestingly, however, Dubé et al. identified positively charged amino acids that are frequently found within the tyrosine motif of subtype B Vpus that determined the efficiency and specificity of trafficking between the TGN and the endosomal system. Since localization of B Vpus at the TGN was shown to be essential for tetherin counteraction, these residues most likely also affect anti-tetherin activity (Dubé et al., 2009). A role of these charged residues for TGN localization of Vpu and tetherin antagonism has been confirmed by Vigan et al. (Vigan and Neil, 2011), whereas Nomaguchi et al. showed that these amino acids are not critical for Vpu function (Nomaguchi et al., 2010). I found that all wild type and chimeric Vpus mainly localized to the TGN irrespective of the presence or absence of the YXXφ motif or positively charged amino acids at the respective positions. Minor differences in localization might be missed due to over-expression of Vpu. However, a general involvement of the YXXφ motif or the more recently characterized positively charged amino acids within this motif seems unlikely.

My results further revealed that anti-tetherin activity and TGN localization were also independent of the di-leucine trafficking motif in the second α-helix. In contrast to the
YXXφ motif that is highly conserved in M and N Vpus, the [D/E]XXXL[L/I/V/M] motif is only preserved in HIV-1 M subtype B. Similar to the tyrosine trafficking motif the di-leucine motif was shown to be responsible for TGN localization and tetherin antagonism (Dubé et al., 2009; Kueck and Neil, 2012; Pacyniak et al., 2005). Interestingly, the poor anti-tetherin activity of HIV-1 N Vpus was also ascribed to the lack of this motif in N Vpus (Sauter et al., 2012). However, all wild type and chimeric Vpus examined in the present thesis mainly localized to the TGN irrespective of the presence or absence of this motif (figure 6). In this regard, it is important to note that only the chimeric Vpus that were able to interact with tetherin relocalized the restriction factor to the TGN whereas the respective wild type Vpus did not. Thus, my data suggest that localization of Vpu at the TGN and its ability to interact directly with tetherin can be sufficient for full anti-tetherin activity. Nevertheless, we cannot exclude that there are additional yet to be identified trafficking motifs in Vpu. For example, an NPX[Y/F] internalization motif is found in many SIVcpz (e.g. SIVcpzPtSM ANT) and most HIV-1 O Vpus (Davis et al., 1987). This tyrosine motif is known to allow other type I transmembrane proteins (e.g. the LDL and insulin receptors) to recruit AP-2 (Backer et al., 1990; Boll et al., 2002; Chen et al., 1990). Its relevance for Vpu function, however, remains to be investigated. Taken together, these findings show that diverse Vpu proteins rely on different sequence requirements to antagonize tetherin. Hence, the requirement of previously described trafficking motifs - as well as more recently described adjacent positively charged amino acids - for Vpu-mediated tetherin counteraction should not be generalized.

The di-serine based β-TrCP binding motif is highly conserved in HIV-1 M Vpus of different subtypes and its role in tetherin counteraction has been subject of several studies. The two serines have been shown to be phosphorylated by casein kinase II and to subsequently bind the adaptor protein β-TrCP (Friborg et al., 1995; Schubert and Strebel, 1994). β-TrCP recruits an E3 ubiquitin ligase complex that ultimately induces the degradation of tetherin (Mangeat et al., 2009; Margottin et al., 1998; Mitchell et al., 2009). Of note, in the initial study on Vpu-mediated tetherin counteraction, these two serine residues were reported to be absolutely required for antagonism (Van Damme et al., 2008). Douglas et al. confirmed the importance of the two serines (Douglas et al., 2009). They expressed a di-serine motif mutant Vpu and did not see an effect on cell surface or total steady-state tetherin level. Notably, however, this study only analyzed tetherin expression levels but not enhancement of virion release. It has been shown that the efficiencies of tetherin degradation or down-modulation and virus release do not always correlate (Dubé et al., 2010; Jafari et al., 2014; Kuhl et al., 2011; McNatt et al., 2013; Miyagi et al., 2009; Neil
et al., 2008; Nomaguchi et al., 2010). Thus, a lack of degradation does not necessarily imply inactivity in promoting virus release. My results confirm that efficient down-modulation of tetherin is not always associated with efficient virus release - or vice versa - demonstrating that it is important to investigate tetherin down-modulation and virus release enhancement (table 3). Importantly, it was been shown only recently that tetherin also acts as a viral sensor and enables antibody-dependent cell cytotoxicity at the surface of infected cells (Alvarez et al., 2014; Arias et al., 2014; Galão et al., 2012; Tokarev et al., 2013). Thus, both enhancement of virus release and down-modulation of tetherin from the cell surface should be considered as means to counteract tetherin functions and it might be interesting to determine whether the chimeric Vpu proteins also inhibit the newly described functions of tetherin.

More importantly, though, I showed that the chimeric Vpu proteins were active although they do not contain a consensus DSGXXS β-TrCP binding motif (tables 2 and 3). In fact, those vpu alleles lacking this consensus motif were the most active ones in enhancing virus release (table 3). In line with these results, older studies that investigated the Vpu-dependent enhancement of virus release before tetherin was identified showed that virus release is only partially dependent on Vpu phosphorylation (Friborg et al., 1995; Schubert and Strebel, 1994; Schubert et al., 1995). Similarly, more recent follow-up studies on Vpu-mediated tetherin counteraction reported a partial or even total recovery of the restriction when expressing a di-serine motif mutant Vpu or knocking-down β-TrCP in the presence of wild type Vpu (Mangeat et al., 2009; Mitchell et al., 2009; Miyagi et al., 2009; Tervo et al., 2011). Of note, Schmidt et al. reported that the DSGXXS motif but not β-TrCP itself was required for tetherin accumulation within the cell. Thus, conclusions about the functional relevance of β-TrCP interaction based on mutations in the DSGXXS must be drawn with caution (Schmidt et al., 2011). Furthermore, some differences observed in the requirement of the DSGXXS motif might at least be in part due to the experimental setup. Schindler et al. reported that the requirement of an intact DSGXXS motif depends on the amount of transiently or endogenously expressed tetherin (Schindler et al., 2010). These discrepancies highlight the need not only to work with cell lines but also with primary cells to minimize the risk of artifacts.

Interestingly, the exact composition of the DSGXXS motif sequence seems to be less strict than reported by most studies. Hattori et al. molecularly dissected the interaction of β TrCP with IkBα and showed that the glycine is dispensable for binding of β-TrCP and even proposed the consensus sequence (D/E)SXXXS as β-TrCP binding motif (Hattori et
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Moreover, Kanemori et al. and others showed that phosphorylation at the serine residues can be mimicked by negatively charged amino acid residues in some cases, particularly aspartic acid residues (Firzlaff et al., 1991; Kanemori et al., 2005). Based on these less stringent definitions, all tested Vpus contain a β-TrCP binding motif. However, Vpus containing the strict DSGXXS motif yielded the strongest signals in the β-TrCP interaction assay and the exchange of glycine to alanine in NL4-3 Vpu led to a loss of interaction (figure 9). Importantly, the most active chimeras NL_AN and NL_BQ that both do not contain the binding motif did not show strong interaction with β-TrCP. These findings show that there is most likely no alternative β-TrCP binding site in the analyzed Vpus. Moreover, it strengthens the hypothesis, that β-TrCP binding is not generally required for Vpu-mediated tetherin counteraction. Finally, the interaction assay also proved that the exchange of the TMD does not alter the ability of Vpu to interact with β-TrCP and hence is not the reason for the gain of function against tetherin (figure 5).

The β-TrCP binding motif is also known to be important for the second main function of Vpu, CD4 degradation (Kimura et al., 1994; Lama et al., 1999; Wildum et al., 2006; Willey et al., 1992). Vpu binds the cytoplasmic tail of CD4 and it has been shown that the DSGXXS motif in Vpu is essential for CD4 counteraction (Bour et al., 1995; Margottin et al., 1996, 1998; Paul and Jabbar, 1997; Schubert and Strebel, 1994; Tiganos et al., 1997; Willey et al., 1992). Moreover, the two α-helical domains within the cytoplasmic domain of Vpu are important for degradation of CD4 (Bour et al., 1995; Tiganos et al., 1997) with the membrane-proximal α-helix I mediating the interaction with CD4 (Tiganos et al., 1997). Additionally, amino acids V21-V25 within the C-terminal part of the TMD of Vpu are reported to be required for CD4 antagonism, although the exact residues are controversial (Magadán and Bonifacino, 2012; Tiganos et al., 1998). Thus, it came as a surprise that most of the wild type SIV Vpus were similar or even more active than the intensively studied HIV-1 M NL4-3 Vpu although they lack the di-serine motif and/or do not contain the previously described amino acids within the TMD. Of note, the interaction assay showed that the altered di-serine motifs in SIVcpzPts and SIVgor Vpu have no strong β-TrCP binding capability, hence, β-TrCP seems not to be involved in CD4 modulation (figure 9). The finding that the DSGXXS motif can be dispensable for CD4 down-modulation is in agreement with unpublished data of our group showing that some primary HIV-1 M Vpu proteins that lack the DSGXXS motif are highly active in down-modulating CD4 (Langer et al., unpublished). Additionally, Gomez et al. showed that two SIVcpzPts vpu alleles lacking
the DSGXXS motif were able to down-modulate CD4. They suggested that an acidic stretch is important instead (EEDEE) (Gomez et al., 2005). Interestingly, both SIVcpz Pts Vpu analyzed in the present study, ANT and TAN, indeed contain an acidic stretch (ANT: EEDEE, TAN: DEDEE) and efficiently down-modulate CD4 (figure 8). However, both SIVgor Vpus were also active although they lack an acidic stretch. Taken together, although the di-serine motif seems to be necessary for down-modulation of CD4 by some HIV-1 M Vpus it is not absolute essential for other Vpu proteins. Thus, it remains to be determined which motifs or positions can substitute the β-TrCP-dependent CD4 down-modulation activity.

I also observed that the exchange of the TMD was often associated with a loss of CD4 down-modulation (figure 8). This finding was unexpected since the NL4-3 transmembrane domain contained all requirements reported to be necessary for CD4 counteraction and, obviously, the cytoplasmic parts of the respective SIVcpz or SIVgor Vpu harbored the intrinsic ability, too (Magadán and Bonifacino, 2012). As mentioned before, the exchange of the TMD did not affect β-TrCP binding, hence, lack of this interaction did not account for this loss of activity (figure 9). Petit et al. who examined chimeras between HIV-1 M and O Vpus made similar observations. Some chimeric proteins were no longer able to reduce CD4 surface expression although both wild type Vpus were able. They concluded that O and M Vpus use different combinations of parts of Vpu to achieve the same effect on CD4 (Petit et al., 2011). These findings point to a complex interplay between the different domains. Of note, Vpu proteins are quite heterogeneous regarding their secondary and tertiary structures and it has been shown that SIVcpz Pts TAN1 and ANT Vpus have predicted secondary and tertiary structures that are aberrant from those of HIV-1 M Vpu (Gomez et al., 2005). Thus, the exchange of the TMD might affect the three-dimensional structure of the cytoplasmic part in the fusion protein thereby abrogating possible interactions with cellular components.

It is important to note that most data on Vpu-mediated tetherin counteraction (and CD4 down-modulation) were derived from three Vpu proteins of T cell-line adapted strains of HIV-1 M subtype B (NL4-3, LAI, HXB2). First studies on Vpus of primary B isolates of transmitted/founder and chronic clade viruses showed, that the results gained from NL4-3 might be misleading and strengthen the hypothesis, that down-modulation of tetherin and enhancement of viral egress are mechanistically separable functions (Jafari et al., 2014). Additionally, Pickering et al. recently analyzed a large set of patient-derived HIV-1 vpu and found that NL4-3 Vpu was one of the least active Vpu proteins in tetherin down-modulation and enhancement of virus release (Pickering et al., 2014). Sequence alignments revealed no
specific differences in the trafficking and β-TrCP binding motifs. However, the primary isolates contained an insertion (three amino acids) and deletion (four amino acids) in their cytoplasmic part and notably, the cytoplasmic N-terminus was considerably diverse from NL4-3 Vpu. Interestingly, the (so far disregarded) N-terminus also became focus of attention in a study by Douglas et al. on chimeras between Vpus of a cell culture-adapted B and primary C isolates (Douglas et al., 2013). They showed, that for complete inactivation of the B vpu allele, both the N-terminus and TMD from the inactive vpu C allele were required. Thus, similar to the results of Petit et al. that suggest a complex interplay between different domains in CD4 down-modulation the data by Douglas et al. indicate a complex interaction of different domains also in tetherin counteraction. These discrepancies between cell-culture-adapted strains and primary isolates clearly highlight the necessity for further analysis of Vpu-mediated tetherin antagonism by primary isolates.

Taken together, my results show the gain of function against tetherin was independent of the previously described trafficking or β-TrCP binding motifs in the cytoplasmic part of Vpu. Thus, the presence or absence of primary sequence congruence with known motifs is not necessarily indicative of functional integrity. Generalization based on the analysis of limited numbers of alleles should be avoided and more in-depth studies of primary isolates are required. My results further unveil the enormous versatility and plasticity of Vpu proteins. Although the cytoplasmic parts of the different SIVcpz and SIVgor Vpus are quite diverse, they generally fulfill the functional requirements for efficient counteraction of human tetherin.

Two important questions remain: (1) why have the descendants of SIVgor not evolved Vpu-mediated tetherin antagonism and (2) why has SIVcpzPts not been successfully transmitted to humans? (1) Just recently, we found that the descendants of SIVgor adapted their accessory proteins Nef to antagonize human tetherin (Kluge et al., submitted). Although it was thought that human tetherin is generally resistant to Nef-mediated counteraction due to the five amino acid deletion I could show that HIV-1 O Nefs have evolved the ability to target a region N-terminal of the deletion region in the cytoplasmic part of human tetherin. Thus, SIVgor may be more prone to evolve Nef- rather than Vpu-mediated anti-tetherin activity in humans. This adaptation might have been sufficient to make it a replication competent virus in humans and to overcome the hurdle imposed by tetherin. Although HIV-1 O Nef-mediated counteraction is less efficient than group M Vpu-mediated counteraction this result may explain why HIV-1 O spread epidemically in Central Africa. (2) Regarding the lack of SIVcpzPts transmission to human, the possibility that the evolutionary hurdle to
gain Vpu-mediated anti-tetherin activity is higher for SIVcpzPts than for SIVcpzPtt still cannot be excluded. While our data indicate that both SIVcpzPtt and SIVcpzPts Vpuls only have to evolve an interaction site in their TMDs to acquire significant anti-tetherin activity, they do not allow to estimate which and how many nucleotide substitutions are required to achieve effective anti-tetherin activity. Taken the amino acids for interaction with the TMD of tetherin of M Vpu as basis, the number of amino acid changes that are required is clearly higher for SIVcpzPts than for SIVcpzPtt (SIVcpzPts ~7 changes (median=7; n=16); SIVcpzPtt changes ~3.3 changes (median=2; n=15) (Li et al., 2012; Skasko et al., 2012; Vigan and Neil, 2010). However, the adaptive changes in the TMD that allow HIV-1 N Vpus to interact with human tetherin are overlapping but distinct and we cannot exclude that there are multiple possibilities to evolve an interaction site (Lim et al., 2010; Sauter et al., 2012). Thus, a disadvantage of SIVcpzPts Vpu to evolve anti-human tetherin activity cannot be ruled out. Additionally, our recent finding that HIV-1 O Nefs counteract human tetherin raises the question about the ability of SIVcpzPts Nef to evolve anti-tetherin activity after zoonotic transmission.

It also has to be considered that SIVcpzPtt and SIVcpzPts are quite diverse, although their host species are closely related. Leitner et al. hypothesized that after the separation of the Central and Eastern chimpanzee subspecies SIV super-infection led to further recombination events that resulted in the emergence of SIVcpzPts (Leitner et al., 2007). Thus, although the hosts of these SIVcpz are closely related the Gag, Pol and Env proteins of these viruses differ about 30-50 % (Vanden Haesevelde et al., 1996; Prado-Martinez et al., 2013). It is therefore possible that an adaptation of SIVcpzPts is in general more difficult and that it is not the ability to acquire tetherin antagonism that underlies the lack of transmission. Bibollet-Ruche et al. studied the replication capacity of different SIVcpz strains in human lymphoid tissue and showed that SIVcpzPts produces about two-fold lower viral titers than SIVcpzPtt in human lymphoid tissues (Bibollet-Ruche et al., 2012). Thus, it is tempting to speculate that SIVcpzPts is inferior in interacting with or counteracting human orthologues of dependency or restriction factors, respectively. However, as the known restriction factors such as TRIM5α or APOBEC3G are highly conserved between humans and chimpanzees it seems unlikely that these restriction factors make the difference but that additional yet-to-be-discovered restriction factors are involved (Sheehy et al., 2002; Stremlau et al., 2004). Additionally, it is conceivable that SIVcpzPts is more prone to sensing by the human immune system than SIVcpzPtt. Efficient immune sensing of the virus after transmission might therefore prevent SIVcpzPts from successful infection of humans.
Moreover, we cannot exclude that the frequency of ape-human interaction in Central and East Africa with the respective chimpanzee species differs (Sharp and Hahn, 2011). Ape hunting, bushmeat butchering and consumption were and are still practiced in the habitats of Central chimpanzees and Eastern chimpanzees but precise data on hunting places and frequencies are missing (Locatelli and Peeters, 2012). Additionally, seroprevalences of SIV infections in Central as well as Eastern chimpanzees in these areas range from 0-50% (Li et al., 2012). Thus, the risk of exposure to contaminated blood seems to vary and exposure to infected Eastern chimpanzees (or bushmeat thereof) might simply be lower in East Africa. Finally, although there are millions of infected individuals one has to keep in mind that the ratio of known transmission events of SIVcpzPtt:SIVgor:SIVcpzPts is 2:2:0, hence, cross-species transmissions are rare events and it may just be coincidence that SIVcpzPts has not been successfully transmitted yet. Of note, transmission of HIV-1 occurred only about 50-100 years ago and HIV can still be considered an emerging virus (Lemey et al., 2004; Wertheim et al., 2009; Worobey et al., 2008). Given the ongoing chase, handling and consumption of apes and bushmeat in Central and East Africa, there clearly is the potential for new SIV transmission events (Locatelli and Peeters, 2012). Thus, considering the enormous plasticity of primate lentiviral proteins it is tempting to speculate that neo-functionalization of these proteins after cross-species transmission might lead to the evolution and manifestation of a new virus in the human population.

In summary, this thesis demonstrates that differences observed in the ability of different SIVcpz and SIVgor strains to evolve Vpu-mediated counteraction of human tetherin and to facilitate spread in humans are most likely not due to predispositions in their respective Vpu proteins. Chimeric SIV Vpus were active against tetherin irrespectively of previously described motifs in their cytoplasmic part. Hence, the presence or absence of primary sequence congruence with known motifs was not indicative of functional integrity. This indicates that the requirements for these motifs for Vpu-mediated tetherin counteraction are highly context dependent and that results obtained from a few T cell line adapted HIV-1 strains cannot be generalized. Importantly, I have shown only recently that SIVgor evolved its Nef protein to counteract human tetherin and this might have been sufficient to overcome the hurdle imposed by human tetherin and to spread epidemically (Kluge et al., submitted). Thus, adaptation of SIVgor Vpu to human tetherin might have not be obligatory. For SIVcpzPts it is not yet clear, why SIVcpzPts related viruses have not been found in humans until now. Adaptation of SIVcpzPts Vpu to human tetherin seems to be easier than
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previously anticipated and the factors underlying the ‘lack’ of transmission remain to be determined.
5 Summary

HIV-1 is a zoonosis that arose after cross-species transmissions of simian immunodeficiency viruses (SIV) from Central chimpanzees (SIVcpzPtt) and Western lowland gorillas (SIVgor) to humans. An important barrier for successful zoonotic transmission and efficient spread is the restriction factor tetherin, an interferon-inducible protein that inhibits virus release by tethering newly formed budding virions to the surface of infected cells. SIVcpz and SIVgor use their accessory protein Nef to antagonize tetherin of their respective host species by targeting a five amino stretch in the cytoplasmic part. However, the human tetherin orthologue contains a deletion of exactly these five amino acids and is thus resistant to counteraction by SIV Nefs. So far, only SIVcpzPtt, the precursor of pandemic HIV-1 group M strains, fully mastered this hurdle by switching from Nef- to Vpu-mediated tetherin antagonism during adaptation to humans. This effective Vpu-mediated counteraction distinguishes pandemic group M strains from the non-pandemic groups N, O and P. Of note, viruses related to SIVcpzPts from Eastern chimpanzees have never been found in humans although this chimpanzee species lives in close proximity to and are as frequently infected with SIVcpz as the Central chimpanzee. Counteraction of human tetherin by Vpu requires a direct interaction between the transmembrane domains of both proteins. Additionally, it has been reported that tyrosine- and di-leucine-based trafficking motifs and a β-TrCP binding domain in the cytoplasmic part of HIV-1 M Vpus are important for efficient counteraction of human tetherin. Interestingly, these motifs are frequently found in the Vpu proteins of SIVcpzPtt but are usually absent in SIVcpzPts and SIVgor Vpus. Thus, I hypothesized that SIVcpzPtt Vpu only required changes in its transmembrane domain to evolve an interaction interface with human tetherin whereas SIVcpzPts and SIVgor Vpus would need additional adaptive changes in their cytoplasmic parts to become active against human tetherin. This might explain why SIVcpzPts has never been transmitted successfully to humans and why SIVgor, the precursor of group O and P, has failed to acquire Vpu-mediated anti-human tetherin activity and spread less efficiently.

To test whether SIVcpzPtt Vpu only requires changes in its transmembrane domain whereas SIVcpzPts and SIVgor Vpus require also changes in their cytoplasmic parts, I generated a set of chimeric Vpus. I exchanged the N-terminal part of selected primary strains of SIVcpz and SIVgor Vpus by that of the HIV-1 M NL4-3 Vpu. The transmembrane domain of this M Vpu is known to interact with human tetherin. The resulting chimeric Vpus that differed only in their cytoplasmic parts were tested for their ability to antagonize human tetherin and to down-modulate CD4. Surprisingly, the exchange of the N-terminal part was
sufficient to confer anti-human tetherin activity to all SIVcpzPtt, SIVcpzPts Vpus and SIVgor Vpus irrespective of the presence or absence of previously reported functional motifs. Strikingly, the chimera with the cytoplasmic part of an SIVcpzPts Vpu (NL_AN) that lacked all motifs enhanced virus release most efficiently. In contrast, both chimeric Vpus with the cytoplasmic part of SIVcpzPtt Vpus (NL_MB, NL_EK) that contained all functional motifs were in fact the least active ones in enhancing virus release. Thus, the gain of anti-tetherin activity was independent of previously reported trafficking and β-TrCP binding motifs in the cytoplasmic part. Unexpectedly, the gain of anti-tetherin activity was often associated with a loss of CD4 down-modulation function.

These results suggest that it is easier for SIVcpzPts and SIVgor to evolve Vpu-mediated anti-human tetherin activity than previously anticipated. The data further demonstrate that the requirements for trafficking and β-TrCP binding motifs in the cytoplasmic part of Vpu for human tetherin counteraction are highly context dependent. Thus, results obtained from the analysis of a few T cell line adapted HIV-1 strains should not be generalized. My findings further demonstrate the enormous plasticity of Vpu proteins and show that predictions of functionality based on sequence data is difficult. It remains to be determined which factors keep SIVcpzPts from successfully crossing the species barrier and SIVgor from adapting its Vpu protein to human tetherin.
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LG
Silvia“
P.s.: „Ich denke an vieles. Und den Rest vergesse ich.“

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“Hier kannst einfach cut ab und übersetzen.”
Teile dieser Dissertation wurden bereits in folgendem Fachartikel veröffentlicht: