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Mechanism and Evolutionary Origins of HIV-1 Virion Entrapment by Tetherin

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MECHANISM AND EVOLUTIONARY ORIGINS OF HIV-1 VIRION ENTRAPMENT BY TETHERIN

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Siddarth Venkatesh

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MECHANISM AND EVOLUTIONARY ORIGINS OF HIV-1 VIRION

ENTRAPMENT BY TETHERIN

Siddarth Venkatesh, Ph.D.

The Rockefeller University 2014

The cellular restriction factor, tetherin, prevents HIV-1 and other enveloped virus particles from being disseminated into the extracellular milieu by infiltrating their envelopes and by physically crosslinking them to the cell surface. However, the mechanisms underlying virion retention have not yet been fully delineated. In this body of work, we employed biochemical assays and engineered tetherin proteins to demonstrate conclusively that virion tethers are composed of the tetherin protein itself, and to elucidate the configuration and topology that tetherin adopts during virion entrapment. We demonstrated that tetherin dimers adopt an “axial” configuration, in which pairs of transmembrane domains or pairs of glycosylphosphatidyl inositol anchors are inserted into assembling particles, while the remaining pair of membrane anchors remains embedded in the infected cell membrane. We used quantitative western blotting to determine that a few dozen tetherin dimers are used to trap each virion particle, and that there is ~3-5 fold preference for the insertion of glycosylphosphatidyl inositol anchors rather than transmembrane domains into tethered virions. Cumulatively, these results demonstrated that axially configured tetherin homodimers are directly responsible for trapping virions at the cell surface. We propose that insertion of glycosylphosphatidyl inositol anchors may be preferred so that effector functions

that require exposure of the tetherin N-terminus to the cytoplasm of infected cells are retained.

Allied to these efforts, we also endeavored to determine the evolutionary origins of tetherin. We used computational and biochemical approaches to build a case that tetherin arose via duplication of the neighboring PLVAP/PV1 gene. The PV1 and tetherin genes are located adjacent to each other in eutherian mammals and encode proteins with a shared and relatively unusual overall architecture. Phylogenetic analyses provided evidence that tetherin is ~147 MY old and that it has evolved under positive selection. Conversely, PV1 is ~400 MY old and has evolved under purifying selection in therian mammals. Using biochemical assays, we demonstrated that PV1 can be endowed with antiviral activity against HIV-1 when it is engineered to encode a C-terminal GPI anchor. Although we did not detect any sequence homology between PV1 and tetherin, *in silico* evolution experiments indicated that it is possible to evolve sequences from an ancestral PV1 sequence that bear no significant homology to contemporary PV1, after simulating the evolutionary processes that contributed to the emergence of tetherin. We found that genes encoding proteins with PV1/tetherin-like architecture are quite rare in the human and mouse genomes, and that the majority of these genes that are adjacent to each other likely arose via gene duplication. Moreover, some, but not all, PV1/tetherin-like proteins have the intrinsic ability to trap virions, when appended with a GPI anchor. Finally, we analyzed the organization of genes in the PV1 locus from amphibians to eutherian mammals and identified an ancestral synteny block that supports a

model in which tetherin arose from a PV1 duplication, that occurred between the times that monotremes and marsupials diverged from other mammals (~166-147 MYA). Cumulatively, these findings suggest that tetherin arose by gene duplication of PV1 and exemplify the remarkable capacity of our genomes to innovate antiviral factors from pre-existing genetic raw material.

This thesis is dedicated to

Santhosh Palani,

for giving me the benefit of his excellent advice and immense wisdom.

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Chapter I Introduction

At present, acquired immunodeficiency syndrome (AIDS) is one of the most devastating global pandemics in human history, and has been responsible for claiming more than an estimated 36 million lives worldwide (UNAIDS, 2013). This is quite remarkable, given that its etiologic agent, human immunodeficiency virus-1 (HIV-1), was only recently identified to be a human pathogen (Barre-Sinoussi et al., 1983). Infected individuals progress to AIDS and eventually succumb to opportunistic infections or tumors as a consequence of a near-complete erosion of their immune systems. The advent of highly active antiretroviral therapy (HAART) marked a turning point in the epidemic, and resulted in reduced morbidity and mortality in First World countries. However, it is sobering to note that most infected people in the Third World still have limited (or no) access to HAART (Richman et al., 2009). In spite of prevention strategies and efforts, the numbers of new infections continue to climb steadily. Also, AIDS patients who are noncompliant become resistant to antiretroviral therapy due to the acquisition of mutations; thus there is an impetus to discover new drug targets and therapeutic modalities.

HIV-1 is a member of a family of enveloped diploid RNA viruses called *retroviridae*. All retroviruses establish persistent infection in mammalian species by depositing copies of their genomic DNA into the chromosomes of host cells (Temin, 1964). HIV-1 belongs to the genus of retroviruses called lentiviruses, which are characterized by the unusually long time that it takes between infection and manifestation of chronic disease. Many of the known lentiviruses are

transmitted between individuals in a horizontal manner, i.e. via extracellular virions. In certain instances, however, they are inserted into the germline as proviruses and can be inherited vertically in a Mendelian fashion (Katzourakis et al., 2007; Martin et al., 1981a; Martin et al., 1981b). HIV-1 is also vertically transmitted from a HIV-positive mother to her child during pregnancy, delivery and breastfeeding.

In general, lentiviruses share many features such as their genetic architecture, particle (or virion) morphology, and life cycles. All retroviruses encode three genes: *gag*, *pol*, and *env*, which direct the production of the structural, enzymatic and envelope components of the virus respectively. In addition, lentiviruses also encode the so-called “accessory genes” that are necessary for efficient replication in certain cell types, but are dispensable in others. HIV-1 also encodes the Tat and Rev proteins that regulate transcription from the viral LTR and export of unspliced and incompletely spliced transcripts to the cytoplasm, respectively.

A lentiviral particle typically comprises of a viral core of approximately 1000-5000 copies of the capsid (CA) protein, which have the propensity of self-assembling into a stable lattice (Briggs et al., 2004; Piatak et al., 1993; Zhu et al., 2003). Protected within this structure are two copies of the viral RNA genome along with an assortment of virion-associated proteins such as matrix (MA), capsid (CA), nucleocapsid (NC), protease (PR), reverse transcriptase (RT), integrase (IN) and viral protein R (Vpr) (Kohl et al., 1988). As such, the core is itself confined within a spherical lipid bilayer that serves as an outer shell, with MA being closely

associated with the inner leaflet of the lipid bilayer. Several copies of the viral envelope protein (Env) are embedded within the bilayer and extend outwards to interact with receptor molecules on cell surfaces.

A comprehensive treatment of the lentiviral life cycle is not feasible because of space constraints; however, a few simple concepts will be presented in the next section.

Overview of the lentiviral life cycle

The lentiviral life cycle (Figure 1.1) begins with the adhesion of virions to target cells by relatively non-specific interactions with various molecules such as integrins or DC-SIGN that are expressed on the cell surface (Cicala et al., 2009; Geijtenbeek et al., 2000). Thereafter, the Env molecules bind a specific receptor molecule on the target cell, and it is the specificity of this interaction that governs host range and tissue tropism. In the case of HIV-1, the Env protein is a trimer of gp120 and gp41 heterodimers. The gp120 subunit is the surface protein and the gp41 is a transmembrane protein (McCune et al., 1988). The gp120 subunit binds the primary receptor CD4 (Maddon et al., 1986; McDougal et al., 1986), which prompts conformational changes in Env, facilitating the engagement of the coreceptors CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996) (Figure 1.1).

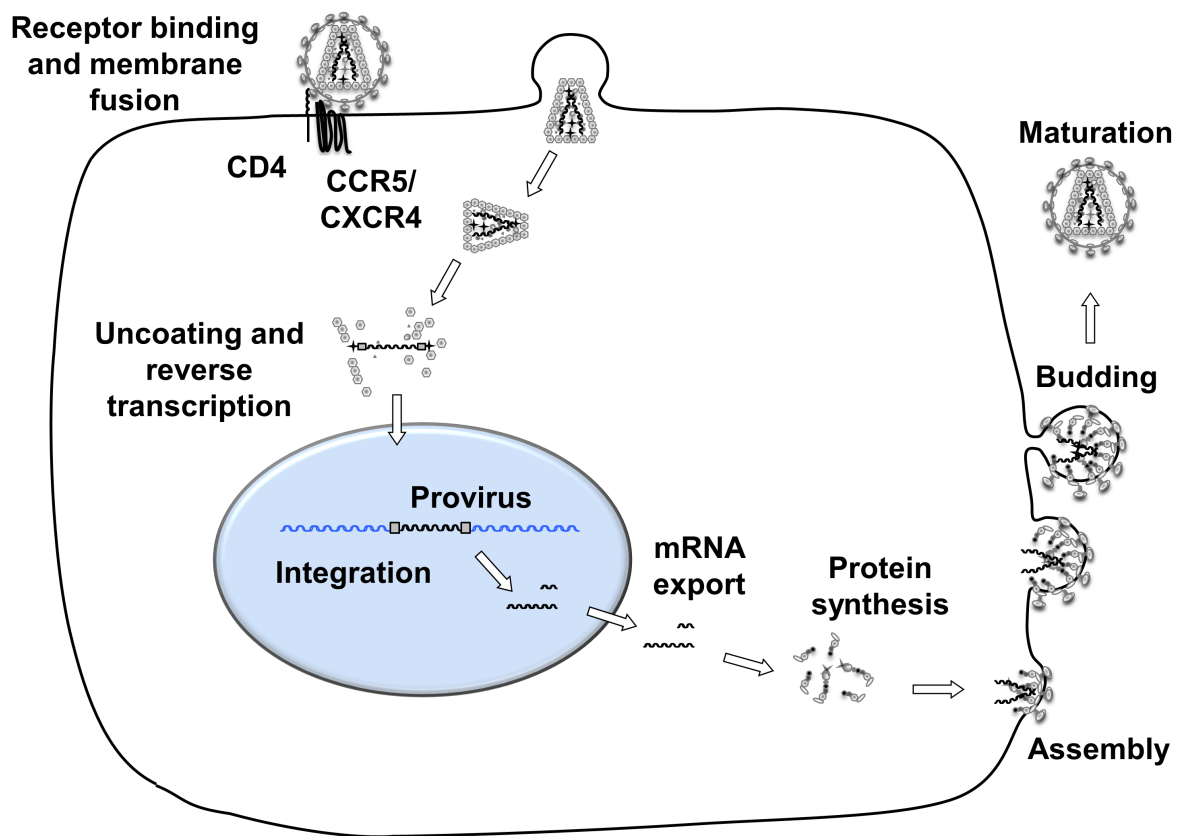


Figure 1.1. Overview of the lentiviral life cycle. Refer text for details.
 Disclaimer: This figure was kindly provided by Theodora Hatzioannou (Aaron Diamond AIDS Research Center, NY).

Next, a series of structural rearrangements within the transmembrane region of Env exposes the hitherto concealed gp41 fusion peptide, which inserts into the target cell membrane and forms a six-helix bundle (Chan et al., 1997; Weissenhorn et al., 1997). Folding of the gp41 molecule to form a thermodynamically favorable state wherein its termini are at the same ends of the folded molecule, drives fusion of the two lipid bilayers. This is believed to proceed via a hemifusion stalk intermediate in which the proximal leaflets merge, and ultimately leads to the formation of a pore (Eckert and Kim, 2001). However, this is a relatively small opening, and pore dilation occurs via poorly understood mechanisms. The pore serves as the conduit through which the viral core is delivered into the cytoplasm (Wilén et al., 2012).

What follows next is a series of nebulous events popularly referred to as “uncoating” (Figure 1.1), wherein the capsid lattice begins a process of disassembly as it traverses through the cytoplasm en route to the nucleus (McDonald et al., 2002; Zhang et al., 2000). This sets the stage for the first of two steps that are peculiar to the retroviral life cycle, i.e. the generation of linear double stranded DNA (dsDNA) from the single-stranded RNA genome via the action of the viral enzyme reverse transcriptase (RT) (Baltimore, 1970; Mizutani et al., 1970) (Figure 1.1). The biochemical mechanisms that underlie the process of reverse transcription are quite well established (Hu and Hughes, 2012). Interestingly, lentiviral RTs have evolved to be processive even under low concentrations of deoxyribonucleotides (dNTPs), so that they can infect a wide range of cell types (Diamond et al., 2004; Lahouassa et al., 2012). The nascent

dsDNA and associated enzymes form a preintegration complex (PIC) that migrates toward the nucleus by engaging the cellular actin cytoskeleton network (Bowerman et al., 1989; Miller et al., 1997). Lentiviral PICs are ushered into the nucleoplasm through nuclear pore complexes; hence they are capable of infecting non-dividing cells (Bukrinsky et al., 1992; Lewis et al., 1992) (Figure 1.1). In contrast, many other retroviruses must wait for the breakdown of the nuclear envelope during mitosis to access chromosomal DNA, and thus depend on the cell cycle for establishing a productive infection (Bieniasz et al., 1995; Roe et al., 1993).

Within the nucleus, the viral enzyme integrase (IN) catalyzes the transesterification reactions that insert the viral dsDNA into the host genome (Brown et al., 1987; Fujiwara and Mizuuchi, 1988; Goff, 1992; Roth et al., 1989) (Figure 1.1). Integration is essentially irreversible and allows the “genomic intruder” to persist as provirus for the lifetime of the cell. Once integrated, the proviral DNA is virtually indistinguishable from host DNA, and the host transcriptional machinery generates viral transcripts that have all the typical attributes of cellular mRNAs. Retroviruses tend to vary in their preference for integration sites, and HIV-1 has a preference for integrating into active transcriptional units (Craigie and Bushman, 2012; Schroder et al., 2002; Wu et al., 2003).

The next phase of the life cycle, i.e. gene expression, is regulated by the Tat and Rev proteins. The Tat protein is responsible for transactivation of LTR-driven gene expression, and Rev mediates the export of the unspliced and incompletely spliced transcripts to the cytoplasm (Dayton et al., 1986; Fisher et al., 1986;

Laschia et al., 1989; Rice and Mathews, 1988; Sodroski et al., 1986; Sodroski et al., 1985a; Sodroski et al., 1985b). At first, basal levels of LTR activity drive the generation of low levels of full-length transcripts. Thereafter, HIV-1 transcripts undergo a complex splicing program to produce the full range of mRNAs that are needed to encode the viral proteins. Note that the unspliced ~9-kb, incompletely spliced ~4-kb and completely spliced ~1.8-kb mRNAs are produced even at early stages of transcription, but in the absence of Rev, the longer transcripts are retained in the nucleus where they are eventually degraded by RNA surveillance mechanisms (Karn and Stoltzfus, 2012; Pollard and Malim, 1998). In contrast, the short completely spliced ~1.8-kb mRNAs are shuttled out of the nucleus and encode the three early gene products, i.e. Tat, Rev and Nef. The Tat protein then stimulates transcription by binding the viral transactivator response (TAR) element situated in the repeat (R) region of the mRNA transcript in the nucleus (Berkhout et al., 1989; Muesing et al., 1987). This results in phosphorylation of the RNA polymerase II C-terminal domain that results in enhanced processivity on the proviral DNA template and multi-fold enhancement in levels of the full-length transcript (Bieniasz et al., 1999; Parada and Roeder, 1996).

When the levels of the Rev protein in the nucleus exceed a certain threshold, Rev molecules bind cooperatively to the Rev response element (RRE) that is present in the unspliced ~9-kb and incompletely spliced ~4-kb mRNAs, and shuttle them out to the cytoplasm (Malim et al., 1989; Pollard and Malim, 1998). The full-length mRNAs are either translated to yield the ~55-kDa Gag and ~160-kDa Gag-Pol precursors, or are assembled into progeny virions as genomes

(Jouvenet et al., 2008; Jouvenet et al., 2006; Jouvenet et al., 2009b) (Figure 1.1). Intriguingly, in most retroviruses, the Gag-Pol precursor is translated as a result of programmed frameshifting at a uridine-rich (UUUUUUA) slippery sequence before the Gag stop codon (Jacks et al., 1988; Yoshinaka et al., 1985a, b). Importantly, this event only occurs about 1 in 20 translation events, resulting in an optimal stoichiometry of the Gag:Gag-Pol precursors.

The ~4-kb mRNAs are translated to yield the other late gene products, i.e. Vpu, Vpr, Vif and Env. The Env glycoprotein is inserted cotranslationally into the ER and is extensively glycosylated and assembled as trimers during anterograde transport to the plasma membrane. Moreover, it is processed by the cellular protease furin in the Golgi compartments to generate the mature gp120 and gp41 subunits, and then delivered to the plasma membrane via the secretory pathway (Goff, 2007; Sundquist and Krausslich, 2012).

The assembly and budding of extracellular virions is an intricate process and is mediated by the Gag polyprotein (Figure 1.1). Remarkably, ectopic expression of the multifunctional Gag protein in a host cell is sufficient for the assembly and release of immature virus-like particles (VLPs). The amino-terminal domain of Gag, known as p17 matrix (MA), is responsible for plasma membrane targeting, mainly via interactions between a string of basic residues near the amino terminus with plasma membrane-specific phospholipids PI(4,5)P₂ (Bieniasz, 2009). This binding event exposes a myristic acid modification at the N-terminus of Gag molecules which tethers them stably to the inner leaflet of the plasma membrane (Dalton et al., 2005; Paillart and Gottlinger, 1999; Zhou et al., 1994).

The p24 CA protein mediates homo-oligomerization, and as mentioned earlier, condenses upon maturation to form the virion core (Borsetti et al., 1998). The p7 nucleocapsid (NC) protein traps genomic RNA dimers during particle assembly via interactions between its zinc finger motif, and a packaging motif (ψ) that is present on the full-length transcripts (D'Souza and Summers, 2005). The carboxy-terminal p6 domain mediates the severing of the thin cytoplasm-filled stalk that separates the viral and cellular membranes by usurping the cellular ESCRT pathway (Martin-Serrano and Neil, 2011). It is well known that retroviruses encode small peptide motifs known as late-budding (L) domains that are essential for this process (Garrus et al., 2001; Gottlinger et al., 1991; Martin-Serrano et al., 2001; Parent et al., 1995; Puffer et al., 1997; Wills et al., 1994; Yasuda and Hunter, 1998; Yuan et al., 2000). In HIV-1, these domains bind Tsg101 (a component of ESCRT-I) and ALIX (an ESCRT-I and ESCRT-III-binding protein) (Bieniasz, 2006). L domain mutants fail to complete the final scission step in budding, thus resulting in clusters of immature virions whose membranes are continuous to the cell membrane, in a manner reminiscent of “beads on a string” (Gottlinger et al., 1991). Importantly, late domains require the Class E vacuolar protein sorting (VPS) pathway to release virions (Bieniasz, 2006; Garrus et al., 2001; Martin-Serrano et al., 2005; Strack et al., 2003).

The maturation of virions requires proteolytic cleavage of the Gag and Gag-Pol precursors by the viral enzyme PR to yield the structural (MA, CA, NC and p6) and enzymatic (PR, IN, RT) proteins (Kohl et al., 1988) (Figure 1.1). The timing of this event is linked to the activation of protease, which may be induced by the

oligomerization of Gag-Pol precursors within the virion. Thus, the mature virion diffuses into the extracellular milieu and begins a new cycle of infection.

In turn, cells have erected rather formidable barriers to guard themselves against viral invasion. We provide an overview of these defense measures in the next section.

Restriction factors: an “intrinsic” immune system in host cells

Many examples are found in nature wherein antagonistic co-evolution results in rapid adaptation of genes, physiology and behavior (Blanco-Melo et al., 2012). Classic examples include those involving relationships between parasites and hosts, or predators and prey. The invasion of host cells by viruses represents another example of an antagonistic evolutionary struggle. Because viral infections are often detrimental to host survival and reproduction, host cells have evolved numerous defense mechanisms to sense, evade, and defend themselves against a variety of viral infections. In animal cells, the sensing of viruses by pattern recognition receptors leads to interferon production and signaling, which induces the expression of hundreds of interferon-stimulated genes (ISGs) in infected and bystander cells (Schoggins and Rice, 2011). Among these are several classes of autonomously acting proteins that are popularly termed “restriction factors”, and are considered to comprise an “intrinsic” immune system (Bieniasz, 2004) or a specialized arm of conventional innate immunity. There are five currently known families of restriction factors that target HIV-1 and

other primate lentiviruses: the APOBEC3 proteins (Sheehy et al., 2002), TRIM5 proteins (Stremlau et al., 2004), Tetherin (Neil et al., 2008; Van Damme et al., 2008), SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011) and Mx2 (Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013). These proteins inhibit the replication of viruses which then adapt to evade and defend themselves against this form of host immunity. In turn, five classes of primate lentivirus proteins: Vif (Sheehy et al., 2002), Vpu (Neil et al., 2008; Van Damme et al., 2008), Nef (Jia et al., 2009; Zhang et al., 2009), Vpx (Hrecka et al., 2011; Laguette et al., 2011) and Env (Gupta et al., 2009b; Le Tortorec and Neil, 2009) have evolved the ability to antagonize a specific antiviral protein. In this way, antagonistic conflict begets defense and counter defense measures, while recursively shaping host and viral functions and genomes.

Restriction factors often possess certain attributes that differentiate them from most other gene products (Malim and Bieniasz, 2012). Importantly, only those gene products that directly engage viruses and inhibit their replication are restriction factors. Specifically, they (i) are dominantly and autonomously acting proteins that exhibit antiviral activity in simple cell-culture based assays, (iii) are often constitutively expressed in some cell types, but are sometimes induced by the expression of interferons, (iv) employ unique and unanticipated mechanisms to inhibit specific processes in viral replication, (v) have unusually diverse amino acid sequences as a consequence of antagonistic co-evolution with viruses, and (vi) are often (but not always) antagonized by viral accessory proteins.

Restriction factors are autonomous inhibitors of viral replication

In general, the antiviral restriction factors that have been identified thus far act as self-sufficient entities, rather than as components of complex pathways. Moreover, they act in a cell-autonomous fashion, i.e. their activity is evident in simple cell-culture based viral replication or infectivity assays. Thus, their existence was indicated by early studies that defined cell lines to be “permissive” or “non-permissive”, depending on whether wild type or mutant viral strains could efficiently replicate therein (Malim and Bieniasz, 2012). Often, cell fusion experiments between permissive and non-permissive cell lines demonstrated that heterokaryons that contained the contents of both cells exhibited the restrictive phenotype, suggesting a dominant mode of action for putative restriction factors (Malim and Bieniasz, 2012). Sometimes, mutant viruses lacking a particular accessory gene failed to complete particular steps in the viral life cycle in restrictive cell lines, and it was posited that the accessory gene was required to antagonize an unknown inhibitor. These concepts underlined the design of experiments that established the identification of several restriction factors. In each case, expression of a single gene could convert the phenotype of a cell line from permissive to non-permissive (Hrecka et al., 2011; Laguette et al., 2011; Neil et al., 2008; Sheehy et al., 2002; Stremlau et al., 2004; Van Damme et al., 2008).

Restriction factors are constitutively expressed or induced by interferons

Adaptive immunity differs from innate immunity in various respects (Medzhitov, 2007). One key difference is the rapidity with which restriction factors can exert their effects. T- and B- lymphocyte receptor diversity is generated via somatic recombination, meaning that only tiny numbers of cells with antiviral potential are present at the onset of a new infection. Effector cells are clonally expanded upon exposure to the pathogen, requiring several days to weeks to accumulate sufficient numbers to mount an effective adaptive immune response. In contrast, fully active forms of restriction factors are encoded in the germ line and are sometimes constitutively expressed. Indeed, prototypical human antiretroviral restriction factors, i.e. APOBEC3G and TRIM5, were found to be constitutively expressed in cell types targeted by primate lentiviruses, leading to the notion that cells are resistant to infection in the apparent absence of any prior exposure or signaling event (Bieniasz, 2004). The expression of another restriction factor, tetherin, was stimulated upon the expression of interferons, indicating that it constituted an antiviral response. While it was subsequently shown that each of the known primate lentivirus restriction factors could be induced by interferon in some cell types, IFN-induction is not essential to observe profound antiviral activities in cells.

Restriction factors have diverse mechanisms of action

In the case of adaptive immune responses and antiviral drugs that target viral proteins, resistance is most often acquired through one or a few amino acid substitutions that prevent recognition by the immune system and drugs. Like antiretroviral drugs, restriction factors target specific steps in the HIV-1 life cycle, but unlike antiretroviral drugs, do so in a way that makes it difficult for HIV-1 to evolve resistance to the inhibitor through simple evasive mutations. In fact, these proteins directly inhibit the replication of viruses via unanticipated, diverse and remarkably elegant mechanisms of action (Figure 1.2).

Some members of the APOBEC3 proteins (APOBEC3F/G/H) inhibit HIV-1 by infiltrating newly assembled virions and cause extensive deamination of deoxycytidines in the nascent complementary DNA (cDNA) strand in target cells during reverse transcription (Figures 1.2 and 1.3). Consequently, there is an accumulation of deleterious deoxyguanine-to-deoxyadenine mutations in the viral genome (Refsland and Harris, 2013). The Tripartite motif containing 5 isoform α (TRIM5 α) protein targets the incoming capsid protein prior to reverse transcription, and triggers premature uncoating and/or capsid degradation (Sastri and Campbell, 2011) (Figure 1.2). Tetherin is a dual-anchored transmembrane protein that inhibits enveloped virions at the cell surface, thus preventing their dissemination (Neil, 2013) (Figure 1.2). The SAM- and HD-domain containing protein 1 (SAMHD1) is an enzyme with triphosphohydrolytic activity that inhibits HIV-1 replication primarily by depleting intracellular nucleotide pools and

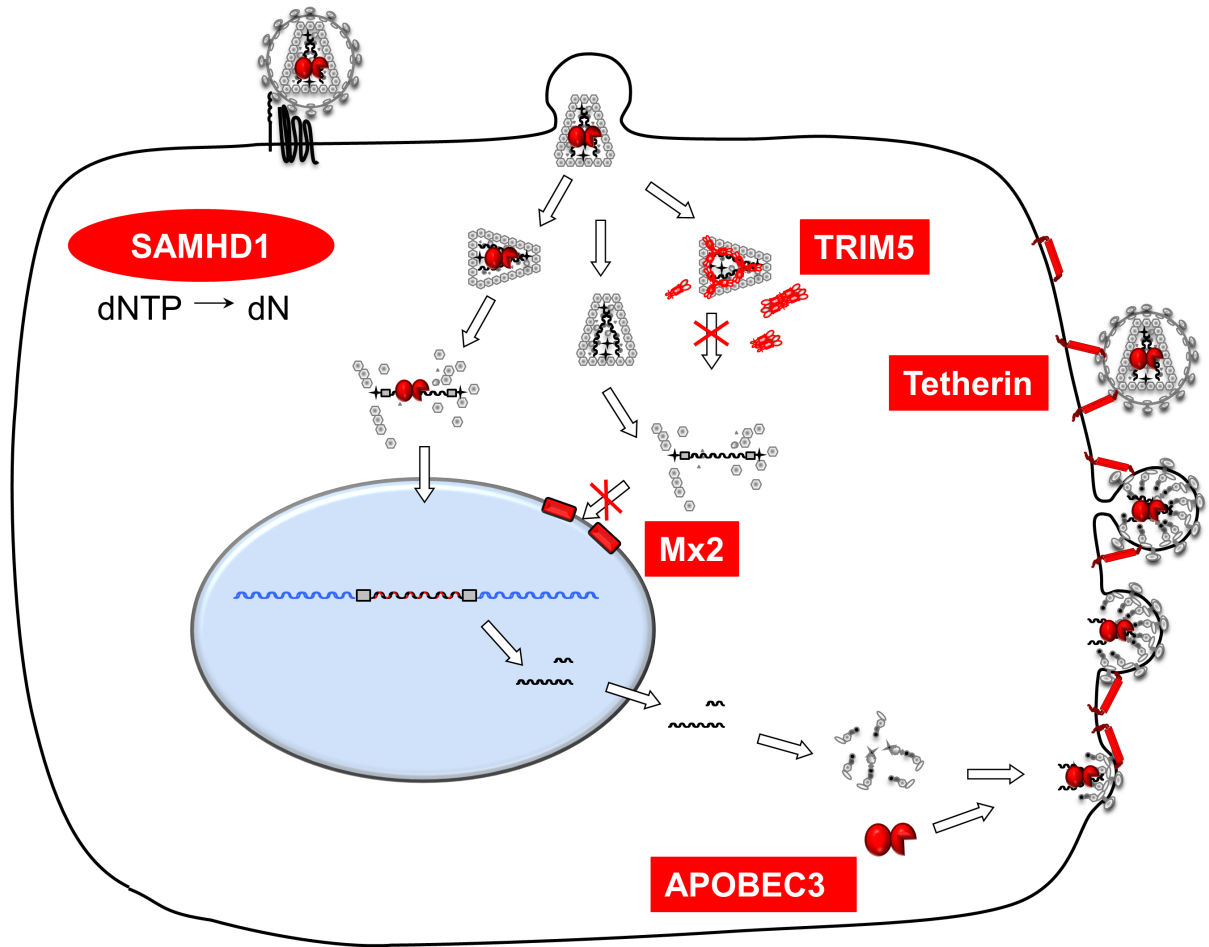


Figure 1.2. Restriction factors directly inhibit the replication of HIV and SIVs. The mechanisms by which restriction factors directly act upon various stages of the retroviral replication cycle are depicted. The restriction factors are indicated as red figures and the APOBEC3-mediated hypermutation of the viral genome is indicated as red wavy lines. Refer text for details.

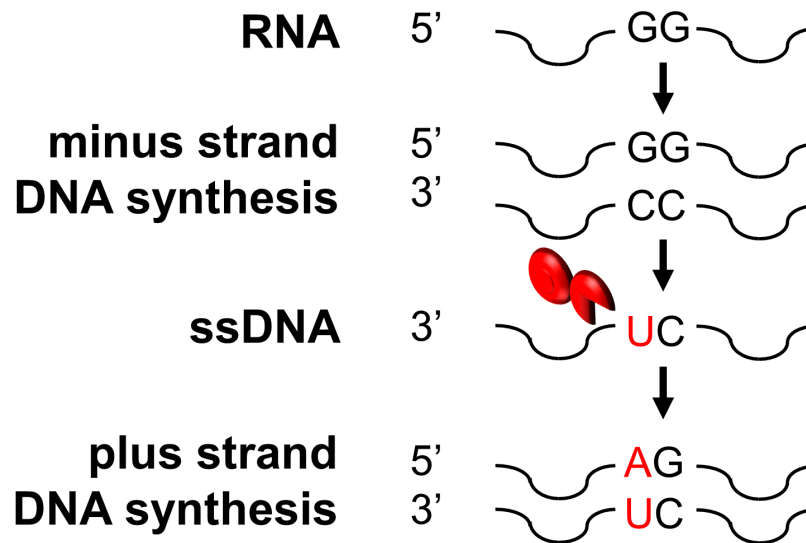


Figure 1.3. Mechanism of the antiviral activity of the APOBEC3 family of proteins. The APOBEC3F/G/H proteins catalyze extensive deoxycytidine deamination (i.e. C to U mutations) in the nascent complementary DNA strand that results in the accumulation of deoxyguanine-to-deoxyadenine mutations in the viral genome. Such hypermutation is lethal as it results in the accumulation of stop codons and aberrant splicing events in the viral genome.

inhibiting reverse transcription (Sze et al., 2013) (Figure 1.2). The myxovirus resistance 2 (Mx2) protein inhibits HIV-1 replication by inhibiting nuclear import pathways, or by destabilizing viral DNA within the nucleus (Goujon et al., 2013; Kane et al., 2013; Liu et al., 2013) (Figure 1.2). While the other restriction factors directly interact with viral components (such as genome, capsid, and lipid envelope), SAMHD1 has an indirect antiviral effect by regulating the concentrations of intracellular nucleotide pools.

Antagonism of restriction factors by viral accessory genes

Presumably because they target steps of the viral replication cycle that are immutable, and are hence difficult to escape via simple evasive mutations, the mechanisms employed by restriction factors often provide an impetus for the virus to evolve new strategies to counter the inhibition. In general, these specialized countermeasures involve binding of the restriction factor by a viral protein, followed by its removal from the cell, or sequestration to a subcellular location where it can no longer execute its antiviral activity. During antagonism, viral proteins often exploit the normal cellular degradation or transport machinery. For example, Vif proteins bind to a number of APOBEC3 proteins and induce their polyubiquitination and degradation by recruiting a cellular ubiquitin ligase complex composed of cullin5, elongins B and C, and Rbx2 (Yu et al., 2003). A similar mechanism is employed by SIV Vpr and Vpx proteins to eliminate SAMHD1 from cells, although in this case, Vpr and Vpx are brought into a newly

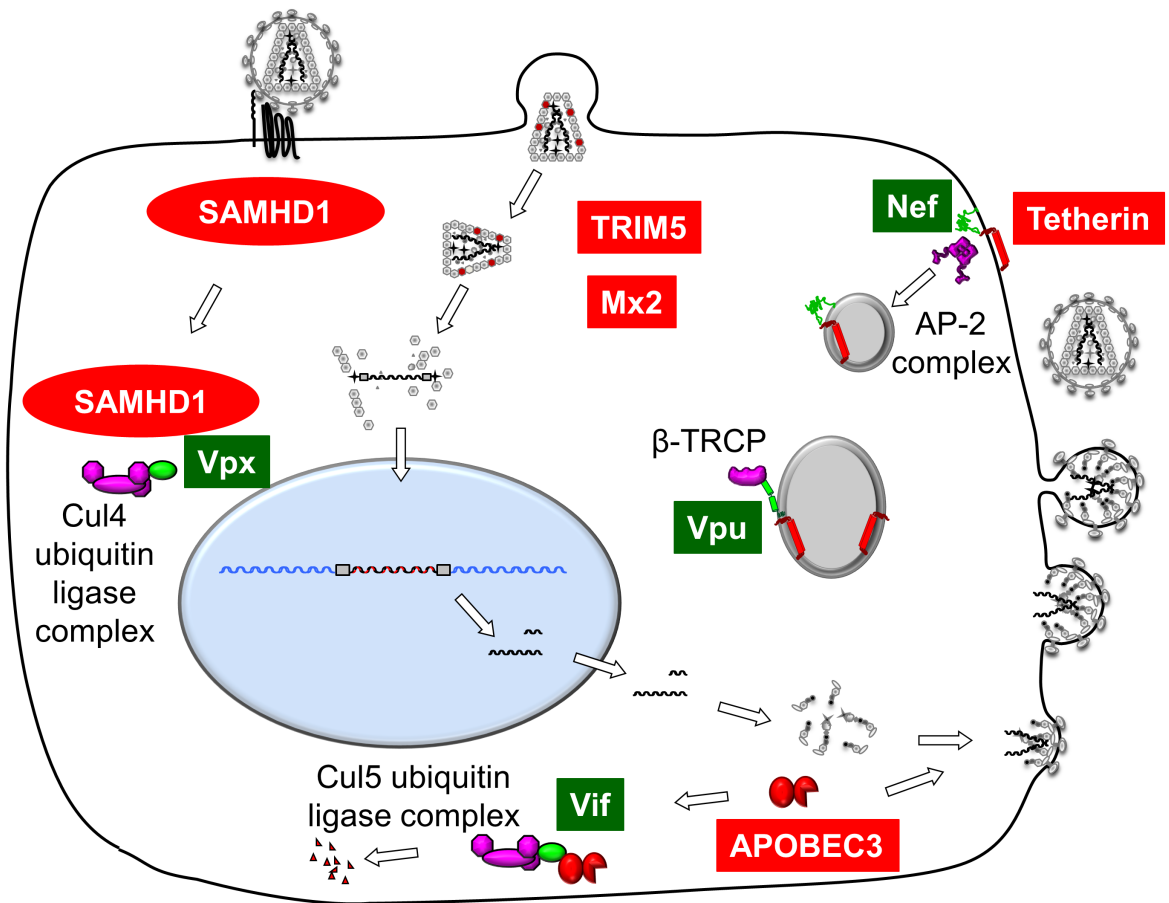


Figure 1.4. Counteraction of restriction factors by viral accessory genes. The mechanisms by which restriction factors are antagonized are depicted. The evasive mutations in the capsid protein that confer resistance to TRIM5 and Mx2 are indicated as brown circles. Restriction factors and their cognate antagonists are indicated in red and green figures respectively. Cellular complexes that are recruited by the antagonists are indicated in black lettering.

infected cell as a component of the incoming virion. They do so by recruiting a cullin4A-based ubiquitin ligase complex through a DDB1–DCAF1 adaptor (Schrofelbauer et al., 2007). Simultaneous Vpr or Vpx binding to SAMHD1 leads to SAMHD1 ubiquitination and degradation by proteasomes (Hrecka et al., 2011; Laguette et al., 2011). Interestingly, three different lentiviral proteins (Vpu, Nef and Env) have evolved the ability to antagonize tetherin. This topic will be dealt with in greater detail in an upcoming section.

Restriction factors have evolved under positive selection

It is likely that antagonistic coevolution of interactions between viruses and their hosts constitute a major driver of evolutionary change in both groups. Restriction factor variants that confer a fitness advantage to an individual are selected by viral infections, and become fixed in the host population. The reduction in viral fitness in the newly adapted host provides the impetus for the selection of viral variants that have mutations or new functions that relieve restriction and restore fitness. Iterative cycles of this genetic conflict constitute a molecular “arms race” and often result in intense Darwinian pressure to explore sequence space at the interaction surfaces of restriction factors and their viral antagonists (Blanco-Melo et al., 2012) (Figure 1.5).

A molecular arms race between protein-coding genes often means that mutations that change the amino acid sequence (non-synonymous mutations) are fixed more frequently than those that do not (synonymous mutations)

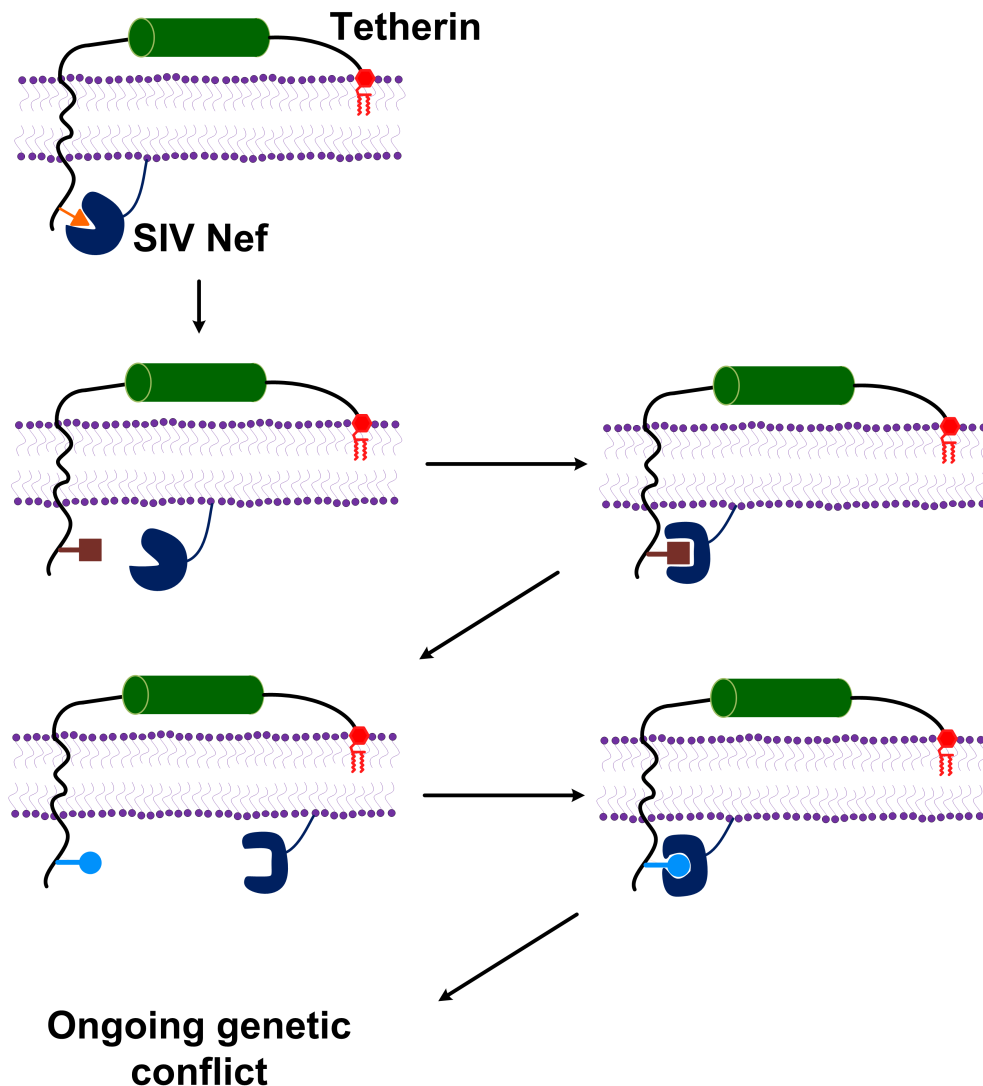


Figure 1.5. Genetic conflict between the tetherin proteins in simian cells and the Nef proteins encoded by SIVs. The tetherin proteins in simian cells are antagonized by the SIV Nef proteins via a motif in the tetherin cytoplasmic tail (indicated as an orange triangle). This selects for tetherin proteins that evade recognition by the Nef proteins via the accumulation of amino acid changes at the interface of molecular recognition (indicated as a brown square and blue circle in subsequent cycles). In turn, this provides the impetus for the selection of viral variants that restore replicative fitness by recognizing the new motif. Thus, iterative cycles of genetic conflict continually shape genomes and functions.

(Daugherty and Malik, 2012). A ratio of non-synonymous substitutions/non-synonymous sites (dN) versus synonymous substitutions/synonymous sites (dS), i.e. dN/dS, greater than 1 indicates that the sequence evolving under positive selection. On the other hand, a dN/dS ratio of less than 1 indicates that natural selection has conserved the ancestral state and that the sequence is evolving under purifying selection. As expected, the majority of human genes have evolved under purifying selection whereas a subset has evolved under positive selection (Meyerson and Sawyer, 2011). Positively selected genes include those involved in sensory perception, likely driven by the requirement to sense environmental changes, food sources, or predators. Predictably, they also include those involved in pathogen defense, including restriction factors. Notably, APOBEC3G and TRIM5 α are among the highest dN/dS ratios of human genes (Sawyer et al., 2004; Sawyer et al., 2005; Song et al., 2005).

Positively selected residues are typically found at molecular recognition interfaces between host and viral proteins that are involved in a long series of genetic conflict. For example, a few amino acids in APOBEC3G that are important for Vif antagonism are known to have evolved under positive selection (Sawyer et al., 2004). Similarly, portions of TRIM5 α that bear the signatures of positive selection are found only in the SPRY domain, which directly binds to the incoming retroviral capsid (Sawyer et al., 2005; Song et al., 2005). Although the overall dN/dS ratio is quite low for tetherin, portions of its cytoplasmic tail and transmembrane domain that are targeted by viral antagonists have evolved under positive selection (Gupta et al., 2009a; McNatt et al., 2009). Similarly,

SAMHD1 sequences that are determinants for Vpx sensitivity also exhibit positive selection. Thus, each of the four classes of restriction factors exhibit signatures of positive selection over portions of their sequence.

Restriction factors are determinants of viral host range

Positive selection causes high interspecies protein sequence variability in restriction factors. For instance, their sequences are unusually divergent across even primate species. Hence, the adaptations that a virus acquires in order to antagonize restriction factor variants in one host species can come at the cost of susceptibility to variants in another species. This diminishes the likelihood of a virus managing to overcome the array of defenses that it confronts, when it gets the opportunity to colonize a new host species. Thus, zoonoses that result in successful cross-species transmissions are probably quite rare events.

Blocks to cross species transmission that are imposed by the prototypical restriction factors, APOBEC3G and TRIM5 proteins appear particularly formidable, perhaps because (i) these two restriction factors are especially potent inhibitors, and (ii) they are constitutively expressed in the natural target cells of primate lentiviruses. HIV-1 and SIV Vif proteins are universally capable of antagonizing APOBEC3G proteins in their natural hosts, but are often impotent when confronted with APOBEC3G proteins from other primates (Bogerd et al., 2004; Mariani et al., 2003; Schrofelbauer et al., 2004; Virgen and Hatzioannou, 2007). Indeed, the inability of many SIV Vif proteins to induce degradation of

human APOBEC3G might explain why many SIVs have been unable to colonize humans (Gaddis et al., 2004). Notably, SIV_{CPZ} Vif and SIV_{SMM} Vif are both active against human APOBEC3G, and both of these lineages have managed successful zoonotic transmissions to humans (as HIV-1 and HIV-2, respectively) (Compton and Emerman, 2013).

Although human TRIM5 α is largely ineffective as an inhibitor of primate lentiviruses, simian variants can be very potent inhibitors, and those found in some species (e.g. African green monkeys) are capable of restricting an impressively diverse array of retroviruses (Hatzioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). Although lentiviruses have not evolved a novel gene specifically to antagonize TRIM5 α , evasion is achieved via the acquisition of amino acid changes in the capsid protein.

Species-specific SAMHD1 degrading activities are also evident among Vpr and Vpx proteins and some Vpx and Vpr appear to have specialized to deal with SAMHD1 variants that are present in the natural host species (Laguet et al., 2012; Lim et al., 2012). However, SAMHD1 may not be a powerful barrier to cross-species transmission, because HIV-1 apparently lacks the ability to antagonize human SAMHD1, yet has spread globally in human populations. Rather, the ability of a particular primate lentivirus to antagonize SAMHD1 may determine the extent to which myeloid cell types are infected within a particular host species.

In contrast, tetherin poses a significant barrier to cross-species transmission of primate lentiviruses. Vpu and Nef proteins of lentiviruses are generally only able to antagonize tetherin from a narrow range of species. For example, most HIV-1 Vpu proteins antagonize human tetherin but are impotent against monkey tetherin proteins (McNatt et al., 2009). Moreover, SIV Nef proteins can antagonize tetherin proteins in their host species, but are incapable of recognizing human tetherin (Jia et al., 2009; Zhang et al., 2009). An intriguing series of adaptations might have been responsible for the successful colonization of humans by HIV-1 (Sauter et al., 2009). Prior to the zoonotic transmission from chimpanzees to humans, SIV_{CPZ} arose through a recombination event between two SIV lineages that are found in simian species that chimpanzees prey upon. Remarkably, one of those SIVs employs Vpu as a tetherin antagonist while the other employs Nef, and thus SIV_{CPZ} became armed with two tetherin antagonists. Adaptation of the recombinant SIV_{CPZ} in chimpanzees resulted in the selection of Nef as its tetherin antagonist and the loss of tetherin antagonist function by Vpu. However, upon transmission to humans, SIV_{CPZ} encountered a tetherin variant that was insensitive to its Nef protein (due to the deletion of a G/D₁₄DIWK₁₈ motif in the cytoplasmic tail of tetherin) and, as a consequence, had to devise an alternative route to nullify tetherin. Thus, Vpu rapidly adapted to recover its lost function (Sauter et al., 2009). These instances underscore the role of tetherin in shaping lentiviral evolution.

Evolutionary origins of restriction factors

Notwithstanding the remarkable advances in the study of restriction factors, we still know very little about their emergence and evolutionary history. Do they arise *de novo* as completely new genes, or do they emerge via fairly minor adaptations of pre-existing cellular genes whose normal function includes the intrinsic potential to inhibit a certain stage of the viral replication cycle? In general, three mechanisms whereby new genes are generated include non-homologous recombination, retrotransposition and gene duplication (Long, 2001). Exons from different genes might form new genes by illegitimate recombination and acquire new functions. Retrotransposition occurs when a message RNA (mRNA) is reverse transcribed to complementary DNA (cDNA) entirely bereft of introns, and then inserted into the genome. Gene duplication is considered an important mechanism for innovation of novel functions. A duplicated daughter gene might have three possible evolutionary fates: (i) it might become a pseudogene due to random mutations incurred via drift, (ii) it might share the functional workload of the ancestral gene, and, (iii) because it is a redundant copy, it might be released from functional constraints, and is thus well-suited to evolve rapidly and acquire new functions.

In general, restriction factors are members of gene families that have undergone dramatic expansions via gene duplication. The emergence of the APOBEC3 family of proteins, which hypermutate viral genomes, likely occurred via sequential gene duplication events from cytidine deaminases that have important roles in somatic hypermutation, class switch recombination and RNA editing (AID

and APOBEC1). Because AID is found in fish, it is suspected that an ancestral AID gene might have duplicated and diverged to produce APOBEC1 (Refsland and Harris, 2013). Thereafter, the APOBEC3 family of proteins might have arisen via duplications of the ancestral AID/APOBEC1 gene locus. Similarly, one could envisage a scenario wherein SAMHD1 arose via gene duplication of one of the metalloenzyme with phosphohydrolytic activity. In this instance, the metabolic function of SAMHD1 (i.e. regulation of cellular deoxynucleotide pools) could have been conveniently harnessed by cells to inhibit the reverse transcription step in the retroviral life cycle. It is unclear exactly how TRIM5 emerged, but it likely evolved by duplication and divergence of one of the members of the ~100 TRIM proteins. Although most of these do not possess intrinsic antiretroviral activity, TRIM1 and TRIM34 exhibit weak antiretroviral activity when overexpressed (Zhang et al., 2006). Remarkably, independent retrotransposition events in Owl monkey and macaque TRIM5 genes have resulted in the replacement of the C-terminal SPRY domain with a complementary DNA sequence encoding cyclophilin A. The antiviral activity of these novel “TrimCyp” proteins against HIV-1 is dependent on their ability to recognize and bind the capsid protein (Sayah et al., 2004; Virgen et al., 2008). Interestingly, humans only contain a single TRIM5 copy, whereas the genomes of cows, rats, and mice contain tandemly clustered arrays of TRIM5 genes, although not all variants have been reported to possess antiviral activity (Fletcher and Towers, 2013). It is difficult to imagine how such TRIM5 arrays, and the few dozen TRIM proteins might have evolved without gene duplication.

One intriguing question about restriction factors is whether they arose purely as antiviral genes. Put another way, do they perform cellular functions that are unrelated to their antiviral activity? A few pieces of evidence are consistent with the notion that restriction factors might have evolved primarily to inhibit the replication of viruses. First, restriction factors are either constitutively active (APOBEC3G and TRIM5) or are strongly upregulated by interferons (tetherin) (Malim and Bieniasz, 2012). Second, each restriction factor typically inhibits the replication of viruses by either targeting a specific component of the virion (genome, capsid, and envelope) or by interfering with a process that is critical to the viral life cycle (reverse transcription, nuclear import, assembly) (Blanco-Melo et al., 2012). Third, viruses evolve novel countermeasures to antagonize restriction factors in their host species, although this renders them susceptible to variants in other species. Fourth, restriction factors have evolved under positive selection and harbor a high number of polymorphisms even within species (Johnson, 2013). Each variant was probably shaped independently by antagonistic interactions with past viruses. Fifth, the genes encoding restriction factors are dispensable for host viability. For instance, mice harboring deleterious lesions in APOBEC3 and tetherin have no obvious developmental abnormalities.

Tetherin- a restriction factor against enveloped viruses

History and discovery of tetherin

Investigations that aimed to elucidate the role of the HIV-1 protein Vpu routinely observed that it was required, in a cell type-specific manner, for the efficient release of progeny virions from the surfaces of infected CD4⁺ T cells and macrophages (Gottlinger et al., 1993; Klimkait et al., 1990). Cell types such as HeLa, Jurkat T and Hep-2 were observed to be “non-permissive” for the replication of Vpu-deficient HIV-1 whilst HOS, 293T, HT1080, COS and CV-1 cells were deemed “permissive” (Gottlinger et al., 1993; Neil et al., 2006; Neil et al., 2007). Fusion experiments between the permissive COS cells and non-permissive HeLa cells generated heterokaryons that also did not support the release of Vpu-deficient HIV-1 virions (Varthakavi et al., 2003). As explained earlier, such a result hinted at the presence of a dominant restriction factor in non-permissive cells that was absent in permissive cells but retained activity in heterokaryons (Varthakavi et al., 2003). Interestingly, the expression of Vpu *in trans* efficiently released virions from these heterokaryons, indicating that it probably reversed the inhibitory effect by antagonizing the putative restriction factor (Varthakavi et al., 2003). Strikingly, the requirement for Vpu was induced in interferon- α (IFN- α) treated HT1080 and 293T cells, suggesting that perhaps this restriction factor was mobilized as part of a global antiviral response, even in permissive cells (Neil et al., 2006; Neil et al., 2007). These studies laid the foundation for the identification of tetherin (also known as BST-2, CD317, or HM1.24) as a HIV-1 restriction factor that is the cellular target of Vpu. A list of

candidate genes were pruned by comparing gene expression profiles between (i) HT1080, 293T and Jurkat T cells that were either untreated or treated with IFN- α , and (ii) non-permissive HeLa cells and permissive HOS cells (Neil et al., 2008). Tetherin emerged as a leading candidate because (i) it was a membrane protein, (ii) it was strongly induced by IFN- α treatment in HT1080, 293T and Jurkat T cells, and (iii) it was expressed in HeLa cells, but was absent in HOS cells (Neil et al., 2008).

Structural features of tetherin

Tetherin is a type II membrane protein whose ectopic expression causes the physical entrapment of nascent mature enveloped virions at the cell surface (Evans et al., 2010; Hammonds et al., 2012; Neil et al., 2008; Swiecki et al., 2012; Van Damme et al., 2008). Structurally, it comprises of a short N-terminal cytosolic tail, a single pass transmembrane helix, an extracellular domain that is predominantly an alpha helical coiled-coil (Hinz et al., 2010; Schubert et al., 2010; Swiecki et al., 2011; Yang et al., 2010), and a C-terminal glycosylphosphatidylinositol (GPI) moiety that is a second membrane anchor (Andrew et al., 2009; Kupzig et al., 2003; Ohtomo et al., 1999) (Figure 1.6). Three invariant cysteine residues in its ectodomain (C53, C63, and C91) stabilize parallel homodimer formation via disulphide bridges (Figure 1.6). Also, tetherin is glycosylated at two asparagine residues, which is responsible for its proper

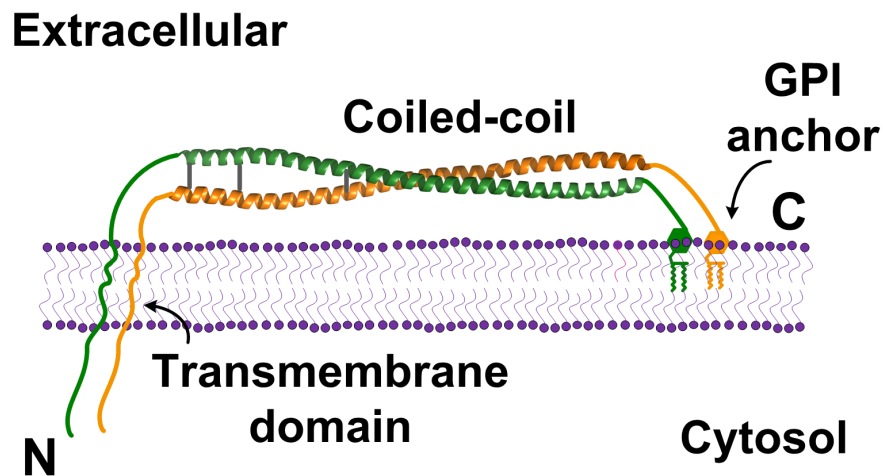


Figure 1.6. Structural features of tetherin. The tetherin molecule comprises of a short N-terminal cytosolic tail, a single pass transmembrane helix, an extracellular domain that is predominantly coiled-coil. It is also modified at its C-terminus with a glycosylphosphatidylinositol (GPI) moiety. Three extracellular cysteine residues drive parallel homodimer formation via disulphide bridges (indicated as grey lines).

folding and anterograde transport to the plasma membrane (Perez-Caballero et al., 2009).

Crystals structures have revealed that the ectodomain is essentially a ~170Å rod-like parallel homodimer, with its two N-termini ends splayed apart (Hinz et al., 2010; Schubert et al., 2010; Swiecki et al., 2011; Yang et al., 2010). The coiled-coil domain is surprisingly quite flexible and is capable of rotating around two pivotal residues. Up to ~25° rotation is permitted at A88 and up to ~18° rotation is permitted at G109 due to packing defects of the coiled-coil domain (Yang et al., 2010). Perhaps the conformational flexibility of the coiled-coil around these two hinge points preserves dimeric integrity when dramatic changes in membrane curvature occur during virion assembly (Swiecki et al., 2011; Yang et al., 2010).

Under reducing conditions, the tetherin ectodomain undergoes tetramerization through a 4-helix bundle interaction (Schubert et al., 2010; Yang et al., 2010). Although its biological relevance is yet to be determined, two lines of evidence suggest that the formation of tetramers is not essential for the antiviral activity of tetherin. First, C53/C53 and C63/C63 disulphide bridges cannot form in the tetramer because they are far too spatially separated (Yang et al., 2010); yet, all three cysteine residues form disulphide bridges in tetherin dimers in cells (Andrew et al., 2009; Perez-Caballero et al., 2009) and are essential for optimal virion retention (Perez-Caballero et al., 2009). Second, a tetherin variant which targets amino acids that are critical for tetramer formation, is only modestly

impaired in its function, confirming that the tetrameric form of tetherin is dispensable for tethering (Yang et al., 2010).

Cellular localization and distribution of tetherin in tissues

Tetherin was originally cloned from a bone marrow stromal cell line (hence the name bone marrow stromal cell antigen 2, or BST-2) derived from a rheumatoid arthritis patient that could facilitate stromal cell-dependent growth of a pre-B-cell line (Ishikawa et al., 1995). It has also been described as a tumor antigen that is expressed on the surface of human multiple myeloma cells (Ohtomo et al., 1999), and on plasmacytoid dendritic cells (Blasius et al., 2006). An early survey of tetherin expression indicated that its transcript is well-expressed in liver, lung, placenta, and heart (Ishikawa et al., 1995). However, a comprehensive evaluation of tetherin expression *in vivo* showed that it is expressed in a wider range of tissues that included spleen, gallbladder, pancreas, kidney, liver, the GI tract and blood vessels (Erikson et al., 2011).

Analyses of an ~2-kb DNA fragment upstream to the transcription start site (TSS) of the tetherin gene have revealed the presence of *cis*-regulatory elements such as interferon stimulated response elements (ISREs) and γ -activated site (GAS), as well as several potential binding sites interspersed in the region, for the signal transducer and activator of transcription (STAT), interferon regulatory factors (IRFs), NF- κ B and NF-AT (Bego et al., 2012; Ohtomo et al., 1999). Notably, a cluster of sequences proximal to the TSS (~150 bp upstream), is required for

induction by IFN- α (Bego et al., 2012). This “interferon response cluster” comprises of an ISRE, a GAS as well as binding sites for IRFs. Interestingly, tetherin expression can also be induced in the absence of type I IFN signaling by IRFs 1, 3, and 7, suggesting that it might be directly activated by viral infection, independently of IFN signaling (Bego et al., 2012). This view is reinforced by the stimulation of tetherin expression upon treatment with agonists of TLR3 and TLR8 and prior to any detectable interferon signaling (Bego et al., 2012).

In cells, tetherin resides at the cell surface and is recycled back to the early endosomes and trans-Golgi network (TGN) via the endocytic pathway (Rollason et al., 2007). Interestingly, its GPI anchor is believed to facilitate its free diffusion to cholesterol- and sphingolipid-rich microdomains at the plasma membrane (Kupzig et al., 2003). It is thought that tetherin molecules oriented such that their GPI anchors are within microdomains might serve as boundaries of such structures (Billcliff et al., 2013). A role for integral membrane proteins in the stabilization of microdomains by mediating interactions with the underlying actin cytoskeleton has been hypothesized by Sheetz (Sheetz, 1983). Indeed, the cytoplasmic tail of tetherin has been shown to interact with the actin cytoskeleton via RICH2 (Rollason et al., 2009), but this interaction is dispensable for antiviral activity. Thus, the ability of tetherin to trap virions might simply be a consequence of its preferential localization to cholesterol-rich microdomains, the site of HIV-1 assembly (Nguyen and Hildreth, 2000). A similar argument can be invoked to explain why tetherin specifically traps virions and not exosomes. The latter are vesicles that are released from the cell when multivesicular bodies fuse with the

plasma membrane (Denzer et al., 2000; Raposo et al., 1996; Thery et al., 2002). Because they might bud non-specifically, rather than from cholesterol-rich microdomains, they probably do not encounter tetherin molecules during budding.

Mechanism of antiviral activity of tetherin

Tetherin inhibits the release of nascent enveloped virions by physically crosslinking them to the plasma membrane, but the molecular mechanisms that underpin the process of virion tethering have been difficult to determine. Accumulating evidence seems to indicate that tetherin acts directly and autonomously to trap virions. First, trapped virions can be liberated from the cell surface by treatment with the protease subtilisin A, indicating that the tether itself is composed of protein (Neil et al., 2007). In such experiments, tetherin fragments can be found in subtilisin-liberated virions (Perez-Caballero et al., 2009). Second, both membrane anchors are essential for antiviral activity, and inactive tetherin proteins that lack either of the two membrane anchors, are efficiently incorporated into virions (Perez-Caballero et al., 2009). Third, fluorescent and electron microscopic analyses demonstrate that tetherin is localized at sites of virion entrapment (Fitzpatrick et al., 2010; Hammonds et al., 2010; Lehmann et al., 2011; Perez-Caballero et al., 2009). Fourth, an artificial tetherin protein assembled from heterologous protein domains that have similar configuration but no primary sequence homology to tetherin, recapitulates tetherin function (Perez-Caballero et al., 2009). Taken together, these findings

suggest that (i) the biological activity of tetherin can be ascribed to its overall configuration rather than its primary sequence and (ii) tetherin does not require specific cofactors or the recognition of specific viral components to cause virion entrapment. These findings are difficult to reconcile with complex models in which tetherin might act as a virion sensor to induce other factors that have tethering activity. Rather, they are more easily explained by the idea that tetherin acts autonomously and directly to trap virions, simply as a consequence of being incorporated into the lipid envelope of virions as they bud through cell membranes. Consistent with these arguments, tetherin exhibits antiviral activity against a broad spectrum of enveloped virions whose proteins have essentially no sequence homology (Dietrich et al., 2011; Jouvenet et al., 2009a; Mansouri et al., 2009; Radoshitzky et al., 2010; Sakuma et al., 2009; Watanabe et al., 2011; Weidner et al., 2010; Yondola et al., 2011).

Because a protected, β -mercaptoethanol-sensitive, dimeric amino-terminal tetherin fragment can be recovered from virions that have been liberated by protease treatment, it appears that at least some trapped virions are infiltrated by both N-termini of a parallel tetherin homodimer (Perez-Caballero et al., 2009). Moreover, a tetherin variant that lacks a GPI anchor preferentially localizes to sites of viral budding, suggesting that the tetherin N-terminus provides the dominant driving force for infiltration into budding virions (Perez-Caballero et al., 2009). These results have also been supported by other studies involving super-resolution microscopy (Lehmann et al., 2011). Nevertheless, it remains a tantalizing challenge to establish unequivocally that tetherin is directly

responsible in trapping virions, and to determine the precise configurations that might be adopted by tetherin homodimers as they engage in virion entrapment.

Upon virion entrapment, the tetherin-virus complexes accumulate in early and late endosomes. The internalization of virions is dependent on endocytosis, because dominant-negative forms of dynamin (K44A) and Rab5a (S34N) that inhibit this process, abolish the intracellular accumulation of virions (Neil et al., 2006). Virion uptake might also be facilitated by interactions with BCA2, a Rab7-interacting protein with E3 ubiquitin ligase activity (Miyakawa et al., 2009). Ultimately, virions infiltrated by tetherin are either sequestered or targeted for degradation in lysosomes (Neil et al., 2006).

Antagonism of tetherin by viral proteins

One argument in favor of the notion that tetherin acts quite nonspecifically to trap enveloped virions arises from the mechanisms that viruses have evolved to evade the action of tetherin. Rather than acquiring viral protein sequence changes that might enable escape from interaction with tetherin, viruses have instead adapted novel genes to gain interaction with, and thereby antagonize, tetherin. For instance, HIV-1 has evolved an accessory protein Vpu to overcome the antiviral effects of tetherin (Neil et al., 2008). Vpu is a type I integral membrane protein that comprises an N-terminal transmembrane domain followed by a cytoplasmic tail that consists of two α -helices separated by an acidic flexible loop that contains a conserved casein kinase II phosphorylation motif

(S/T₅₂xxD/ES₅₆) (Cohen et al., 1988; Strebel et al., 1988). Domain replacement experiments have shown that the transmembrane domain of tetherin carries critical determinants of sensitivity or resistance to Vpu (McNatt et al., 2009). Strikingly, two mutations (Δ GI/T₄₅I) in the transmembrane domain that mimic tetherin proteins in rhesus macaques and African green monkeys, render human tetherin completely insensitive to Vpu (McNatt et al., 2009). Recent crosslinking studies have unequivocally demonstrated that Vpu and tetherin interact directly via their transmembrane domains (McNatt et al., 2013).

Phosphorylation of the two serine residues in the S/T₅₂xxD/ES₅₆ motif (Schubert et al., 1994) governs the recruitment of the adaptor protein β -TrCP and its associated Skp1/Cullin/F-Box (SCF) E3 ubiquitin ligase complex (Margottin et al., 1998). Vpu physically associates with tetherin via their transmembrane domains (Dube et al., 2009; Iwabu et al., 2009; Kobayashi et al., 2011; McNatt et al., 2013; McNatt et al., 2009; Vigan and Neil, 2010), and employs surface downregulation (Dube et al., 2009; Hauser et al., 2010; Schindler et al., 2010; Schmidt et al., 2011; Skasko et al., 2011; Van Damme et al., 2008) and degradation (Douglas et al., 2009; Goffinet et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009) to antagonize tetherin.

Notably, a mutant of Vpu that harbors serine-to-alanine mutations is impaired at carrying out both activities, but still retains a modest ability to antagonize tetherin at the cell surface (Douglas et al., 2009; Iwabu et al., 2009; Schindler et al., 2010). It does so by merely binding and displacing tetherin from sites of viral assembly (McNatt et al., 2013). In this regard, an E₆₃xxxLV₆₈ motif in the Vpu

cytoplasmic tail is essential for downregulation and degradation of tetherin, as well as its displacement from sites of virion assembly (Kueck and Neil, 2012; McNatt et al., 2013).

A number of reports have demonstrated that Vpu alters the anterograde transport of de novo synthesized tetherin molecules or those that are recycling between the trans-Golgi network (TGN) and the plasma membrane (Dube et al., 2010; Dube et al., 2009; Kueck and Neil, 2012; Neil, 2013). By stationing itself in the TGN, Vpu intercepts tetherin molecules en route to the plasma membrane and diverts them to the endosomal compartments via clathrin-dependent transport (Lau et al., 2011). Ubiquitination of tetherin at multiple residues in its cytoplasmic tail, and in particular a S₃TS₅ motif, targets it for degradation in late endosomes (Skasko et al., 2011). Vpu-dependent recruitment of the ESCRT-0 subunit HRS, which binds ubiquitinated molecules, enhances the sorting of tetherin-Vpu complexes into the multivesicular bodies of late endosomes (Janvier et al., 2011). Thus, this redirection of Vpu-tetherin complexes to the late endosomes prevents them from recycling to the cell surface, and most importantly, ensures that tetherin can no longer be salvaged for the purpose of virion tethering.

Most viruses that do not encode Vpu exploit their Nef proteins to antagonize tetherin (Jia et al., 2009; Lim et al., 2010; Sauter et al., 2009; Zhang et al., 2011; Zhang et al., 2009). Nef is a multifunctional viral protein that regulates the levels of a variety of cell surface proteins such as CD4 (Kirchhoff et al., 2008). The mechanism of antagonism involves a direct physical interaction between Nef and tetherin (Serra-Moreno et al., 2013), and as mentioned earlier, the binding

determinants map to a G/D₁₄DIWK₁₈ motif that is present in the cytoplasmic tails of simian tetherin proteins (Jia et al., 2009; Zhang et al., 2009). Nef induces the internalization of tetherin by binding to AP-2 clathrin adaptor complexes (Zhang et al., 2011). Remarkably, human tetherin lacks this motif, which imposed a strong selection pressure on HIV-1 and HIV-2 to adapt their Vpu and Env genes for tetherin antagonism, after zoonoses from chimpanzees and sooty mangabeys respectively.

Other viruses employ their Env proteins to antagonize tetherin. The HIV-2 Env protein has evolved to antagonize tetherin by promoting its internalization and accumulation in the TGN (Gupta et al., 2009b; Hauser et al., 2010; Le Tortorec and Neil, 2009). The residues that are specifically involved are not well defined and are situated in the ectodomains of both proteins. Antagonism is achieved by clathrin-mediated endocytosis, via a conserved endocytic motif in the cytoplasmic tails of gp41 proteins. Additionally, the SIV_{MAC} Env (Serra-Moreno et al., 2011) and Ebola glycoprotein (Kaletsky et al., 2009; Lopez et al., 2010) proteins have adapted to antagonize tetherin proteins in their hosts by targeting different portions of the cytoplasmic tail or the ectodomain respectively.

The diverse ways in which viruses have adapted to antagonize tetherin emphasize the incredible plasticity of their genomes and their ability to innovate in response to selective pressures. It is difficult to speculate as to why three different viral proteins have been deployed to antagonize tetherin. One possible reason could be that tetherin is the only restriction factor that resides in three different compartments, i.e. in the cytosol, plasma membrane and in the

extracellular region, and is thus susceptible to the viral proteins that also localize to these compartments. Second, during a zoonotic transmission, it is possible that a virus might confront a tetherin protein that cannot be easily overcome by its present antagonist. In such an instance, it might be less challenging for any of the other two accessory genes to evolve or regain this function. Thus, it is clear that tetherin has a role in shaping viral evolution.

Role of tetherin in various modes of transmission

It is believed that viruses disseminate within hosts via two distinct modes of transmission between cells either by freely diffusing particles that have been released into the cell-free supernatant (i.e. cell-free transmission), or via direct contact between cells (i.e. cell-to-cell transmission). Clearly, cell-free transmission is unencumbered by the need of intimate cell-to-cell contact and is thus useful in establishing long-range infections within and between hosts. However, in certain scenarios, cell-free transmission might be rather inefficient for the spreading of virus. For instance, virions released into the extracellular milieu might be labile or might be subjected to the action of neutralizing antibodies. In such instances, viral spread to neighboring cells via cell-to-cell transmission could be imagined to be far more efficient.

Cell-to-cell transmission involves the concerted action of adhesion molecules, cytoskeletal rearrangements, and polarization of receptor molecules to promote the transfer of virions across a “virological synapse” (VS) (Chen et al., 2007; Jolly

et al., 2004; Sattentau, 2008). These structures are formed due to interactions between the viral envelope on the infected (donor) cell and the receptors on the target (acceptor) cell, and are very reminiscent of immunological synapses that are bridged by interactions between ICAM-1 and LFA-1 (Jolly et al., 2004; Jolly et al., 2007; Rudnicka et al., 2009). Viral particles can be transferred across long filopodial membrane bridges that connect donor and target cells (Helenius et al., 1980; Hubner et al., 2009; Sherer et al., 2007; Smith and Helenius, 2004). However, unlike neural and immunological synapses, the existence and biological relevance of virological synapses has not been convincingly demonstrated *in vivo*. In fact, an argument that is oftentimes made is that cell-to-cell transmission is actually cell-free transmission over extremely short distances, and that the greater efficiency of the latter mode might reflect the high multiplicities of infection that are achieved due to the increased availability of released virions into a confined area.

Thus far, the majority of investigations on tetherin have focused on its role in inhibiting cell-free transmission, but a few studies have attempted to delineate its impact on cell-to-cell transmission. It has been reported that when HIV-1 is selected to disseminate rapidly in culture, i.e. in the time frame wherein cell-to-cell transmission would be more efficient, lesions in *vpu* are common (Gummuluru et al., 2000). It should be noted, however, that cells were frequently washed during this study, and such an artificial system is not physiologically relevant. After the discovery of tetherin, a number of studies examined its role in modulating cell-to-cell transmission and came to opposing conclusions. Two of

these studies suggested that tetherin inhibits cell-to-cell transmission (Casartelli et al., 2010; Kuhl et al., 2010), while the third indicated that tetherin plays a supporting role (Jolly et al., 2010). These discrepancies could be attributed simply to the different surface expression levels of tetherin in the cell lines used in these investigations. In low tetherin-expressing Jurkat T cells, it is possible that a small amount of virions are released and can productively infect bystander cells (Jolly et al., 2010). On the other hand, in transiently transfected 293T cells, wherein tetherin is expressed at amounts far greater than endogenous levels, hardly any virions are released to infect neighboring cells (Casartelli et al., 2010). Thus, it is difficult to conclude whether tetherin aids or inhibits cell-to-cell transmission, due to the conflicting nature of these studies. Furthermore, the relative importance of cell-to-cell transmission is yet to be firmly established in vivo.

Various approaches such as the transwell coculture system and agar overlay assays might be employed to assess the contribution of tetherin to cell-to-cell transmission. In the transwell coculture system, viral spread would be expected to occur via cell-free rather than cell-to-cell transmission. In contrast, viral spread would be expected to occur predominantly via cell-to-cell rather than cell-free transmission in the agar overlay assays. Thus, the role for tetherin could be analyzed by comparing the ability of Vpu-deficient virus to establish spreading infections in tetherin-deficient versus tetherin-expressing cells. In the transwell coculture system, a Vpu-deficient virus would be expected to establish a spreading infection more efficiently in tetherin-deficient cells compared to

tetherin-expressing cells. This is intuitive, given that transmission occurs solely via the cell-free mode. In contrast, if tetherin aids cell-to-cell transmission, then spreading infections would be more efficient in tetherin-expressing cells rather than the tetherin-deficient cells, and this could be analyzed using the agar overlay assays.

The biological relevance of tetherin in vivo

Accruing evidence supports an important role for tetherin in limiting viral burden and dissemination *in vivo*. First, the ability of the Vpu proteins of the HIV-1 group M strains to efficiently antagonize human tetherin as well as to degrade CD4 seems to have contributed to the success of this lineage in colonizing human populations as compared to the HIV-1 group N, O and P strains, whose Vpu proteins are either severely impaired or impotent against tetherin (Sauter et al., 2009). Second, an inspection of natural Vpu alleles found in infected individuals at different stages of disease found that although there was considerable T-cell driven variation in the Vpu proteins, only a minority of these alleles were defective for antagonism of tetherin (Pickering et al., 2014). The strict maintenance of this function at all stages of infection indicates a selection pressure imposed by tetherin. Third, a tetherin allele with an insertion polymorphism in its promoter that results in lower surface levels of protein expression correlates with rapid disease progression in HIV-1 patients (Laplana et al., 2013). Fourth, a Nef-deficient of SIV that is incapable of tetherin

antagonism reverts to a pathogenic phenotype after serial passages in rhesus macaques (Serra-Moreno et al., 2011). Importantly, the determinants of resistance to rhesus tetherin map to the cytoplasmic tail of gp41. Antagonism of rhesus tetherin is achieved via internalization and sequestration, in a manner reminiscent of how human tetherin is antagonized by HIV-2 Env. Fifth, two mutations confer HIV-1 Nef with the ability to antagonize chimpanzee tetherin after a single passage in chimpanzees (Gotz et al., 2012).

Investigations in mice have also documented a role for tetherin *in vivo*. In one study with Tetherin deficient mice, it was shown that Tetherin inhibits Moloney MLV and a pathogenic MLV known as LP-BM5 *in vivo* (Liberatore and Bieniasz, 2011). Because Moloney MLV replicates without inducing a type I IFN response, stimulation with poly (I:C) was required to induce tetherin expression and attenuate viral replication in WT mice compared to tetherin-deficient littermates. In contrast, LP-BM5 induces immune activation, polyclonal B-cell proliferation, and impaired immune responses very reminiscent of HIV/AIDS. Importantly, it induces type I IFN in infected tissues ~8 weeks after replication and this led to the attenuation of LP-BM5 plasma viremia and pathogenesis in wild type mice as compared to the tetherin-deficient mice.

In another study that correlated genotype, phenotype and virus replication *in vivo*, a single nucleotide polymorphism (SNP) was identified in the tetherin gene of NZW mice wherein the start codon was mutated, resulting in the expression of a shorter isoform of tetherin (Barrett et al., 2012). Because its cytosolic tail is truncated and lacks the YxY endocytosis motif, the variant is retained at higher

levels at the surfaces of splenocytes, dendritic cells, B cells and erythroblasts, leading to diminished levels of infectious plasma viremia and viral loads. Thus, tetherin can control virus replication in vivo.

More recent work using a humanized mouse model of acute R5-tropic HIV-1 infection provides clear evidence that tetherin antagonism by Vpu is critical for viral dissemination (Dave et al., 2013). Mice infected with a Vpu-deficient HIV-1 showed attenuated viral loads and lower numbers of infected T cells in splenocytes. It is interesting to note that mice infected with HIV-1 Vpu carrying mutations in the β -TrCP binding domain had intermediate viral loads as compared to mice that were infected with WT or Vpu-deficient viruses, supporting the view that down regulation of tetherin from the cell surface is only one of the myriad ways that Vpu counteracts tetherin.

Auxiliary functions of tetherin

The primary function of tetherin is to physically inhibit the release of enveloped virions from the cell surface, but it might also have auxiliary functions that augment its antiviral activity. In fact, prior to its identification as an antiretroviral restriction factor, tetherin was identified as an activator of NF- κ B in a whole-genome cDNA screen (Matsuda et al., 2003). Thus, analogous to the Toll-like receptors (TLRs) that act as pattern recognition molecules (Iwasaki, 2012; Medzhitov and Janeway, 2000), tetherin might initiate proinflammatory signaling cascades upon directly engaging the virus. In support of this view, three studies

have recently revealed a signaling function of tetherin that is genetically separable from, and downstream to, its antiviral activity (Cocka and Bates, 2012; Galao et al., 2012; Tokarev et al., 2012).

The accumulation of tetherin-virus complexes in late endosomal compartments might be expected to increase the visibility of virion components to TLRs in intracellular compartments and activate NF- κ B signaling via the IRF pathway. However, the emerging view is that tetherin triggers the expression of proinflammatory cytokines upon trapping viral particles at the cell surface (Galao et al., 2012). Mutations in its N-terminal YDY endocytosis motif and a proximal CRV motif abolishes NF- κ B signaling (Galao et al., 2012; Tokarev et al., 2012). Accumulation of trapped virions at the cell surface leads to the YDYCRV-dependent recruitment of the E3 ubiquitin ligase TRAF2/6 and the E2 ubiquitin conjugating enzyme Ubc13, which activate the TAK1 complex (Galao et al., 2012; Tokarev et al., 2012). This, in turn, induces phosphorylation and proteasomal degradation of I κ B via IKK that results in the activation of NF- κ B signaling.

In another study, it was demonstrated that two isoforms of tetherin, derived by translation initiation from different methionine residues, are produced in both cultured human cell lines and primary cells (Cocka and Bates, 2012). Notably, the shorter isoform lacks the N-terminal YDYCRV motif, and fails to induce NF- κ B activation. It is also more resistant to antagonism by Vpu and is thus more effective at trapping virions. However, the physiologic relevance of the shorter

isoform of tetherin *in vivo*, if any, is unclear because Vpu is a potent tetherin antagonist even in primary cells.

Thus, the attributes of tetherin that endow it with the ability to act as a virion sensor remain to be determined. It has been suggested that tetherin facilitates the organization of lipid rafts or microdomains that might act as scaffolds for NF- κ B signaling (Billcliff et al., 2013). Indeed, the mere clustering of tetherin molecules is sufficient to trigger NF- κ B signaling, albeit not as potently (Galao et al., 2012).

Virion retention at the cell surface might inadvertently have other consequences, i.e. it could potentiate antibody-dependent cellular cytotoxicity (ADCC), a humoral immune response mediated by natural killer cells (Ohtomo et al., 1999). The clustering of tetherin molecules might increase the visibility of viral antigens, leading to their opsonization and engagement by effector cells. ADCC ultimately results in the killing of HIV-1 infected cells via cytolytic granules. In support of this idea, tetherin-expressing cells infected with Vpu-deficient HIV-1 tend to be more susceptible to ADCC *in vitro* (David Evans, unpublished results). Thus, the antagonism of tetherin might be useful in shielding infected cells from ADCC.

Chapter II Materials and methods

Plasmid construction

All tetherin and PV1 proteins were transiently expressed using pCR3.1 (Invitrogen) based plasmids or stably expressed using pLHCX (Clontech) based retroviral vectors. A human tetherin protein internally tagged with an HA epitope at amino acid 155 and, expressed using pCR3.1 or LHCX vectors, has been described previously (Neil et al., 2008). Eight copies of a peptide linker sequence, each comprising the amino acid sequence GGGGS, were inserted immediately C-terminal to the HA tag, to generate the C8 modified tetherin protein (Figure 1D). Similarly, five GGGGS linker units were inserted immediately C-terminal to the tetherin transmembrane domain at amino acid position 50, to generate the N5 modified tetherin protein. Because the BamHI recognition site (GGATCC) encodes a glycine and serine, we incorporated its sequence into the fourth and third linker units for the C8 and N5 proteins respectively. We then used these BamHI sites for the subsequent insertion of a Factor Xa cleavage site (IEGR) to generate the C8Fac and N5Fac proteins (Figure 1D). Thereafter the Flag N5Fac protein was generated by inserting three copies of a FLAG epitope tag at the N-terminus of the N5Fac protein (Figure 1D).

The protein standard used for quantitative western blotting was generated by appending the C-terminus of HIV-1 p24 CA protein with three FLAG epitope tags and an HA epitope tag. Specifically, the p24 CA coding sequence was amplified from the proviral plasmid pNL4-3 using oligonucleotides that encoded the epitope

tags, and inserted as an EcoRI-NotI fragment into the multiple-cloning site of pCRV-1, a previously described hybrid expression vector that is derived from pCR3.1 and from a highly modified HIV-1 provirus (V1B).

All mutagenesis was accomplished by using overlap-extension PCR. A Vpu-deficient version of pCRV-1 was generated by substituting the start codon of the Vpu gene with a BamHI site. The chimeric Gag protein ZWT-p6 construct was a kind gift from Heinrich Göttlinger (University of Massachusetts Medical School, MA). The ZWT- Δ p6 construct was generated by using PCR primers directed against the corresponding sequence. The three Gag constructs: ZWT-p6, its p6-ZWT- Δ p6 and NL4-3 Gag were amplified and inserted as EcoRI-NotI fragments into pCRV-1 Δ Vpu.

The panel of cysteine crosslinkable mutants (E51C, A52C, R54C, D55C, G56C, R58C, A59C, M61C, E62C, Q142C, V143C, S145C, V146C, R147C, A149C, D150C, K152C, Y153C and Y154C) was generated by using PCR primers directed to the corresponding sequences in the C8Fac protein.

To generate the chimeric tetherin proteins (such as Tropo), an intermediate construct was created by introducing NheI sites at codons 100 and 154. The coiled-coil sequences from tropomyosin, golgin-84, CASP, golgin-97, TATA modulatory factor 1, and early endosome antigen 1 were amplified from complementary DNAs (cDNAs) prepared from 293T and THP-1 cells and inserted as SpeI-NheI fragments into the previous construct. The reverse primers were also used to introduce a HA tag C-terminal to the coiled-coils. The

glycosylation-deficient coiled-coil constructs were generated by first identifying the asparagine residues that are predicted to undergo glycosylation (NetNGlyc 1.0 Server, Technical University of Denmark) and mutating them to serine using primers directed against the corresponding sequences.

The panel of mutants in which the tropomyosin coiled-coil is inserted into linker sequences was generated by amplifying the tropomyosin coiled-coil and inserting it as a BamHI-BamHI fragment into the third, fourth, fifth and sixth linkers in the C8Fac protein.

The Tasmanian devil tetherin gene sequence was retrieved from Ensembl (Release 71, Gene ID: ENSSHAG00000002838). The artificial tetherin protein, comprising of the N-terminal cytosolic tail, transmembrane domain and part of the extracellular region of the transferrin receptor, followed by the extracellular coiled coil domain of the dystrophin myotonic protein kinase protein, a HA epitope tag and the GPI anchor addition motif of the urokinase plasminogen activator receptor, has also been described previously (Perez-Caballero et al., 2009).

The human PV1 and the Tasmanian devil tetherin genes were constructed using PCR-based methods (Lee and Bieniasz, 2007; Soll et al., 2010). Overlapping 60mer oligonucleotides were designed using the Genedesign online software from Johns Hopkins University (<http://slam.bs.jhmi.edu/gd>). The target oligonucleotide length and overlap melting temperature was set to 60 nucleotides and 56°C, respectively. XhoI and NotI sites were added to the first and last

oligonucleotides respectively for cloning into the pCR3.1 vector. For each gene construction, two sequential polymerase chain reactions were carried out. The first contained a mixture of the overlapping oligonucleotides prepared by combining 1 μ L of each 100 μ M stock solution and then diluting ten-fold. A 50 μ L PCR reaction contained 3 μ L of the diluted oligonucleotide mixture and gene synthesis was carried out for 20 cycles using Phinzymes Phusion (1 min. 98°C, 1 min. 50°C, and 2 min. 72°C). The second PCR contained 2 μ L of crude product from the first in a 50 μ L reaction with 200nM external primers. Amplification proceeded for 25 cycles (1 min. 98°C, 1 min. 55°C, and 2 min. 72°C) and the resulting product was gel-purified using the Qiagen gel extraction kit and cloned into pCR3.1 using XhoI and NotI enzymes.

The PV1-GPI construct was generated by using a reverse primer that encoded the tetherin GPI anchor sequence (amino acids 160 to 180) and a NotI site. The DelCT variant (PV1-DelCT-GPI), which lacks the N-terminal 23 amino acids, was generated by using a PCR primer directed to the corresponding sequence.

Sequence-validated cDNA clones (Thermo Scientific) for human CD72 (Accession no. BC030227) and mouse CLEC1A (Accession no. BC052840) were amplified and inserted as XhoI-NotI fragments into pCR3.1. The rest of the panel of modified PV1-like proteins was generated as above, by using PCR primers to introduce HA-tags, tetherin GPI anchors and to truncate the N-termini.

Cell culture

Human embryonic kidney (HEK) 293T cells and HeLa-TZM cells expressing CD4/CCR5 and a LacZ reporter gene under control of the HIV-1 LTR (Platt et al., 1998; Wei et al., 2002) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and gentamycin (2µg/ml, Gibco). HEK293T cells were transduced using pLHCX based retroviral vectors expressing genes of interest and selected with hygromycin (50 µg/ml, MediaTech, Inc.) to generate cell lines expressing either the empty vector or epitope-tagged WT or modified tetherin proteins.

Flow Cytometry

The 293T cells stably expressing the modified tetherin proteins and HeLa cells were harvested in PBS plus 5mM EDTA, washed in FACS buffer (PBS plus 2% BSA), and stained with PE anti-human CD317 (tetherin) antibody (Biolegend). Dead cells were excluded by DAPI staining. All data were acquired on an LSR II flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Virus production

A HIV-1 proviral plasmid that expresses green fluorescent protein (GFP) in place of Nef has been described previously (Zhang et al., 2002). 293T cells were

seeded in 10 cm plates at a concentration of 3×10^6 cells/plate and were cotransfected the following day using polyethylenimine (PolySciences) with 10 μ g of wild-type (HIV-1(WT)) or Vpu-deficient (HIV-1(Δ Vpu)) GFP reporter plasmids, along with 1 μ g of a VSV-G expression plasmid. The culture medium was replaced the following day. At 48 hours post transfection, the culture supernatants were harvested, clarified by centrifugation at 3000 rpm, and filtered through a 0.2 μ m PVDF membrane (Millipore). The viruses were stored at -80°C. Infectious virus titers were determined by inoculating sub-confluent monolayers of 293T cells that were seeded in 96 well plates at 30,000 cells/well with 100 μ l of serially diluted supernatants. At 48 hours post infection, the cells were dispersed with trypsin, fixed in 4% paraformaldehyde and analyzed by flow cytometry.

Virion yield assays

293T cells were seeded in 24-well plates at a concentration of 2×10^5 cells/well and were co-transfected the following day using polyethylenimine (PolySciences) with 350ng of wild-type (HIV-1(WT)) or Vpu-deficient (HIV-1(Δ Vpu)) proviral plasmids along with varying amounts of a plasmid expressing tetherin, PV1, or the panel of modified PV1-like proteins (25ng to 100ng). To monitor transfection efficient, a plasmid expressing YFP (75ng) was also co-expressed in all samples. In all transfection experiments, the total amount of DNA was held constant by supplementing the transfection with an empty expression vector. The culture medium was replaced the following day. At 48 hours post transfection, the culture

supernatants were harvested, clarified by centrifugation at 3000 rpm, and filtered through a 0.2 μ m PVDF membrane (Millipore). Infectious virus yield was determined by inoculating sub-confluent monolayers of HeLa-TZM cells that were seeded in 96 well plates at 10,000 cells/well with 100 μ l of serially diluted supernatants. At 48 hours post infection, β -galactosidase activity was determined using GalactoStar reagent, in accordance with the manufacturer's instructions (Applied Biosystems). Physical particle yield was determined by layering 700 μ l of the virion containing supernatant onto 1 ml of 20% sucrose in PBS followed by centrifugation at 20,000xg for 90 minutes at 4°C. Virion pellets were then analyzed by Western blotting.

Recovery of trapped virions

Cells (HEK293T) stably expressing WT or engineered tetherin proteins were infected with VSV-G-pseudotyped HIV-1(WT) or HIV-1(Δ Vpu) GFP at 1 infectious unit per cell in 10 cm dishes. The inoculum was removed 6 h later. At 48 hours post transfection, the culture supernatants were harvested, clarified by centrifugation at 3000 rpm, and filtered through a 0.2 μ m PVDF membrane (Millipore). Physical particle yield was determined as outlined above. Simultaneously, the cells were washed with Factor Xa reaction buffer (20 mM Tris·Cl, pH 6.5; 50 mM NaCl; 1 mM CaCl₂) and incubated with 50 μ g of Factor Xa in 5 ml of Factor Xa reaction buffer for 2 hours at 37°C. Alternatively, the cells were washed with subtilisin A buffer (10 mM Tris, pH 8.0; 1 mM CaCl₂; 150 mM

NaCl), and treated with 5 ml of 1 µg/ml of subtilisin A (Sigma) for 3 min at room temperature. Subtilisin treatment was stopped using DMEM containing 10% FCS, 5 mM PMSF, and 20 mM EGTA. Thereafter, the supernatants were centrifuged, filtered and virions pelleted as described above, and the cells were lysed for analysis of viral protein expression by Western blotting.

Peptide-N-glycosidase-F digestion of tetherin

Lysates of cell and liberated virions were denatured with 0.5% SDS at 100°C for 10 minutes and then treated with 1% NP-40 to neutralize the SDS. The lysates were incubated with (or without) 500 U of peptide-N-glycosidase-F (New England Biosciences) at 37°C for 3 hours. Thereafter, the reactions were quenched with SDS-PAGE loading buffer and the samples were analyzed with western blotting.

Western blot assays

Pelleted virions and cell lysates were resuspended in SDS-PAGE loading buffer, in the presence or absence of β-mercaptoethanol, and resolved on NuPAGE Novex 4-12% Bis-Tris Mini Gels (Invitrogen) in MOPS running buffer. Proteins were blotted onto nitrocellulose membranes (HyBond, GE-Healthcare) in transfer buffer (25 mM Tris, 192 mM glycine). The blots were then blocked with Odyssey blocking buffer and probed with mouse monoclonal anti-HIV-1 capsid (NIH), rabbit polyclonal anti-HA (Rockland), mouse monoclonal anti-FLAG (Sigma) and

mouse monoclonal anti-PV1 (Abcam) primary antibodies. For quantitative western blotting, the bound primary antibodies were detected using fluorescently labeled secondary antibodies (IRDye 800CW Goat Anti-Mouse Secondary Antibody, IRDye 680LT Goat Anti-Rabbit Secondary Antibody and IRDye 680LT Goat Anti-Mouse Secondary Antibody; LI-COR Biosciences). Fluorescent signals were detected using a LI-COR Odyssey scanner and quantitated with Odyssey software (LI-COR Biosciences).

Phylogenetic analyses

Sequences for Tetherin and PV1 genes were retrieved from Ensembl (Release 71) and their cDNA sequence was used to construct multiple alignments using MUSCLE followed by manual refinement based on a translated amino acid sequence. The multiple alignments were used to construct maximum likelihood (ML) phylogenetic trees using RaxML version 7.2.8 with the following parameters: rapid bootstrap analysis with 100 replicates under GTRCAT followed by a ML search under GTRGAMMA to evaluate the final tree topology (-m GTRCAT -# 100 -x 13 -k -f a). The PV1 tree was rooted using the platypus sequence as out-group. The tetherin tree was estimated using a reduced sequence alignment that is composed of the transmembrane and coiled-coil domains (codons 22 to 133 of human tetherin), and it was finally midpoint rooted.

Analysis of PV1 and Tetherin sequences

Analysis of introns and neutrally evolving sequences were carried out using the mVISTA visualization tool for global sequencing alignments. FASTA and annotated sequence files were uploaded to the mVISTA website (<http://genome.lbl.gov/vista/mvista/about.shtml>). A VISTA-Point alignment was generated and a screenshot of all the alignments was captured.

Maximum likelihood analysis on PV1 and Tetherin genes was performed using CODEML from the PAML suite of programs. Due to the high divergence of the N- and C- termini of tetherin sequences across mammalian species, the following analyses were performed on a reduced sequence alignment that is composed of the transmembrane (TM) and coiled-coil domains (codons 22 to 133 of human tetherin). Likelihood ratio tests were used to compare paired, nested models of sequence evolution that allow, or do not allow, variable selective pressure among sites (NSsites models 0 vs. 3), or positive selection (NSsites models 1 vs. 2 and 7 vs 8). The F3X4 model of codon frequencies was used for all analyses in CODEML. The chi-square test was performed using 4 degrees of freedom for M0 vs. M3 or 2 degrees of freedom for M1 vs. M2 and M7 vs. M8. The degrees of freedom are based on the number of parameters for each model. For both PV1 and tetherin alignments used, sites where only one or two taxa contain data (i.e. the vast majority of the sites contain gaps) were removed from the alignment. The platypus sequence was also removed from the PV1 sequence alignment used in this analysis.

In silico evolution

The ancestral PV1 root sequence was inferred using BASEML from the PAML suite of programs under the REV (GTR) model of nucleotide substitutions. We allowed the ancestral PV1 sequence to evolve under distinct neutral substitution rates for a period of 18.5 MY using the EvolveAGene 3 software with the following parameters: two taxa, 0.05 mean branch length, no selection against amino acid replacements and no selection against insertions/deletions (-n2 -b0.05 -a1 -i1 -d1). The EvolveAGene 3 software selects against frameshift insertions/deletions and non-sense mutations allowing us to obtain sequences that maintained full coding capacity. The EvolveAGene 3 output for one run is composed of two sequences where the actual length of each branch will be a random number between 0 and twice the mean branch length selected. A Perl script was written to collect the output of 100 sequences for each neutral substitution rate. Thereafter, the original ancestral PV1 sequence and each of the 100 sequences per neutral substitution rate used were utilized as input for Seq-Gen [29] to simulate tetherin evolution for the remaining mammalian tree (using a tetherin phylogenetic tree constructed from a full length tetherin multiple sequence alignment as input). Parameters for Seq-Gen were estimated by ModelTest using the Bayesian Information Criterion (BIC) likelihood test on a previously constructed full length tetherin multiple sequence alignment. The BIC likelihood ratio test selected the following parameters: HKY+G model; nucleotide frequencies of 0.2519, 0.2668, 0.3012 and 0.1801 for A, C, G and T,

respectively; alpha (for the shape of the gamma rate heterogeneity distribution) = 1.4289; proportion of invariable sites = 0.0039, transition-transversion ratio = 1.0225 (-mHKY -n100 -f0.2519,0.2668,0.3012,0.1801 -a1.4289 -i0.0039 -t1.0225). For each sequence used as input, Seq-Gen produced 100 simulated tetherin data sets (each data set is composed of simulated sequences representing the taxa diversity of the tetherin phylogenetic tree used as input). Thus, for each substitution rate used we obtained 10,000 *in silico*-evolved data sets. For each data set, we compared the simulated sequences for human, mouse and dog with the contemporary PV1 protein sequences using tBLASTn.

Identification of PV1/Tetherin-like proteins

All 105,281 human and 54,447 mouse annotated protein sequences were downloaded from Ensembl (Release 71) using BioMart. A Perl script was written to automate a pipeline to find protein sequences that contain one TM domain followed by coiled-coil domains. Protein sequences were scanned for TM domains using tmhmm 2.0. Sequences with only one TM domain and a probability > 0.5 were selected and scanned for coil-coiled segments C-terminal to the TM domain using COILS (probability > 0.5). A complete list of all human and mouse gene locations was retrieved from Ensembl (Release 71) using BioMart. A Perl script was written to compare the locations of the resulting PV1/Tetherin-like proteins and the entire gene location list to establish the

identity of the adjacent genes. Homology between PV1/Tetherin-like proteins that are adjacent to each other was established by BLASTp searches.

Reconstruction of the PV1 locus

The PV1 loci across animals were retrieved using the Genome Browser (<http://genome.ucsc.edu/index.html>) at the University of California at Santa Cruz and synteny across eutherian mammals was assessed using the Genomicus Browser (<http://www.genomicus.biologie.ens.fr/genomicus-70.01/cgi-bin/search.pl>). The tetherin protein in opossums and PV1 proteins in reptiles and amphibians were not annotated in Ensembl and UCSC Genome Browser and were identified using orthologous protein queries from closer related species in BLAT searches.

Chapter III Mechanism of HIV-1 virion entrapment by tetherin

In the following chapter, we describe the development of biochemical approaches to dissect the molecular mechanisms that underpin the antiviral activity of tetherin against enveloped viruses such as HIV-1. Investigations into the role of tetherin heretofore have been hampered by the dearth of experimental tools that probe tetherin molecules that have infiltrated the envelopes of assembling viral particles. To address this, we used a combination of biochemical assays and engineered tetherin proteins to specifically probe tetherin molecules as they engage in virion entrapment. The results presented herein have been published in a research article (Venkatesh and Bieniasz, 2013), which was the first study in the literature to demonstrate unequivocally that tetherin is directly responsible for the retention of virions at the cell surface. In particular, these biochemical analyses have been invaluable in elucidating the configurations that tetherin homodimers adopt during HIV-1 restriction.

Putative configurations that might be adopted by tetherin during HIV-1 virion entrapment

It is now well-established that the cellular restriction factor, tetherin, prevents HIV-1 and other enveloped virus particles from being disseminated into the extracellular milieu by infiltrating their envelopes and by physically crosslinking them to the cell surface. However, the mechanism by which tetherin traps virions is poorly understood. As described in the Introduction (Chapter 1), a

preponderance of the evidence seem to support the notion that tetherin traps virions via a direct tethering mechanism, i.e. by infiltrating the lipid envelope of assembling particles (Fitzpatrick et al., 2010; Hammonds et al., 2010; Perez-Caballero et al., 2009). Previous biochemical (Perez-Caballero et al., 2009) and structural analyses (Hinz et al., 2010; Schubert et al., 2010; Swiecki et al., 2011; Yang et al., 2010) suggest that tetherin consists of two pairs of membrane anchors that are spatially separated by rod shaped coiled-coil domains. Although the exact configuration adopted by the tetherin protein is still unknown, its unique architecture does lend itself to a number of possibilities. For instance, one can envisage a scenario wherein a pair of anchors partitions into the lipid envelope of assembling particles, whilst the other pair remains rooted in the plasma membrane of the infected cell (*axial configuration*, Figure 3.1A). In this configuration, each tetherin dimer could potentially link viral and cell membranes in either “polarity”, i.e. with N-termini inserted into either the infected cell or the assembling particle. Other obvious possibilities by which entrapment might be achieved would be via the separate partitioning of dimerized tetherin molecules into virion and cell membranes (*equatorial configuration*, Figure 3.1B) or the non-covalent oligomerization of tetherin dimers that have both pairs of anchors embedded in either virion envelopes or cell membranes (Figure 3.1C). In order to dissect the molecular mechanisms that underpin the antiviral activity of tetherin, we asked the following questions: (i) Is tetherin directly responsible in trapping virions? (ii) Which, if any, of the aforementioned configurations are adopted

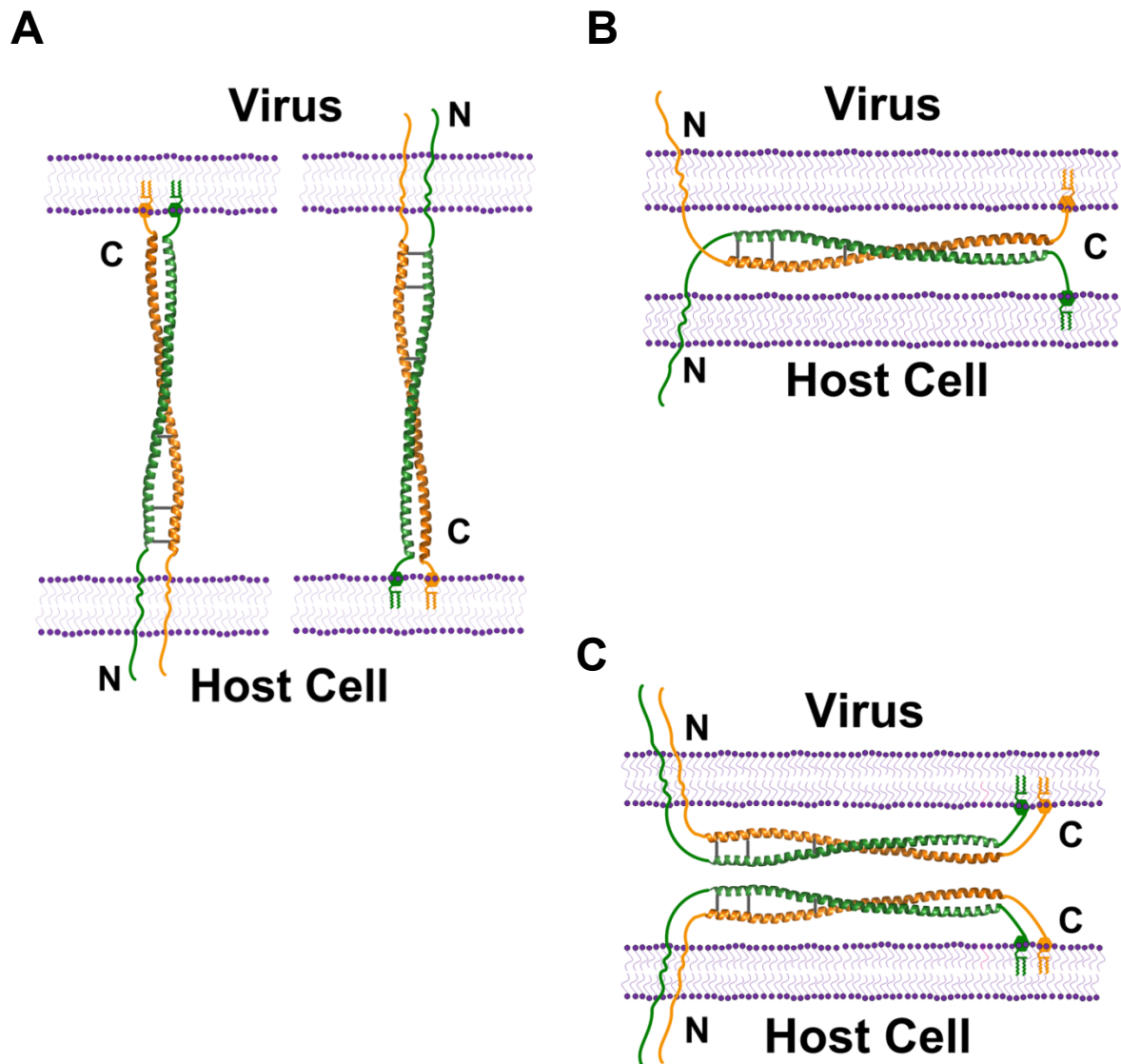


Figure 3.1. Models illustrating the possible configurations adopted by tetherin during virion entrapment. (A) Tetherin dimers might trap virions at the cell surface via the infiltration of one pair of membrane anchors into the viral envelope, while the other pair remains rooted in the infected cell membrane (*axial configuration*). ‘N’ and ‘C’ represent the N- and C- termini of tetherin, respectively. **(B)** Tethering might also be achieved through the separate partitioning of dimerized tetherin molecules into virion and cell membranes (*equatorial configuration*). **(C)** Tethering might be mediated by the non-covalent association of tetherin dimers that have both pairs of anchors embedded in virion envelopes or cell membranes.

during the retention of virions, and, (iii) Does the contribution of any one configuration outweigh that of the others?

A panel of modified tetherin proteins designed to deduce the configuration of tetherin during HIV-1 virion entrapment

To address the above questions, we set out to develop genetic and biochemical tools to probe tetherin molecules that have infiltrated tethered virions. It should be noted that a variety of approaches, including hydropathy analyses, fusion with reporter enzymes, or the insertion of target sites for proteases, antibodies and chemical modifiers, have been used to deduce integral membrane protein topology (Manoil et al., 1988). For example, the insertion of Factor Xa cleavage sites into hydrophilic loops has proven to be useful for such investigations (Ota et al., 1998; Wilkinson et al., 1996). Factor Xa is an endopeptidase that recognizes the tetrapeptide motif Ile-Glu-Gly-Arg with strict specificity and cleaves the protein backbone C-terminal to the arginine residue. To clarify the configuration of tetherin molecules in infected cell and viral membranes, we adapted the aforementioned approaches by engineering modified human tetherin proteins that carried (i) single cleavage sites for Factor Xa and (ii) epitopes such as hemagglutinin (HA) and FLAG tags positioned either N- or C-terminal to the Factor Xa site (Figure 3.2). Importantly, previous experiences with tagged versions of tetherin proteins led to the expectation that such minor alterations

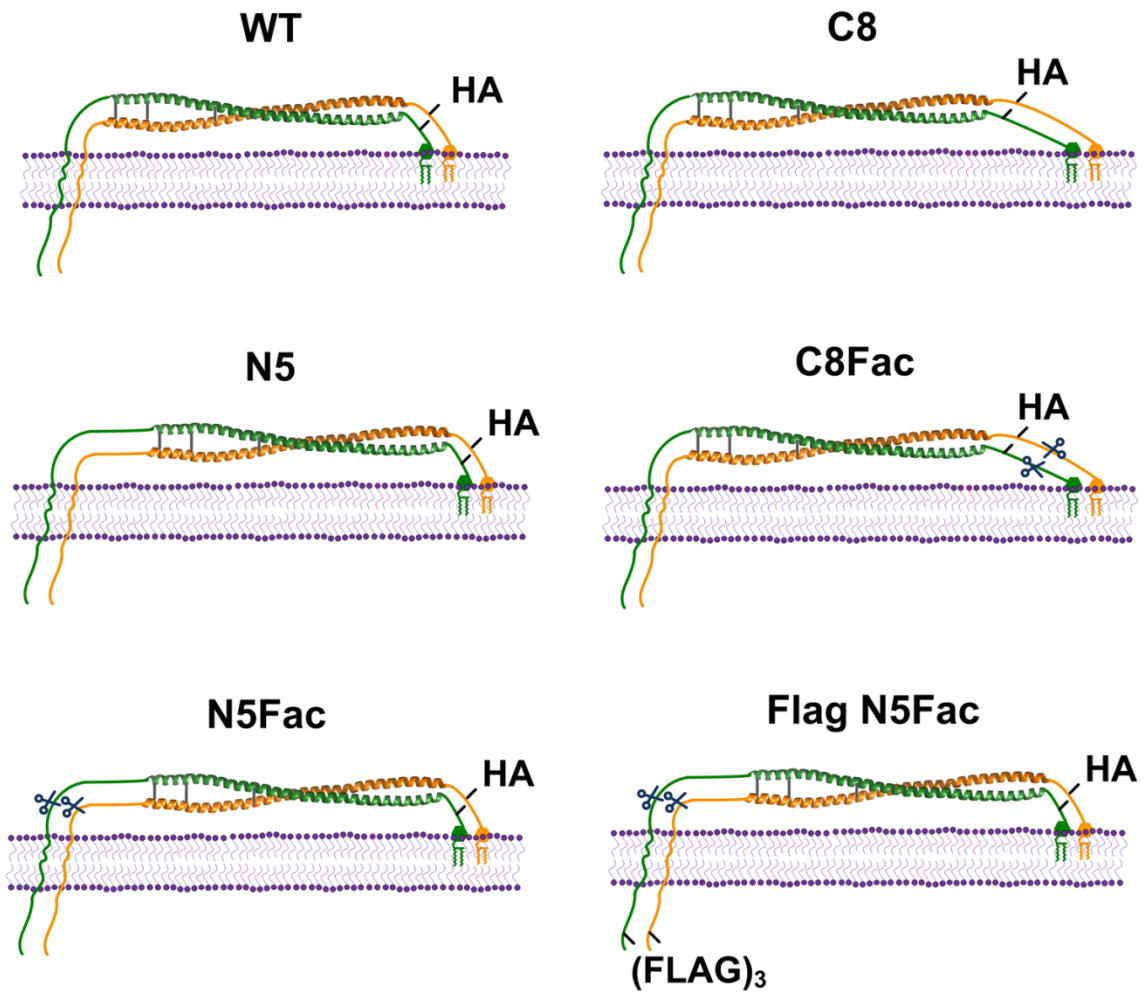


Figure 3.2. Schematic representation of the panel of modified tetherin proteins designed to deduce the configuration of tetherin during HIV-1 virion entrapment. The C8 and N5 proteins have linker sequences inserted at the C- and N-terminus of the tetherin extracellular domain, respectively. The Factor Xa cleavage site is indicated by scissors, and the cysteine residues that stabilize homodimerization are indicated as lines. Diagrams were constructed using PDB entry 3MQC to represent the tetherin extracellular domain.

should have either no or modest effects on antiviral activity (Neil et al., 2008; Perez-Caballero et al., 2009).

Initial experiments in which Factor Xa sites alone were incorporated into Tetherin resulted in proteins that were somewhat refractory to proteolysis (unpublished observations). Hence, we reasoned that the introduction of flexible linkers into its primary sequence might facilitate access to the cleavage site, and increase the efficiency of proteolysis. To test this hypothesis, we generated a panel of linker modified tetherin proteins in which we inserted two, five and eight GGGGS peptide linker units into the extracellular domain of tetherin, either N-terminal (at amino acid 50) or C-terminal (at amino acid 157) to the predicted coiled-coil domain. The GGGGS pentapeptide is predicted to be unstructured because the glycine residues impart flexibility, and the polar serine residue permits hydrogen bonding to the solvent (Argos, 1990; Huston et al., 1991). As an initial line of inquiry, this panel of proteins was screened for their ability to inhibit replication-competent HIV-1, in order to identify candidate proteins for the insertion of the Factor Xa site.

Antiviral activity of modified tetherin proteins

Among the panel of linker modified tetherin proteins, we determined that the proteins with eight linker units C-terminal to the coiled-coil (C8, Figure 3.2) and five linker units N-terminal to the coiled-coil (N5, Figure 3.2) were expressed at comparable levels to WT tetherin (Figure 3.3). Note that Tetherin is

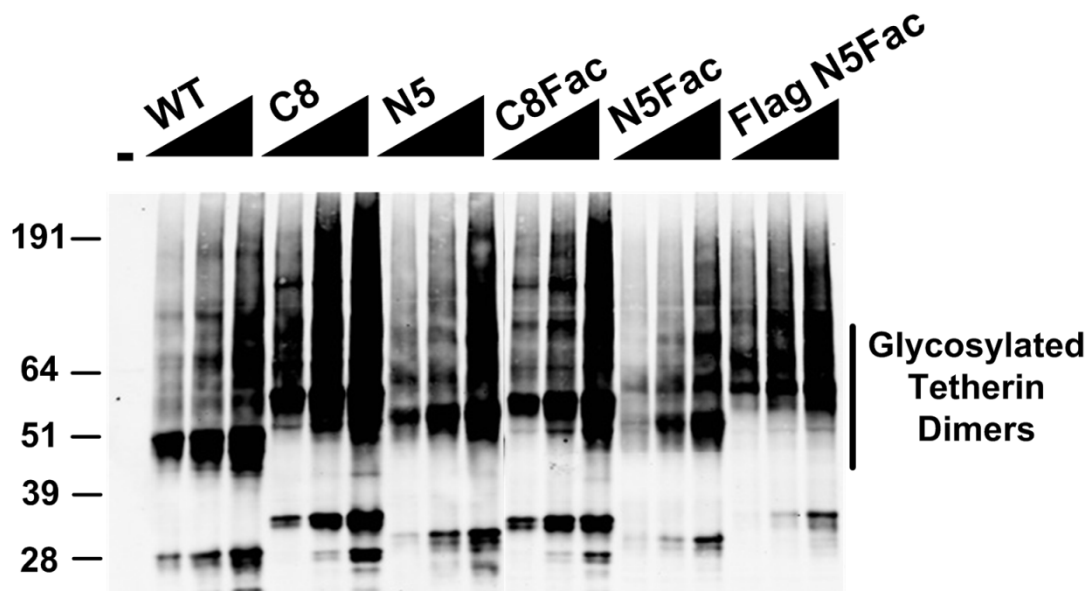


Figure 3.3. Expression profiles of the panel of modified tetherin proteins. Western blot analyses of the 293T cell lysates that were co-transfected with a Vpu-deficient (HIV-1 Δ Vpu) proviral pNL4-3 plasmid along with varying amounts of the indicated modified tetherin proteins. All samples were lysed in non-reducing buffer and probed with an anti-HA antibody.

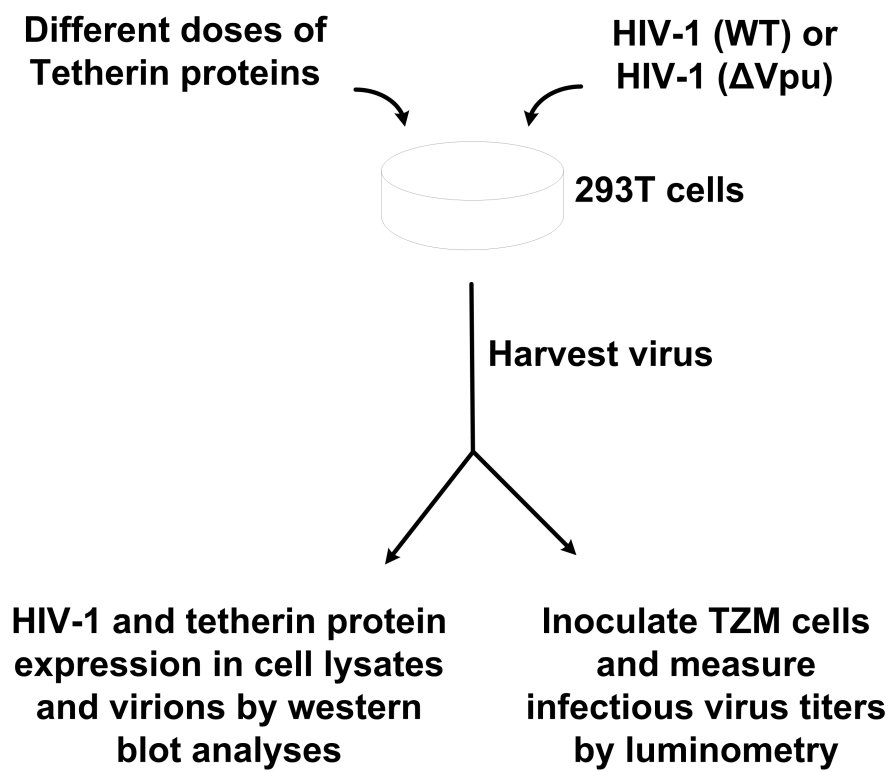
heterogeneously glycosylated, and because the cells were lysed in non-reducing buffer, the tetherin proteins migrated primarily as a smear of dimeric species (Perez-Caballero et al., 2009) (Figure 3.3).

To examine the antiviral activity of the linker-modified tetherin proteins, 293T cells were transfected with either a HIV-1 proviral plasmid (HIV-1(WT)) or its Vpu-deficient counterpart (HIV-1(Δ Vpu)), along with varying amounts of plasmids expressing wild type (WT) tetherin or one of the modified tetherin proteins. 293T cells were chosen because they are a tetherin-negative cell line. Hereafter, WT tetherin (Figure 3.2) refers to a previously described construct that harbors an HA epitope tag at amino acid 155 in the extracellular domain, but retains the antiviral activity of the untagged, endogenous protein (Neil et al., 2008). As expected, WT tetherin potently inhibited the release of HIV-1 (Δ Vpu) in a dose-dependent manner, while only marginally affecting the release of HIV-1 (WT) (Figures 3.4A and 3.4B). Importantly, the C8 and N5 tetherin proteins were only modestly impaired in their antiviral activity compared to WT tetherin as determined by infectious virion yield and extracellular particulate CA protein measurements (Figures 3.4A and 3.4B) and the levels of cell-associated Gag protein were unaffected by the expression of the tetherin proteins (Figure 3.4B). This data indicates that the insertion of linker sequences into tetherin was well tolerated with little effect on antiviral activity.

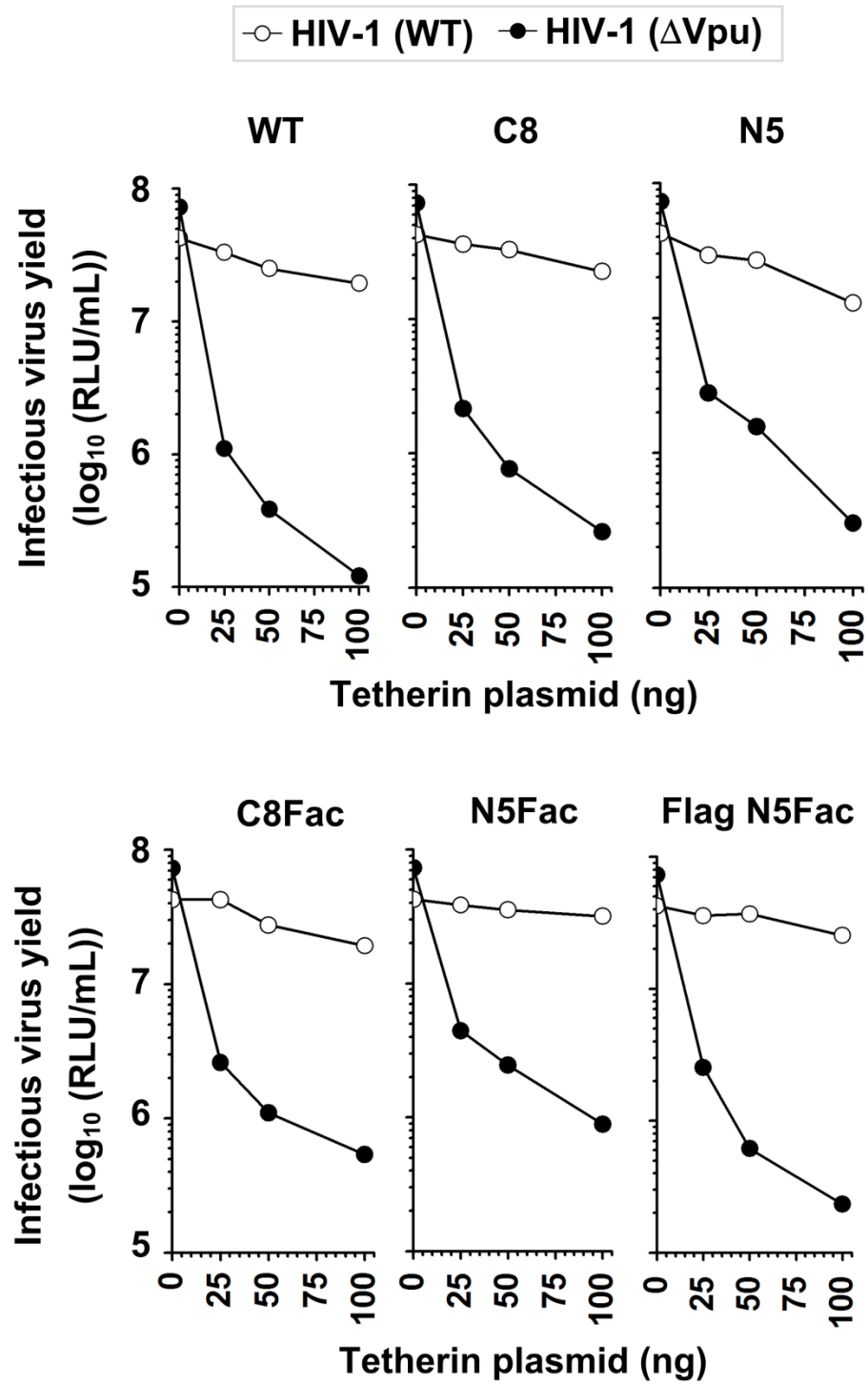
We next programmed the C8 and N5 tetherin proteins with single Factor Xa cleavage sites. The rationale was that these proteins (referred to hereafter as

Figure 3.4. Antiviral activity of the panel of modified tetherin proteins. (A) Schematic representation of the experimental strategy to determine the antiviral activity of the modified tetherin proteins. **(B)** 293T cells were cotransfected with wild-type (HIV-1 (WT)) or Vpu-deficient (HIV-1 Δ Vpu) proviral pNL4-3 plasmids along with varying amounts of the indicated modified tetherin proteins. Infectious virion yield was measured by inoculating HeLa-TZM indicator cells with culture supernatant and is given as the logarithm to the base 10 of the relative light units (RLU). **(C)** Western blot analyses of transfected 293T cell lysates and virions corresponding to the above panel. All samples were probed with an anti-CA antibody. The numbers at the bottom represent measurement of CA protein levels in virion pellets (LI-COR).

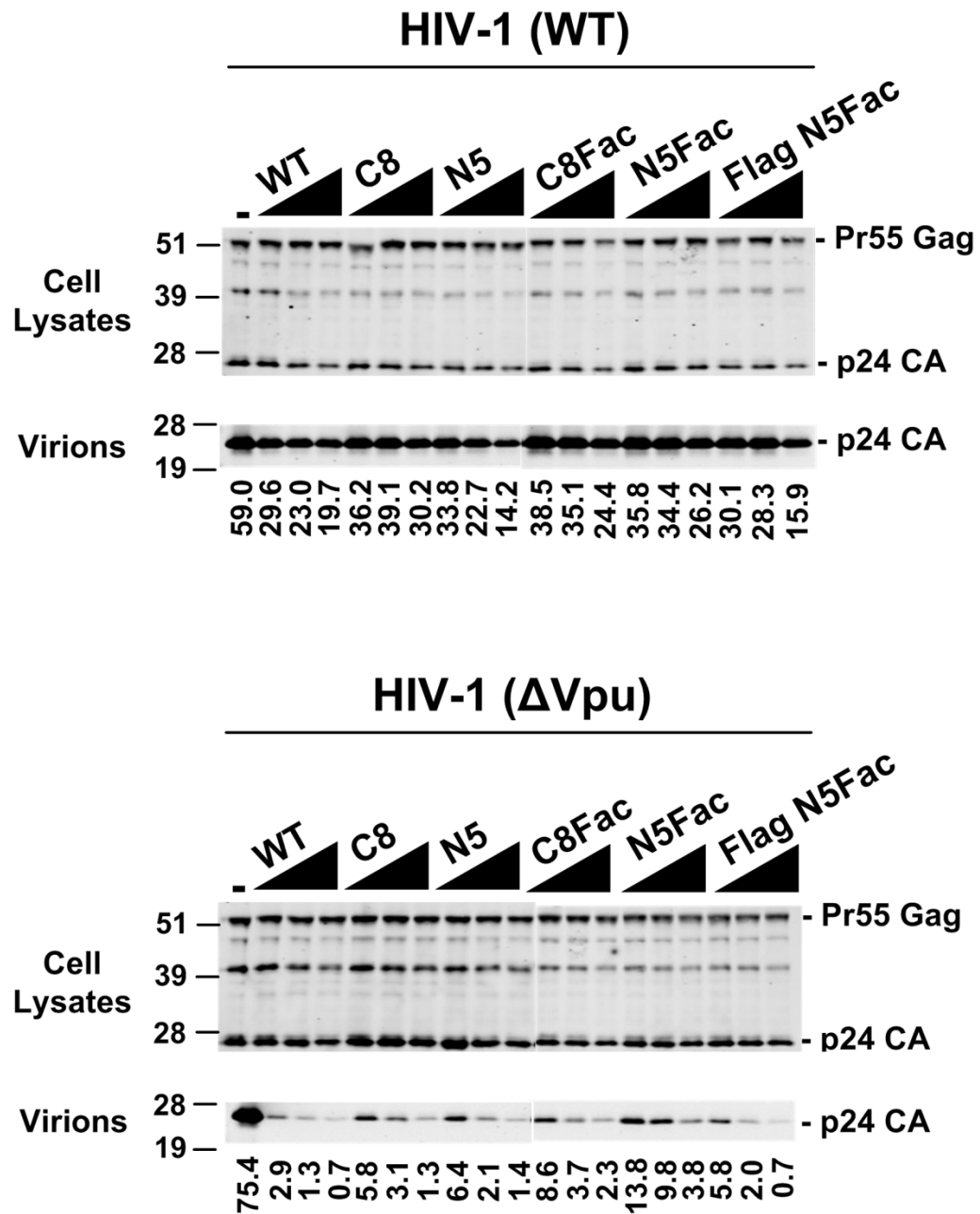
A



B



C



C8Fac and N5Fac respectively, Figure 3.2) would differ in the relative ordering of the HA epitope tag and the protease site. Thus, the epitope tag is positioned N-terminal to the protease site in the C8Fac protein, whereas it is positioned C-terminal to the protease site in the N5Fac protein. In addition to the C8Fac and N5Fac proteins that carried only one epitope tag, we also appended the N-terminus of the N5Fac construct with three tandem FLAG epitope tags. This manipulation results in FLAG and HA epitope tags flanking the protease site (Flag N5Fac, Figure 3.2). The use of three FLAG tags in tandem reportedly enhances signal intensity by ~10-20-fold (Hernan et al., 2000).

Analysis of the antiviral activity of the Factor Xa site-modified tetherin proteins revealed that the C8Fac and N5Fac proteins were only slightly impaired in their ability to trap virions as compared to WT tetherin, while the Flag N5Fac protein was nearly indistinguishable its activity to WT tetherin (Figures 3.4A and 3.4B). Western blotting analyses of the C8Fac and N5Fac proteins indicated that they were expressed at slightly lower levels than the C8 and N5 proteins respectively (Figure 3.3), and were thus proportionately impaired in antiviral activity (Figures 3.4A and 3.4B). Interestingly, in spite of harboring more tags as compared to any of the other modified tetherin proteins, the Flag N5Fac protein was virtually as potent as WT tetherin, and was expressed at levels indistinguishable from WT tetherin. Vpu antagonized all modified tetherin proteins and restored the yield of extracellular virions (Figures 3.4A and 3.4B). Thus, all modified tetherin proteins mimicked WT tetherin, i.e. they trapped virions, and were sensitive to the action of HIV-1 Vpu.

Antiviral activity of modified tetherin proteins in stable cell lines

Thus far, the antiviral activity of the panel of modified tetherin proteins was analyzed using transient transfection assays. In principle, transient transfections yield high expression levels of the protein over short time intervals, after which the plasmids are rapidly lost during cell division. It should be noted that the results from a transfection assay should be interpreted with caution because only a fraction of the cells in culture tend to bias most of the observed phenotype. Another confounding factor is the high expression levels of the protein that is not physiologically relevant. Such issues are ameliorated by the use of stable transduction assays, wherein every cell in culture expresses lower levels of the protein. Furthermore, stable cell lines selected through limited dilution provide a genetically homogenous and clonal population.

To confirm and extend our observations, we generated a panel of 293T cells that stably expressed the epitope-tagged Factor Xa-cleavable tetherin proteins. The levels of cell surface tetherin in these stable cell lines was assessed by flow cytometry using a monoclonal antibody that recognizes the extracellular region of human tetherin. Importantly, the surface expression levels of the WT and modified tetherin proteins were quite similar to each other, varying over a 2.5-fold range (mean fluorescent intensities were 6200, 15000, 8800, and 12000 for WT, C8Fac, N5Fac and Flag N5Fac tetherin proteins, respectively; Figure 3.5A) and were only 1.5 to 3-fold greater than that of the endogenous protein in HeLa cells,

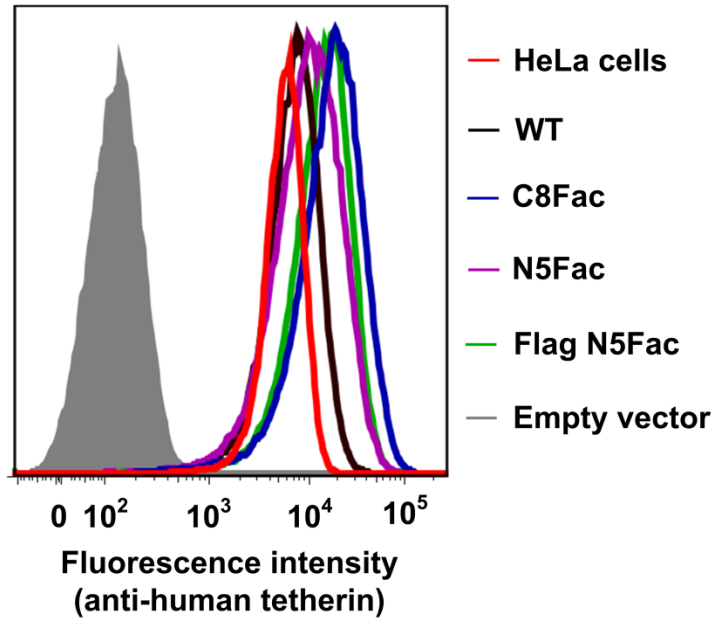
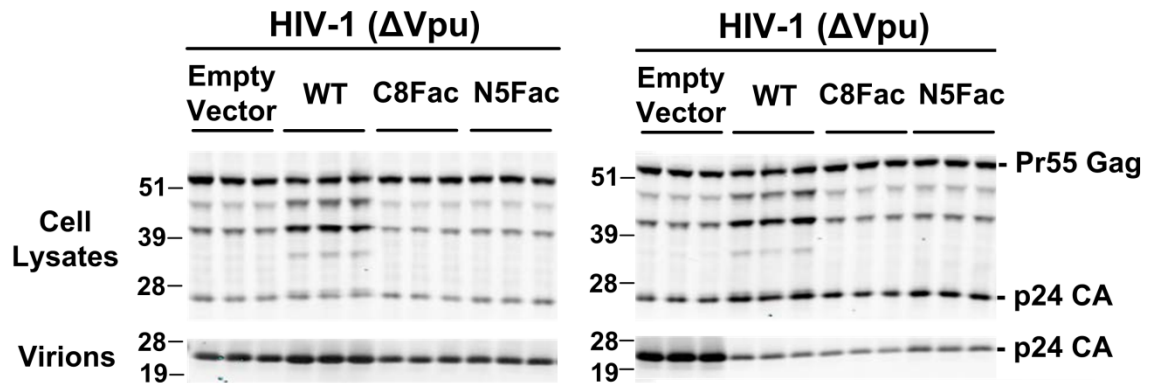
A**B**

Figure 3.5. Antiviral activity of modified tetherin proteins in stable cell lines. (A) 293T cells that stably express the modified tetherin proteins were analyzed by flow cytometry to determine the relative surface expression levels of tetherin, using a mouse anti-human tetherin antibody. (B) Western blot analyses of the stable 293T cell lysates and virions harvested from them, following infection with HIV-1 or its Vpu-deficient counterpart at a MOI of 1. All samples were probed with an anti-CA antibody. The three lanes for each tetherin protein are replicates of the experiment.

a prototype tetherin-positive cell line (mean fluorescent intensity =5000; Figure 3.5A).

We next verified the antiviral activity of the engineered tetherin proteins in the stable cell lines using single-cycle HIV-1 replication assays (Figure 3.5B). The stable cells were infected with HIV-1 or its Vpu-deficient counterpart, with both viruses harboring the GFP protein for quantification of infected cells. Control cells that stably expressed the empty vector were also included for the purpose of comparison. As expected, both the WT and the modified tetherin proteins inhibited the release of particles from infected cells, but did not affect cell associated Gag protein expression (Figure 3.5B). Moreover, the expression of Vpu reversed the inhibitory effect of the modified tetherin proteins, resulting in the release of capsid protein into the supernatant (Figure 3.5B). Thus, it was reassuring that the panel of stable cells recapitulated our transient transfection results.

An assay to probe the configuration of tetherin during HIV-1 particle entrapment

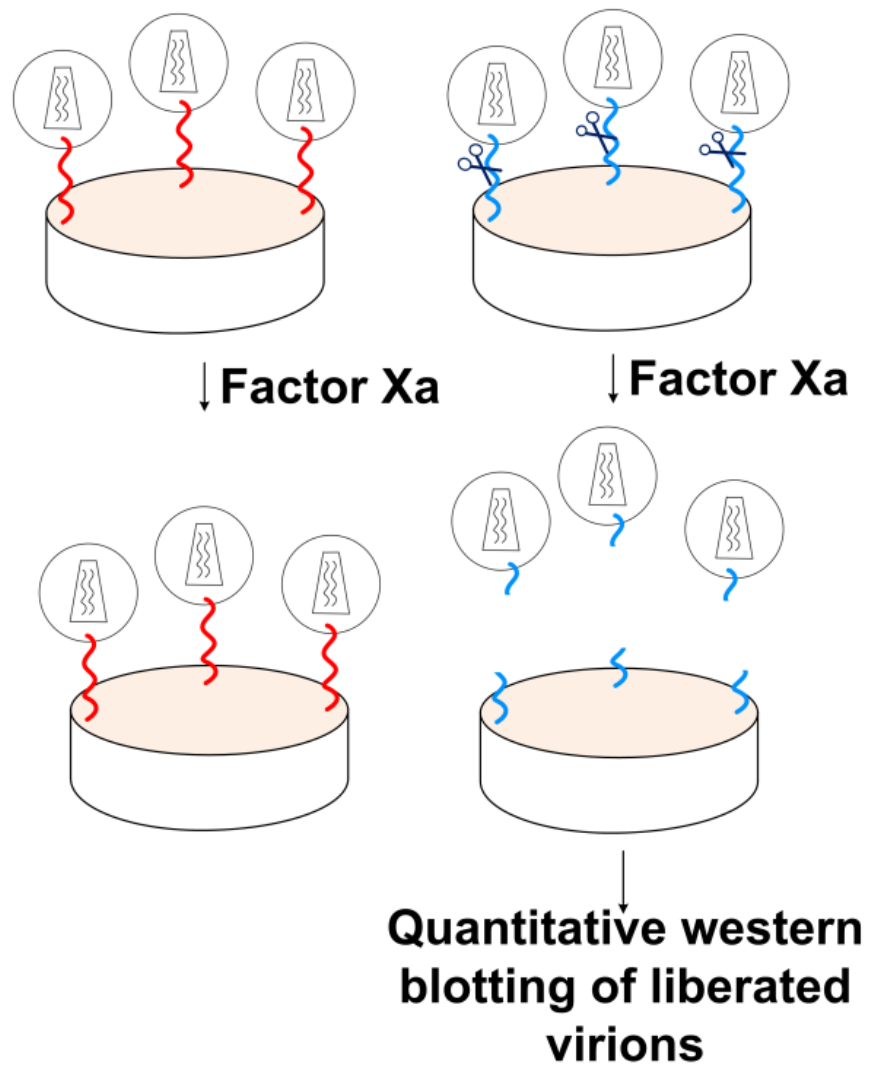
Previous studies from our laboratory have employed a protease “stripping” assay (Neil et al., 2006; Neil et al., 2007) in which a relatively nonspecific protease (subtilisin A) was used to demonstrate that the ectopic expression of tetherin causes virions to become entrapped on cell surfaces by a protein based tether. The logic underpinning the “second generation” assay described herein is that if the tetherin protein itself is directly responsible in trapping virions, then treatment

of cell surfaces with a specific protease (Factor Xa) would trigger the release of virions only when tetherin was programmed with a Factor Xa cleavable site (Figure 3.6A). Moreover, cleavage should result in partitioning of the epitope-tagged proteolytic fragments either into the liberated virions or the infected cells. Because the epitope tags were strategically positioned relative to the protease site, topological information could then be deduced about tetherin in its functional state (Figure 3.6B). However, it is expected that only a minority of the tetherin molecules on the cell surface would actually be involved in tethering virions, and hence, only fragments that are found in virions should be regarded as informative with respect to tetherin topology during virion entrapment. Nevertheless, we also assessed the proteolytic cleavage of the tetherin proteins in whole cell lysates for multiple reasons. First, tetherin molecules that are *en route* to the cell surface would be insensitive to the action of Factor Xa, which is cell-impermeable. If not, one could infer that Factor Xa treatment is resulting in undesirable cell lysis. Second, it is obviously important that the size of the cleaved proteolytic fragments in cells should exactly match that in the liberated virions, if indeed tetherin cleavage is necessary for liberation of virions.

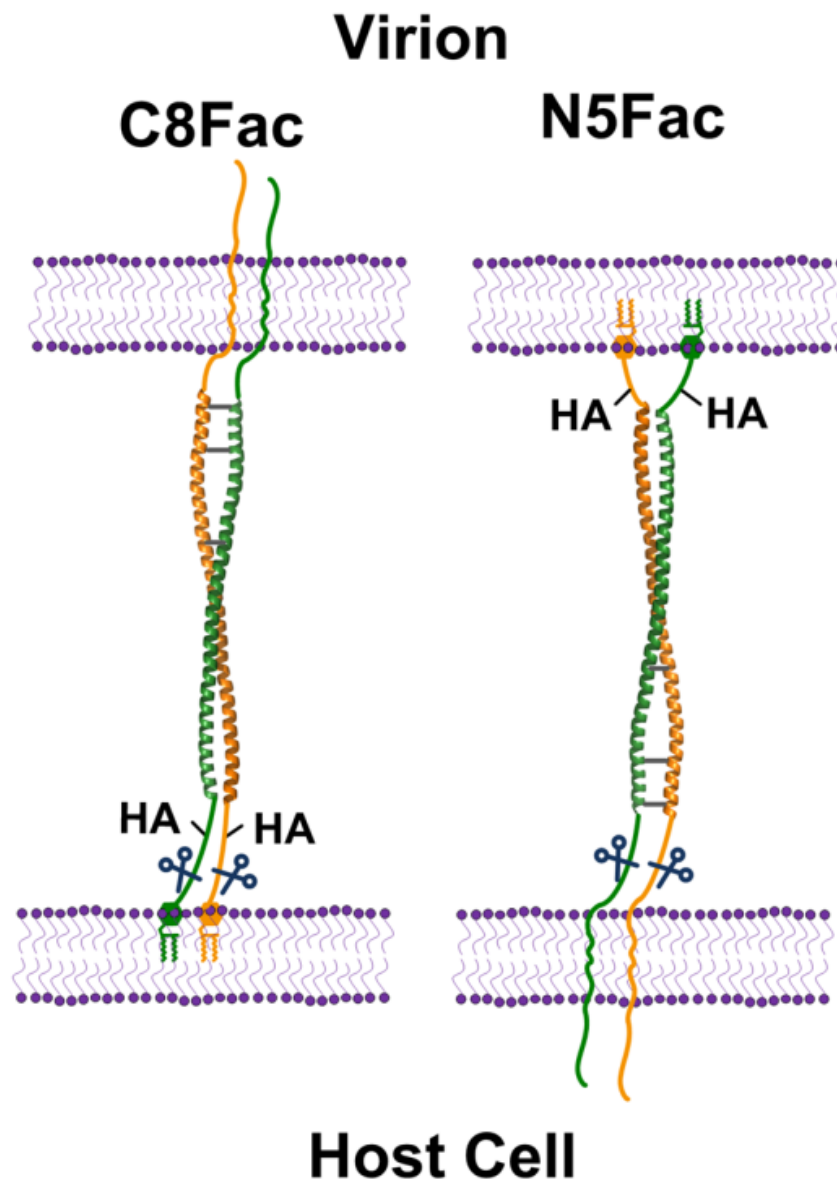
Note that if tetherin adopts the equatorial configurations depicted in Figure 3.1B or 3.1C then we would not expect Factor Xa cleavage to result in virion release, because the cleavage sites are positioned outside the region of coiled-coil interaction. Indeed, the cleavage sites are positioned in artificially introduced linker sequences whose insertion did not perturb tetherin function (Figures 3.2 and 3.4). Conversely, if tetherin adopts the axial configuration in virion tethers,

Figure 3.6. A biochemical assay to probe the configuration and polarity of tetherin homodimers that are engaged in virion entrapment. (A) Schematic representation of the protease-induced virion release assay. Modified tetherin dimers programmed with a protease cleavage site (scissors) are indicated as orange helices, while the WT tetherin dimers are indicated as green helices. If trapped viruses are liberated upon protease treatment only when tetherin is programmed with a protease site, it confirms a role for tetherin as a direct tether in virion entrapment. The liberated virions are subjected to quantitative western blotting analyses to estimate the numbers of tetherin dimers associated with a single virion and their orientation. **(B)** Schematic representation of the polarities that would be adopted by the C8Fac and N5Fac proteins if HA-tagged proteolytic fragments are observed to partition with liberated virions.

A



B



then Factor Xa cleavage should result in virion release. Moreover, if as depicted in Figure 3.6B, the HA-tagged proteolytic fragments partition with virions that are liberated from Factor Xa-treated, C8Fac-expressing cells, it would suggest that tetherin dimers exist with their N-termini inserted into the interior of the virion. Conversely, if HA-tagged proteolytic fragments partition with virions that are liberated from the N5Fac cell line, we would deduce that tetherin dimers exist with their GPI anchors embedded in the virion membrane (Figure 3.6B). If, however, tetherin dimers adopt both polarities, then HA-tagged proteolytic fragments would be observed in virions liberated by Factor Xa from both C8Fac and N5Fac expressing cell lines.

We first investigated the utility of this approach using cell lines expressing the single epitope tagged C8Fac and N5Fac tetherin proteins. Cells were infected with Vpu-deficient HIV-1, and constitutively released particles were harvested from culture supernatants at 48h post infection. Thereafter, the monolayer of cells was treated with Factor Xa for 2h, and then the cell lysates and any liberated virions were also harvested. The amount of Factor Xa and the incubation time were determined empirically. Samples were removed at various time points and analyzed to estimate the extent of Factor Xa digestion. At 2h post-treatment, the cleavage of Factor Xa-modified tetherin molecules was complete.

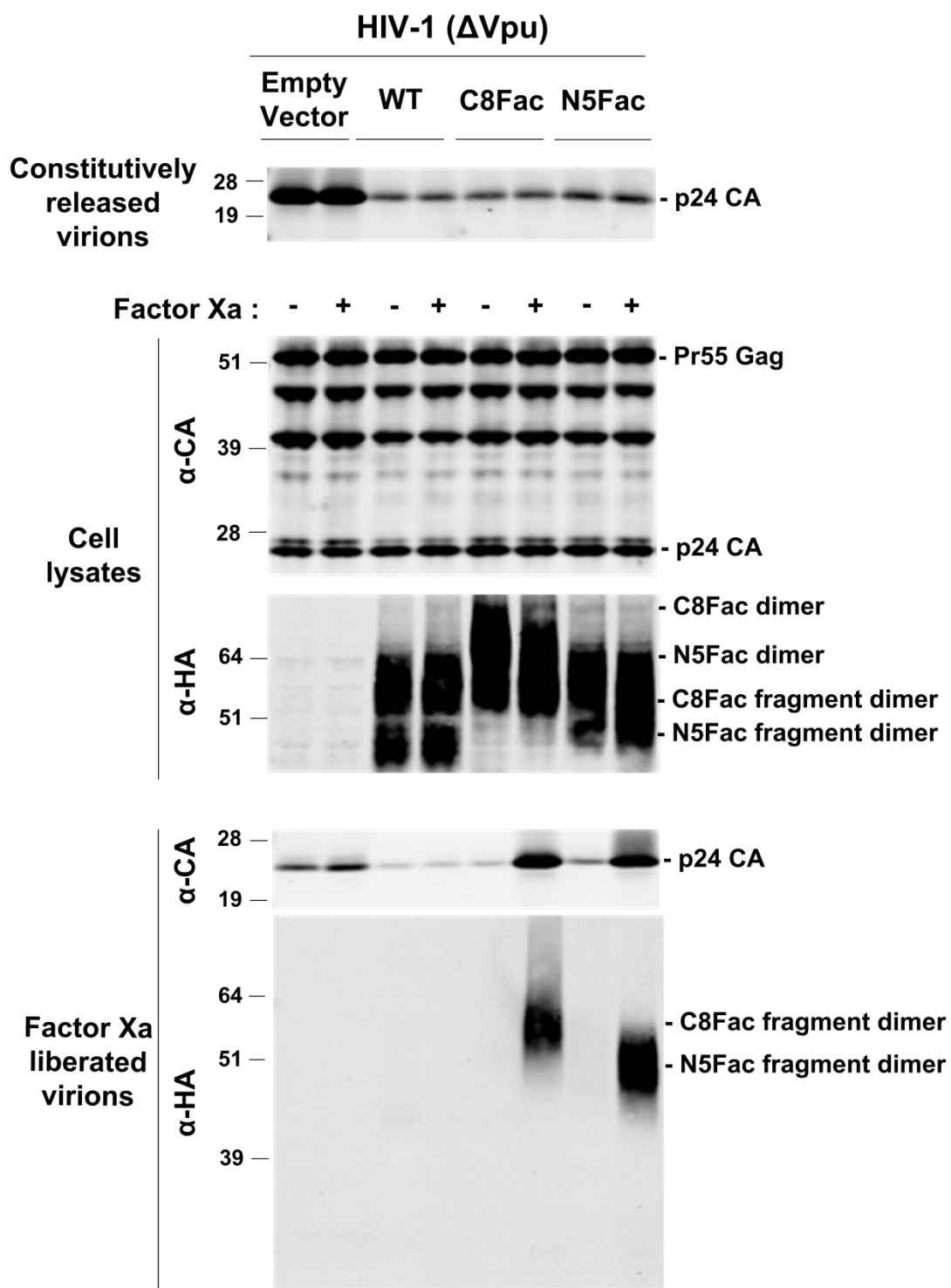
As before, infected control tetherin-negative cells constitutively released substantially high levels of virions into the culture supernatant, while virion yield from cells expressing WT, C8Fac or N5Fac tetherin proteins was markedly

reduced (Figure 3.7A). Importantly, the expression levels of HIV-1 Gag expression in cell lysates were similar, indicating that levels of infection were uniform across all samples (Figure 3.7A).

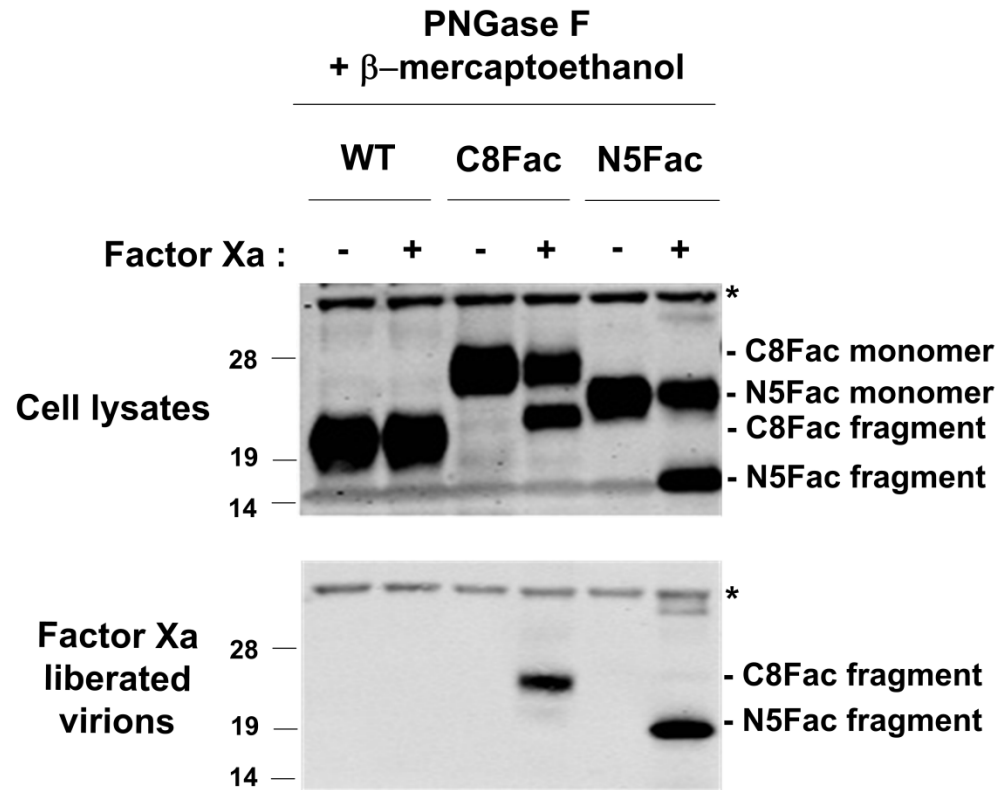
Incubation in Factor Xa cleavage buffer alone resulted in the release of only low levels of pelletable CA from tetherin-deficient cells (Figure 3.7A). This might represent virion particles that were constitutively released during the 2 hour incubation period, or virions that were loosely adhered to the cell surface (Figure 3.7A). Even lower levels of particles were released from cells expressing the WT, C8Fac or N5Fac tetherin proteins that were incubated in Factor Xa cleavage buffer alone (Figure 3.7A). Strikingly however, Factor Xa treatment of the C8Fac and N5Fac resulted in the release of substantial amounts of particulate CA (Figure 3.7A). Crucially, Factor Xa treatment of tetherin-negative or WT tetherin expressing cells did not increase particle release over the low background levels that were observed in the absence of protease, underscoring the tight specificity of the assay (Figure 3.7A). Notably, the proteolytic fragments of tetherin that were observed in Factor Xa-liberated virions from both C8Fac and N5Fac expressing cells and virion-associated fragments were consistent with dimer formation, and were the only tetherin species that were detectable on non-reducing SDS PAGE gels (Figure 3.7A).

Figure 3.7. Axially configured tetherin homodimers directly trap virions, with both polarities contributing to restriction. (A) Western blot analyses of virions, 293T cells stably expressing C8Fac and N5Fac tetherin proteins, and virions liberated upon Factor Xa treatment. The samples were probed using anti-HA and anti-CA antibodies. **(B)** Western blot analyses of PNGase-F-treated cells and liberated virions from the above panel. The samples were probed using an anti-HA antibody. Stars indicate non-specific bands.

A



B



Because tetherin is intrinsically heterogeneous, due to variable glycosylation as well as dimer formation, it was difficult to assess the extent of Factor Xa cleavage in cell lysates (Figure 3.7A, cell lysates), or to unambiguously demonstrate that only cleaved tetherin fragments were present in Factor Xa liberated virions (Figure 3.7A, Factor Xa liberated virions). Therefore we treated cell and virion lysates with Peptide-N-Glycosidase F (PNGase-F) and repeated the western blot analyses under reducing conditions (Figure 3.7B). PNGase-F is an endoglycosidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose and complex oligosaccharides from N-linked glycoproteins (Maley et al., 1989). We observed that the HA-tagged proteolytic fragments (predicted molecular weights of ~20.8 kDa and ~17 kDa for C8Fac and N5Fac respectively) could be resolved from the full-length molecules (~24.7 kDa and ~23.8 kDa for C8Fac and N5Fac respectively) (Figure 3.7B). This analysis revealed that about half of the cell-associated C8Fac and N5Fac protein was cleaved by Factor Xa that was applied to the cell surface (Figure 3.7B). The incomplete cleavage may have been due to the intracellular localization of a fraction of the tetherin protein. As expected, no proteolysis of the WT tetherin protein was observed (Figure 3.7B). Notably, only the cleaved tetherin protein was found in PNGase-F-digested virion lysates, consistent with the notion that tetherin cleavage by Factor Xa was necessary for virion release in this assay (Figure 3.7B).

Overall, these results strongly suggested that tetherin is directly responsible in trapping virions, and that it does so by adopting the axial configurations depicted

in Figure 3.1A. Moreover, because HA-tagged proteolytic fragments from both C8Fac and N5Fac tetherin proteins partitioned with virions, these data suggested that both polarities depicted in Figure 3.1A are adopted during virion entrapment.

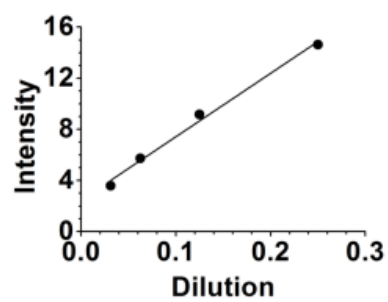
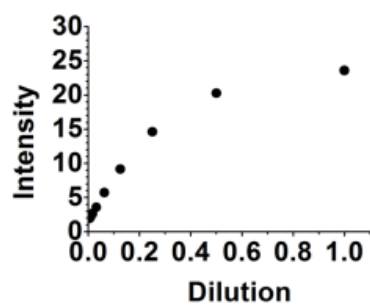
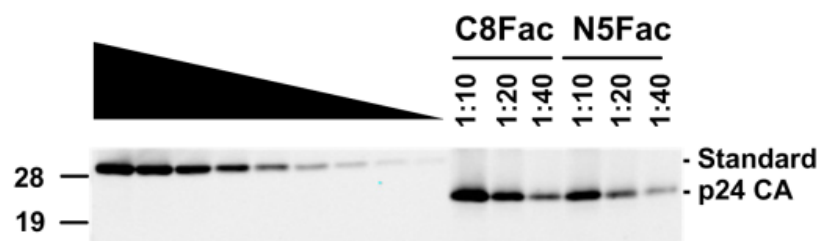
Estimates of the number of tetherin dimers that are required to trap a single virion

Because our biochemical analyses are devised to capture tetherin molecules in the act of retaining virions, we surmised that it would be possible to obtain reasonable estimates of the number of tetherin dimers that were involved in the entrapment of a single virion. Towards that purpose, we employed a quantitative western blotting approach and PNGase-F-digested virion lysates to measure the relative number of CA and HA epitopes in virions that had been trapped by the C8Fac and N5Fac proteins, and then liberated upon Factor Xa cleavage. First, we generated an appropriate internal standard protein to enable relative quantitation. This standard consisted of a fusion protein that comprised the HIV-1 p24CA protein, appended at its C-terminus with three tandem FLAG tags and an HA epitope tag. Thus this single protein included each of the epitopes that we planned to probe, at a stoichiometric ratio of 1:1:1 and could be used as a standard to compare the relative numbers of HA and CA epitopes in tethered virions released from C8Fac and N5Fac expressing cells. Specifically, serial dilutions of cell lysates expressing the HA-Flag-CA protein were run on SDS-PAGE gels, blotted onto membranes and probed with antibodies against CA and HA. The band intensities were analyzed using a LiCOR Odyssey scanner

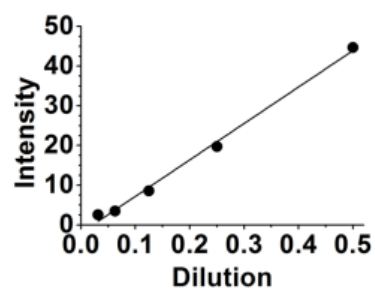
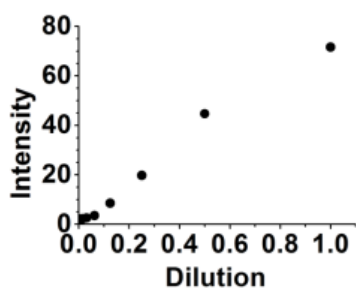
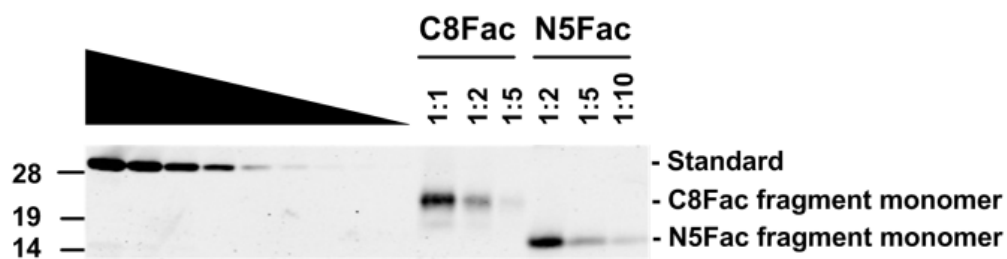
(Figures 3.8A and 3.8B), and regression analysis was performed over the linear range of signal intensities (Figures 3.8A and 3.8B). Dilutions of the PNGase-F-treated virion lysates recovered from C8Fac and N5Fac expressing cells that also yielded band intensities in the linear range of the assay were resolved on the same gel as the standard, and the relative amounts of CA and HA epitope in each samples were deduced by interpolation using the standard curves (Figures 3.8A and 3.8B).

Figure 3.8. Quantitative Western blot analyses of Factor Xa-liberated virions. **(A)** Western blot analyses of serially diluted lysates of 293T cell expressing the CA-HA-Flag protein along with the PNGase-F-treated, Factor Xa-liberated virion lysates (top panel). The samples were probed with an anti-CA antibody. The CA band intensities for the control protein were analyzed using a LiCOR Odyssey scanner and were plotted against the dilution (lower left panel). Regression analysis was performed over the linear range of signal intensities (lower right panel) and the unknown amounts of CA in the PNGase-F-treated liberated virion lysates were deduced by interpolation of the standard curve. **(B)** Same as **(A)**, except that the samples were probed with an anti-HA antibody.

A



B



HIV-1 virions have been reported to contain between 1000-5000 copies of the Gag protein, of which only a fraction contributes to core formation (Briggs et al., 2004; Layne et al., 1992; Piatak et al., 1993; Vogt and Simon, 1999; Zhu et al., 2003). We calculated our estimates of tetherin dimers per virion based on the extremities of this range (Table 3.1). Thus, if each virion contains 1000 CA protein molecules, we estimate that 16 ± 5 dimers of the N-terminus of C8Fac and 71 ± 26 dimers of the C-terminus of N5Fac tetherin dimers were associated with a single tethered virion (Table 3.1). Conversely, if a single virion contains 5000 CA epitopes, then we estimate that 80 ± 25 dimers of the N-terminus of C8Fac and 355 ± 130 dimers of the C-terminus of N5Fac tetherin dimers were associated with a single tethered virion (Table 3.1). Essentially, these estimates suggested a preference (~ 4 to 5-fold) for the insertion of the GPI-anchored tetherin C-terminus, rather than the N-terminal transmembrane domain into virions (Figure 3.6B). Note that the larger number of HA tags associated with virions in the case of N5Fac cannot be explained by differences in tetherin expression levels. In fact, there were slightly lower levels of N5Fac on cell surfaces (MFI = 8800, Figure 3.5A) as compared to the C8Fac protein (MFI = 15000, Figure 3.5A).

Table 3.1. Quantitative Western blotting analyses of virions tethered by the C8Fac and N5Fac proteins.

	Tetherin dimers per virion (assuming 1000 CA molecules/virion)		Tetherin dimers per virion (assuming 5000 CA molecules/virion)	
	C8Fac	N5Fac	C8Fac	N5Fac
Experiment 1	16	42	80	210
Experiment 2	21	80	105	400
Experiment 3	11	92	55	460
Mean \pm S.D.	16 \pm 5	71 \pm 26	80 \pm 25	355 \pm 130

Preferential insertion of tetherin C-termini into HIV-1 particles during virion entrapment

The aforementioned experiments indicated that tetherin directly tethers HIV-1 particles in an axial configuration (Figure 3.1A) and suggested that both polarities contribute to antiviral activity. However, it was possible that the two different estimates for the numbers of tetherin molecules inserted into virions with each polarity might reflect intrinsic differences in the properties of the two different tetherin molecules used (C8Fac and N5Fac). Therefore, we quantitated tetherin insertion into virions in a second set of experiments employing a single tetherin molecule that harbored two different epitope tags flanking the Factor Xa cleavage site (Flag N5Fac, Figure 3.2).

Additionally, we have previously found that Vpu-defective HIV-1 virions that accumulate on the surface of cells as a result of the action of tetherin can sometimes be tethered to each other as well as to the cell surface (Neil et al., 2008; Perez-Caballero et al., 2009) (Figure 3.9). This scenario could well be the result of virion assembly at sites on the cell surface already occupied by trapped virions, which would result in both anchors of a tetherin molecule becoming incorporated into assembling particles (Figure 3.9). It should be noted, however, that chains of tethered virions are typically observed in EM experiments wherein cells are infected at high MOIs (>5) and might thus be an experimental artifact (Figure 3.9). Over time, the accumulation of virions could potentially mask the propensity of either one of the N-or C-termini of the tetherin protein to be

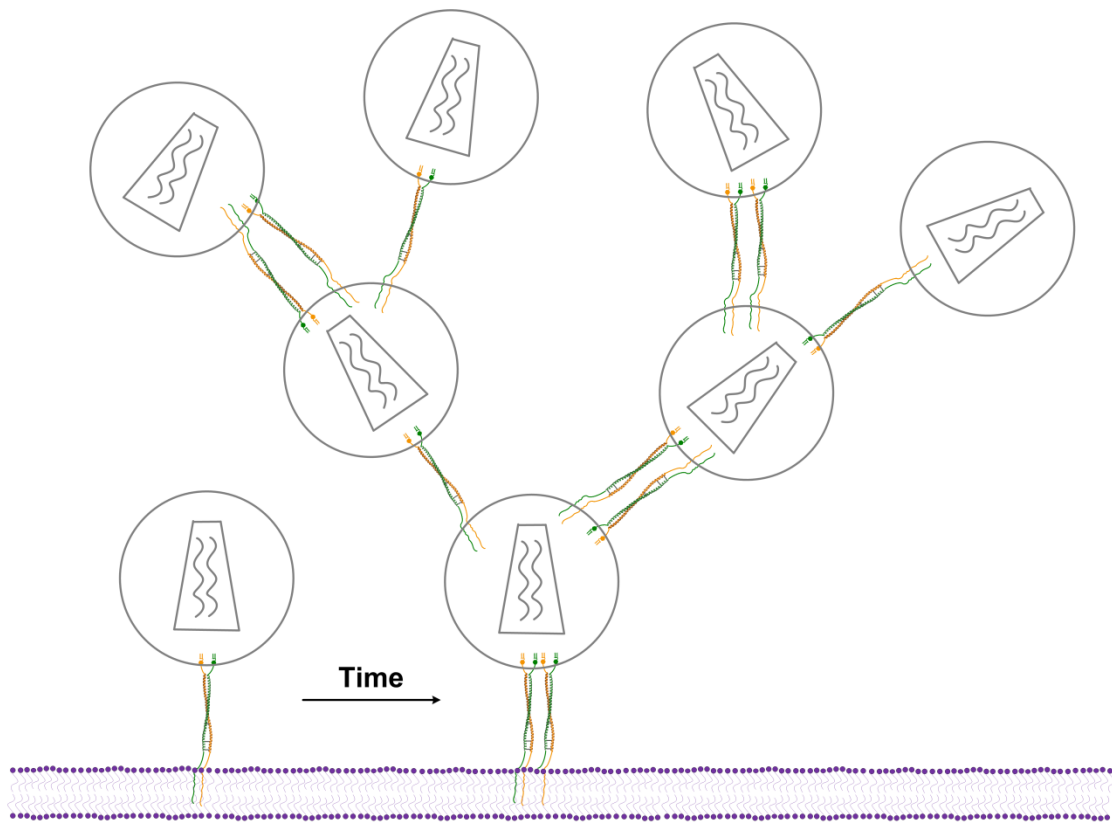


Figure 3.9. Schematic representation of the budding of HIV-1 viral particles. Because virions are not only linked to the plasma membrane, but to each other, tetherin molecules incorporate both anchors into trapped virions. Thus, a preference for inserting the C-terminal GPI anchor into virions during particle assembly would eventually get lost over time.

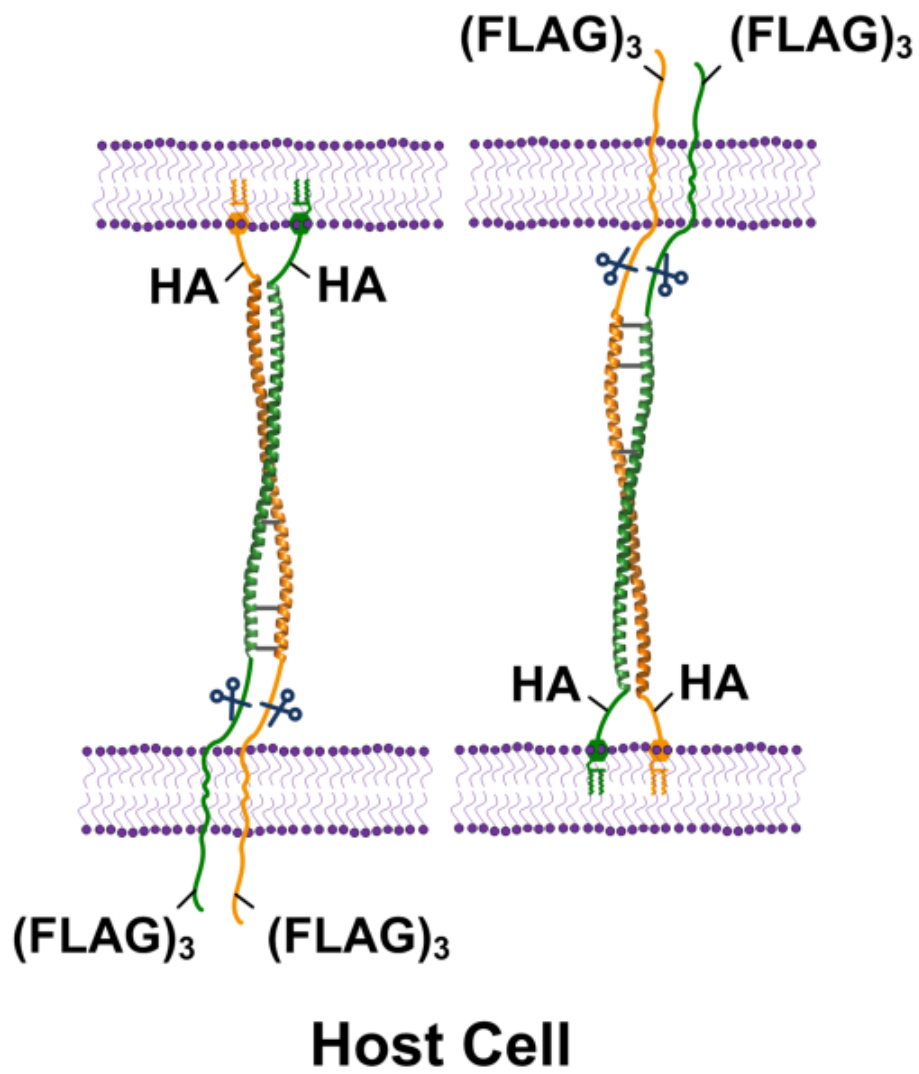
preferentially inserted into virions (Figure 3.9). Thus, we treated the surface of cells expressing Flag N5Fac with Factor Xa at predetermined time intervals following infection with HIV-1 Δ Vpu, and quantified HA- and FLAG-tagged proteolytic fragments in liberated virions.

The Factor Xa site is positioned N-terminal to the sites of N-linked glycosylation as well as to the extracellular cysteine residues in the Flag N5Fac molecule (Figures 3.2 and 3.10A). We observed that Factor Xa cleavage of the 65-70 kDa dimeric, glycosylated Flag N5Fac protein yields a cell associated dimeric, glycosylated ~50-55 kDa α -HA reactive species as well as a cell associated monomeric, nonglycosylated 10 kDa α -FLAG reactive species (Figure 3.10B). This is intuitive given that the extracellular and intracellular redox environments are non-reducing and reducing respectively. The HIV-1 Gag protein became detectable in infected Flag N5Fac-expressing cell lysates at ~24h after infection and levels progressively increased with time thereafter (Figure 3.10B). Treatment of these infected cells with Factor Xa resulted in a time dependent increase in the amount of recovered virions (Figure 3.10B). Notably, both the dimeric glycosylated ~50-55 kDa α -HA reactive species and the 10kDa α -FLAG reactive species were observed in virions, and their levels in the virion fraction increased with time, approximately in parallel with the increasing yield of Factor Xa liberated virions (Figure 3.10B).

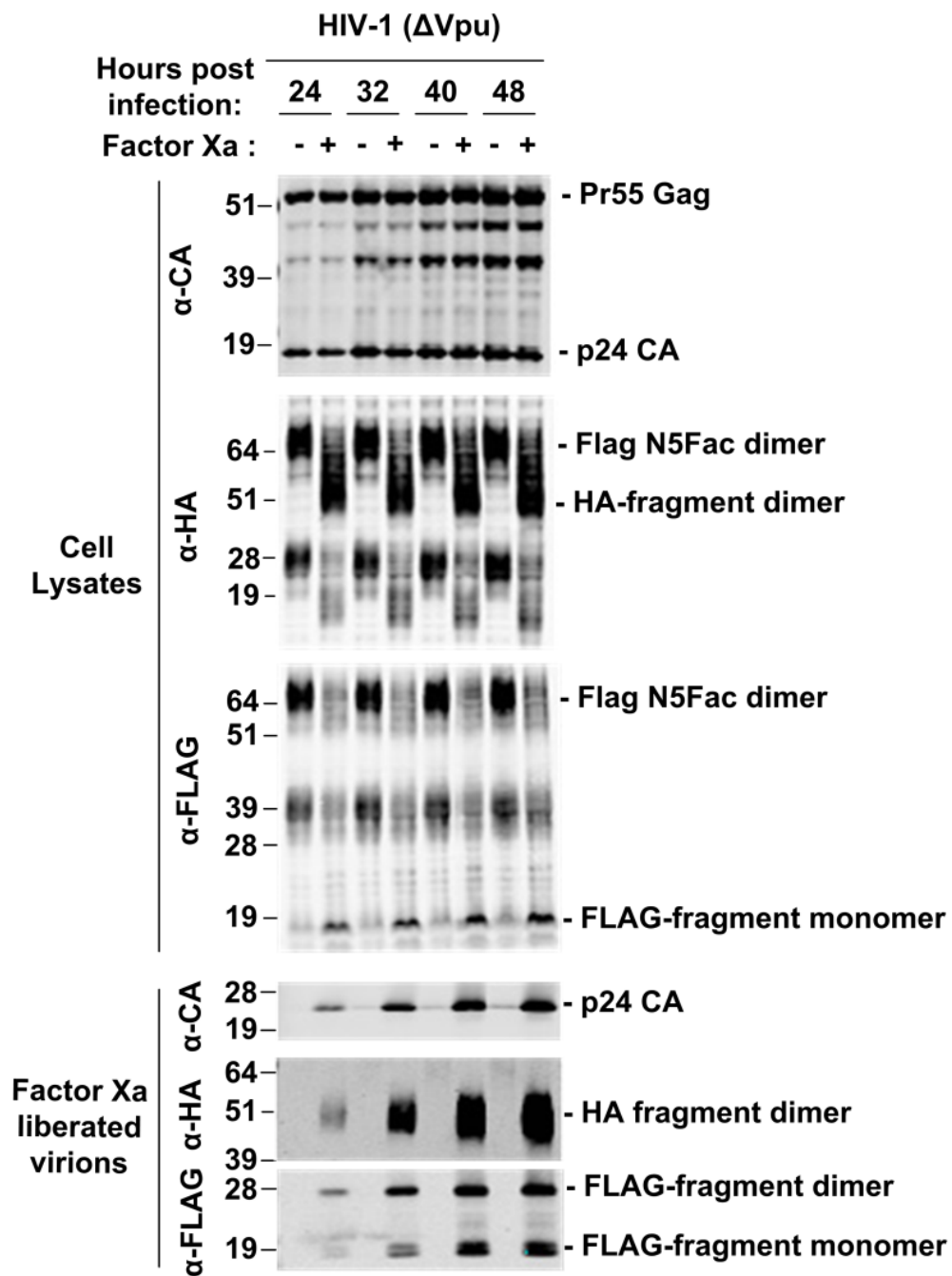
Figure 3.10. Factor Xa-induced liberation of virions trapped by the FlagN5Fac tetherin protein. (A) Schematic representation of the polarities that would be adopted by the Flag N5Fac protein if HA- or FLAG-tagged proteolytic fragments are observed to partition with liberated virions. **(B)** Western blot analyses of 293T cells and liberated virions, obtained by the protease treatment of Flag N5Fac cells at various time points following infection. The samples were probed using anti-HA, anti-FLAG and anti-CA antibodies.

A

Flag N5Fac Virion



B

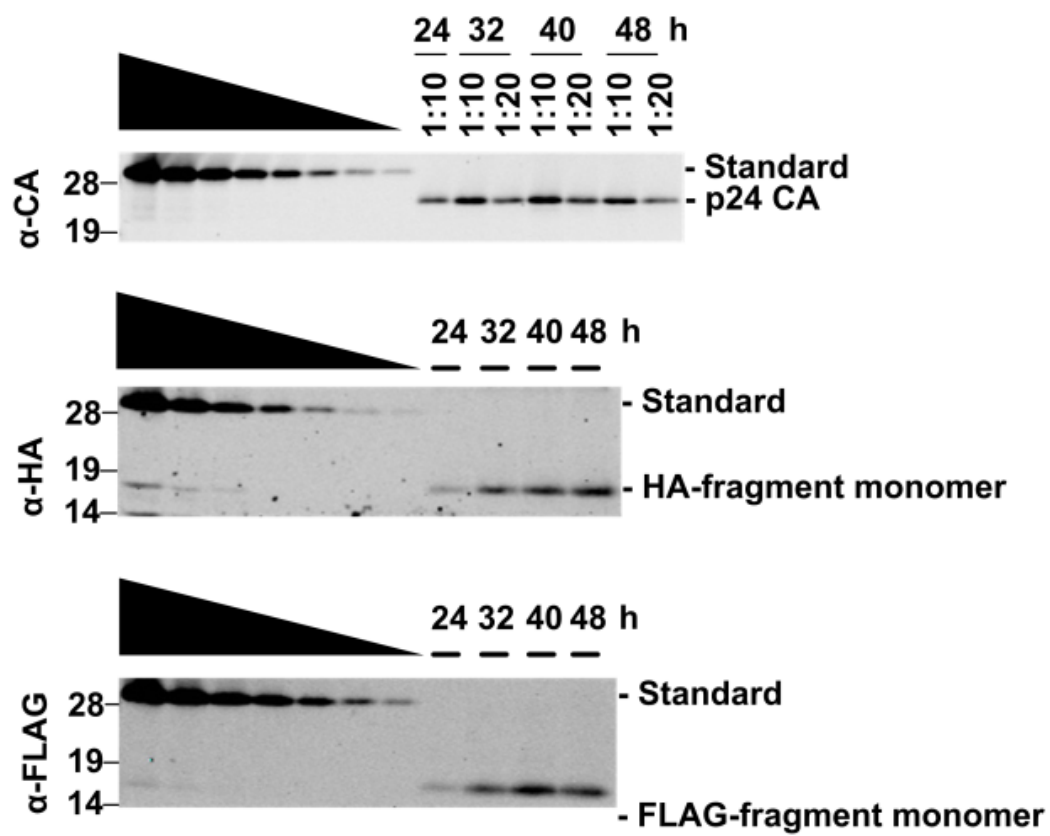


It is interesting that the N-terminal FLAG tagged fragment of the Flag N5Fac protein was also found in virions in a form that was consistent with the formation of dimers (Figure 3.10B). We hypothesize that the cytoplasmic tail of tetherin contains two cysteine residues that might form disulphide bonds in the interior of virions, which is presumably an oxidizing environment. Consistent with this idea, only the smaller of the two Flag tagged species was observed when virion lysates were subjected to SDS PAGE gel electrophoresis under reducing conditions (Figure 3.11A). Additionally, the dimeric glycosylated ~50-55 kDa α -HA reactive species collapsed to a single ~17 kDa band when samples were deglycosylated with PNGase and reduced (Figure 3.11A).

We used quantitative western blot analyses of PNGase-F-digested virion lysates to estimate the number of copies of HA- and FLAG-tagged proteolytic fragments per trapped virion (Figures 3.11A and 3.11B). Again, we used the FLAG-HA-CA fusion protein as an internal standard to determine the relative numbers of HA, FLAG and CA epitopes in the virions liberated from Flag N5Fac expressing cells. We observed that tethered virions could be recovered from the surface of Flag N5Fac expressing cells beginning at 24h after infection, i.e. when the HIV-1 Gag protein was detectable in cell lysates. However, we could not make reliable estimates of the HA and FLAG fragments at this time point, as they were present at low levels that were close to the limit of detection. We were able to make reasonably robust estimates of the levels of incorporation of HA- and FLAG-tagged fragments into virions beginning at 32h after infection (Figures 3.11A and 3.11B).

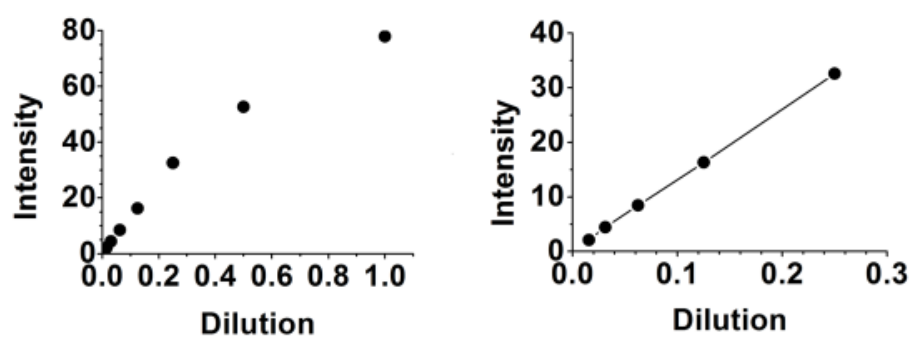
Figure 3.11. Quantitative Western blot analysis of Factor Xa-liberated Flag N5Fac virions. (A) Western blot analyses of 293T cells expressing serial dilutions of the CA-HA-Flag protein and the PNGase-F-treated liberated virion lysates. The samples were probed with anti-CA, anti-HA and anti-FLAG antibodies. (B) The CA, HA and FLAG band intensities were determined using a LiCOR Odyssey scanner and were plotted against the dilutions (left panels). Regression analysis was performed over the linear range of signal intensities (right panels), and the unknown amounts of CA, HA and FLAG epitopes in the PNGase-F-treated, Factor Xa-liberated virion lysates were deduced by interpolation from the standard curves.

A

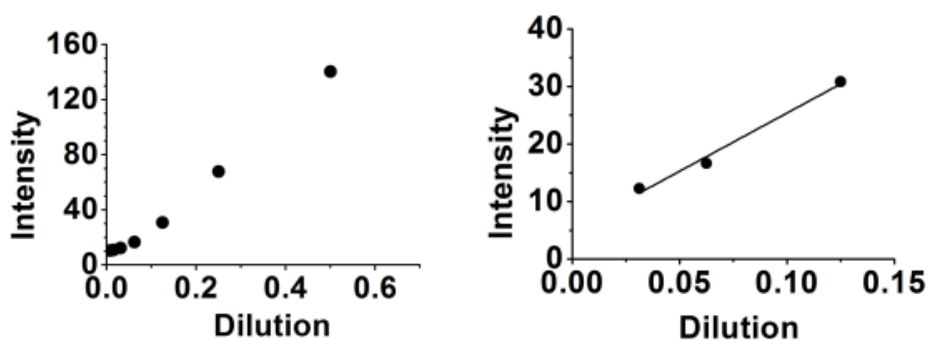


B

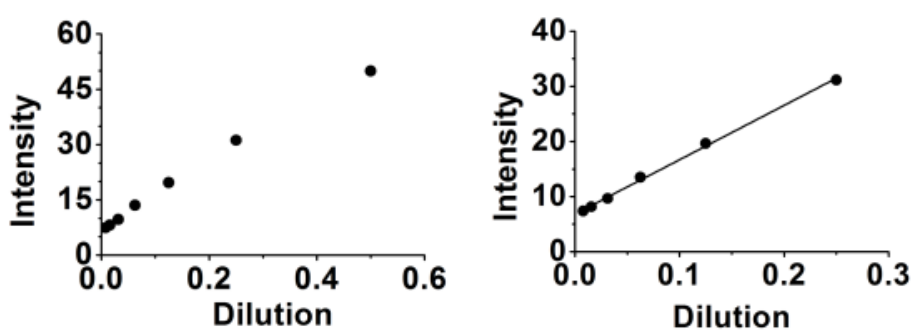
CA



HA



FLAG



Crucially, the number of FLAG-tagged dimers that were estimated to be present in virions (assuming 1000 CA molecules per virion) tethered by Flag N5Fac (11 ± 3 [at 32h] to 16 ± 6 [at 48h], Table 3.2) correlated quite well with the number of HA-tagged dimers present in virions tethered by C8Fac (16 ± 5 [at 48h], Table 3.1). Similarly, the number of HA-tagged dimers in tethered virions recovered from the Flag N5Fac expressing cells (34 ± 18 [at 32h] to 55 ± 28 [at 48h]) (Table 3.2) correlated quite well with the number of copies of HA-tagged dimers in tethered virions recovered from the N5Fac expressing cells (71 ± 26 at 48h) (Table 3.1). These data suggested that our earlier analyses could not have been biased by intrinsic differences between the C8Fac and N5Fac molecules.

In particular, we estimated that the virions liberated from Flag N5Fac expressing cells carried ~3 to 4-fold more HA tags than FLAG tags, again suggesting that axially configured tetherin dimers infiltrate assembling particles, with a modest tendency to embed their C-termini rather than their N-termini in liberated virions. Also noticeable was a marginal trend for the appearance of increasing numbers of tetherin molecules per virion over time. This trend was not statistically significant and could be due to some unknown bias in the measurements. Another interpretation is that virions with fewer numbers of tetherin molecules are constitutively released, leading to the accumulation of virions with greater numbers of tetherin molecules on the surface of cells.

Table 3.2. Quantitative Western blotting analyses of virions tethered by the Flag N5Fac protein.

Tetherin dimers per virion (assuming 1000 CA molecules/virion)						
Hours post infection	HA tags			FLAG tags		
	32	40	48	32	40	48
Experiment 1	47	39	66	14	18	22
Experiment 2	42	43	76	10	11	13
Experiment 3	14	18	23	9	10	12
Mean \pm S.D.	34 \pm 18	34 \pm 14	55 \pm 28	11 \pm 3	13 \pm 4	16 \pm 6

Efficient proteolysis and liberation of virions by Factor Xa

One potential caveat of our experiments is that alternate configurations of tetherin might exist that are resistant to the action of Factor Xa. For instance, higher order configurations of tetherin that are tightly packed might limit the accessibility of Factor Xa to its cleavage site. Such subpopulations of tetherin would then continue to trap virions at the cell surface, and would be unaccounted for in our analyses. To investigate this further, we treated the stable cells expressing the panel of modified tetherin proteins with Factor Xa or subtilisin A, and compared the yield of liberated virions via western blotting analyses. Because subtilisin A works rather non-specifically, it is expected to degrade all configurations of tetherin and would thus release all trapped virions. Reassuringly, we observed that similar levels of virions were liberated by Factor Xa as compared to subtilisin A at 48h after infection for all the modified tetherin proteins (Figure 3.12). These findings suggest that Factor Xa is efficient at liberating virions from the cell surface and discount the likelihood that alternatively configured tetherin molecules continue to trap virions.

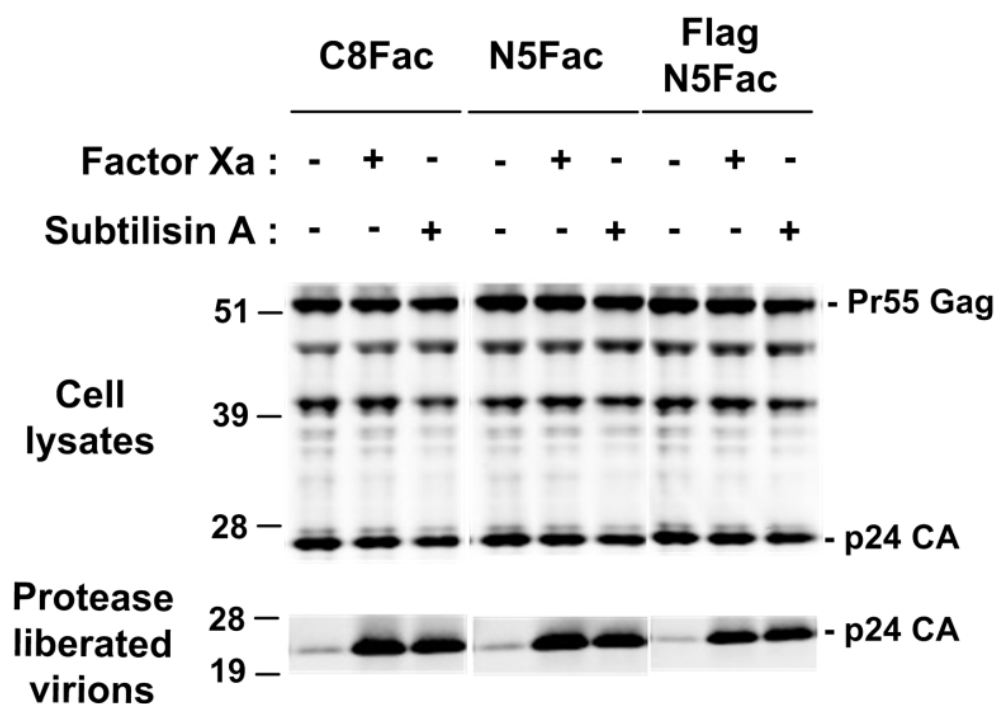


Figure 3.12. Efficient proteolysis and liberation of virions by Factor Xa. Western blot analyses of 293T cells stably expressing the modified tetherin proteins and virions liberated upon Factor Xa or subtilisin A treatment. All samples were probed using an anti-CA antibody.

The ESCRT pathway is dispensable for virion entrapment

Previously, it has been hypothesized that various components of the ESCRT pathway might be required for tetherin to carry out its biological activity, presumably because they are active during similar stages of particle assembly (Grover et al., 2013). However, an artificial tetherin construct that bears no sequence homology to WT tetherin, is proficient at trapping virions, making it highly unlikely that recognition of a cofactor is necessary for tetherin function (Perez-Caballero et al., 2009). Furthermore, the ESCRT pathway is recruited to sever the neck of the stalk that separates the cell and viral membranes, whereas tetherin acts after budding and traps nascent virions whose membranes are discontinuous with that of the infected cell membrane. Thus, the ESCRT pathway and tetherin function at different stages of viral assembly and are not expected to influence each other.

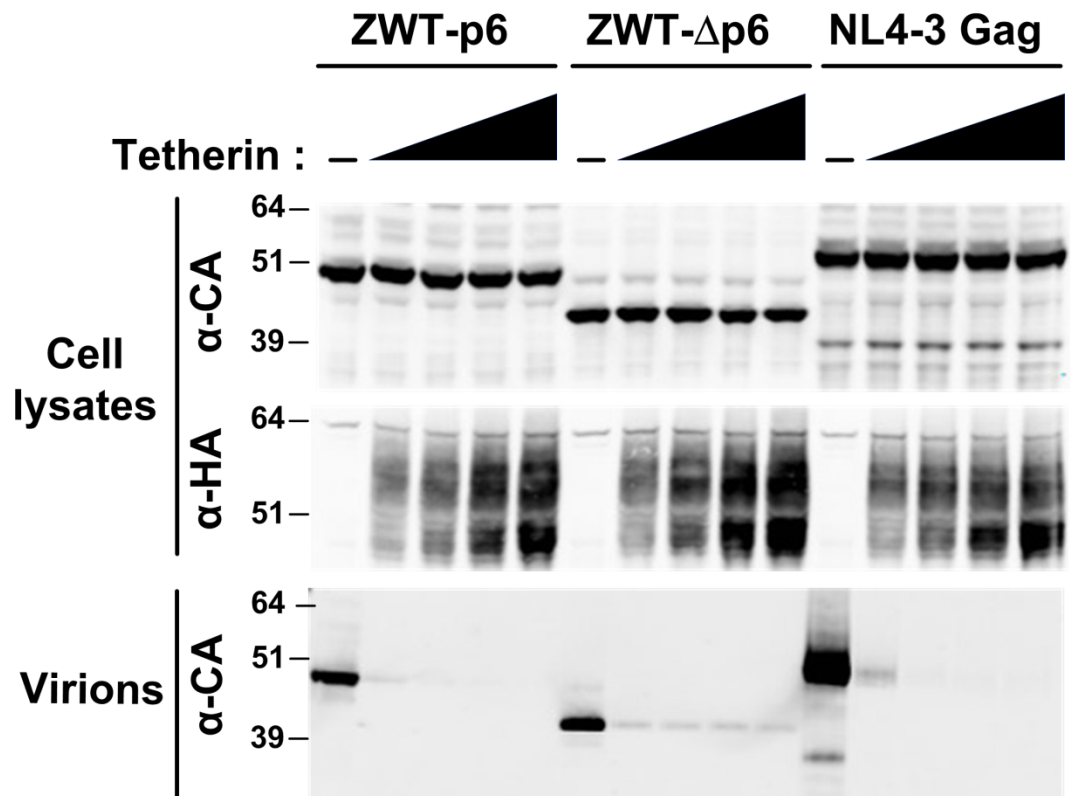
To resolve this controversy, we endeavored to determine if tetherin-mediated virion entrapment was dependent on the ESCRT pathway. To that end, we asked if tetherin is proficient at restricting virions that have been assembled from a novel Gag protein and released thereafter in an ESCRT pathway-independent fashion. Such a protein (ZWT-p6), in which the nucleocapsid and p1 proteins are replaced with the leucine zipper domain of the yeast transcription factor Gcn4, has the propensity to multimerize and form virus-like particles (VLPs) (Accola et al., 2000) (Figure 13.3A). We also generated its p6-deficient counterpart (ZWT- Δ p6; Figure 13.3A) by substituting the start codon of p6 with a stop codon. Thus, both the ZWT-p6 and ZWT- Δ p6 proteins can assemble efficiently without the

Figure 3.13. A minimal Gag protein bearing the leucine zipper domain of the yeast transcription factor Gcn4 and lacking late domains is efficiently restricted by wild type tetherin. (A) Schematic representation of the NL4-3 Gag construct and its derivative ZWT-p6 in which the nucleocapsid protein is replaced by the leucine zipper domain. The ZWT- Δ p6 has a stop codon that abolishes p6 expression. The leucine zipper domain is indicated using red and green helices. **(B)** Western blot analyses of 293T cell lysates and virions generated following cotransfection of 293T cells with 150 ng of the indicated Gag construct and increasing doses of tetherin plasmid. The samples were probed with anti-CA and anti-HA antibodies.

A



B



ESCRT pathway, but the ZWT- Δ p6 protein is impaired in its ability to recruit the ESCRT pathway (Figure 13.3A). The underlying logic is that the ESCRT pathway would be dispensable if tetherin could restrict both Gag proteins with equal efficiency. We included NL4-3 Gag as a control (Figure 13.3A).

All three Gag proteins were expressed by using a Vpu-deficient version of the expression vector pCRV-1. pCRV-1 is a hybrid expression vector of pCR3.1 and the HIV provirus V1-B. In this system, Gag, Rev and Tat are the only viral proteins that are expressed. 293T cells were co-transfected with the panel of Gag proteins along with varying doses of a plasmid expressing tetherin. The expression levels of the Gag proteins were uniform in cell lysates, and the p6 lesion in ZWT- Δ p6 was reflected by a protein of faster electrophoretic mobility (Figure 13.3B).

In the absence of tetherin, there was a substantial accumulation of VLPs of all three Gag proteins in the supernatant (Figure 13.3B). As expected, NL4-3 Gag VLPs were potently restricted by tetherin in a dose dependent manner (Figure 13.3B). Notably, tetherin was equally efficient at trapping the ZWT-p6 and ZWT- Δ p6 VLPs as compared to NL4-3 VLPs (Figure 13.3B). Thus, this result indicates that the ESCRT pathway is dispensable for virion entrapment.

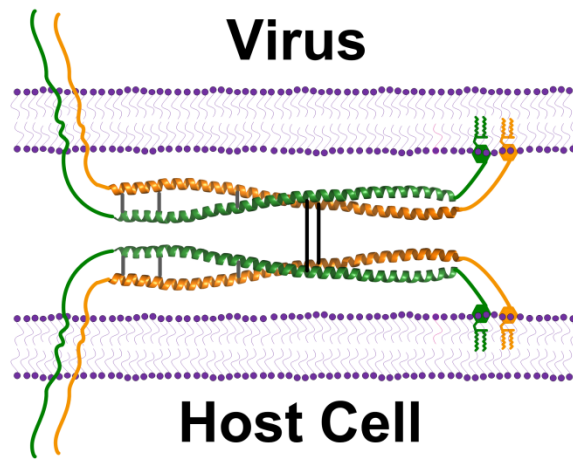
Crosslinking analyses to assess whether higher order multimers contribute to virion entrapment

A number of structural studies have suggested that tetherin forms higher order multimers (Schubert et al., 2010; Yang et al., 2010). However, as described in the Introduction (Chapter 1), the functional relevance of higher order multimer formation is strongly disputed. To determine whether higher order tetherin multimers form, and whether they might contribute to tethering in equatorial or axial configurations, we formulated an approach that coupled cysteine-scanning mutagenesis and the Factor Xa stripping assay. Specifically, we generated a panel of 19 tetherin proteins in which certain amino acid residues in the extracellular coiled-coil domain of tetherin were substituted to cysteine. These mutants were made in the C8Fac background to avail of the Factor Xa cleavage site. Our mutagenesis strategy, guided by structural analyses (Yang et al., 2010), targeted amino acids that are present on the exposed surfaces of the coiled-coil domain. These amino acids can potentially promote the association of higher order multimers via inter-dimer disulphide bridges between proximal cysteine residues.

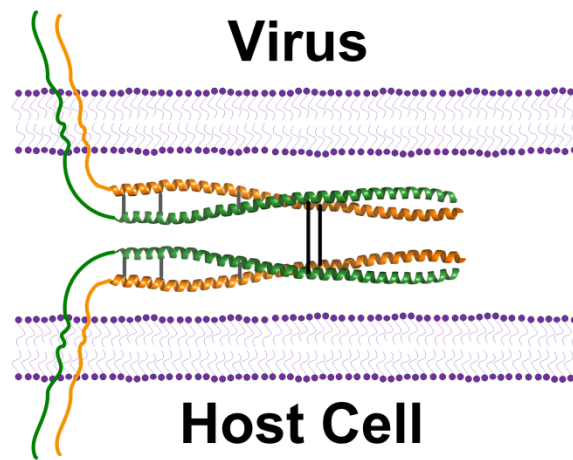
Our earlier efforts have already demonstrated that virions trapped by axially configured tetherin molecules are liberated upon Factor Xa treatment. In contrast, if higher order multimers exist, they would remain intact due to the disulphide bridges and would only be disrupted upon β -mercaptoethanol (β -ME) treatment (Figure 3.14A). Thus, cells that stably express the cysteine mutants were treated with Factor Xa followed by β -ME and the yield of virions was

Figure 3.14. Factor Xa-induced liberation of virions trapped by a crosslinkable mutant of tetherin. **(A)** Experimental strategy to assess if the tetherin molecules in the equatorial configuration contribute to HIV-1 virion entrapment. HIV-1 virions that are trapped by axially configured tetherin molecules would be released upon treatment of Factor Xa alone. Conversely, equatorially configured tetherin molecules will only be released upon treatment with Factor Xa followed by β -mercaptoethanol (β -ME). The cysteine residues that drive inter-dimer interactions are indicated as black lines. **(B)** Western blot analyses of 293T cell lysates following transfection with 150 ng of the indicated plasmids. The samples were probed with an anti-HA antibody. **(C)** Western blot analyses of virions, 293T cells stably expressing Fac4 and Q142C tetherin proteins, and virions liberated upon Factor Xa and β -ME treatment and probed using anti-CA and anti-HA antibodies.

A



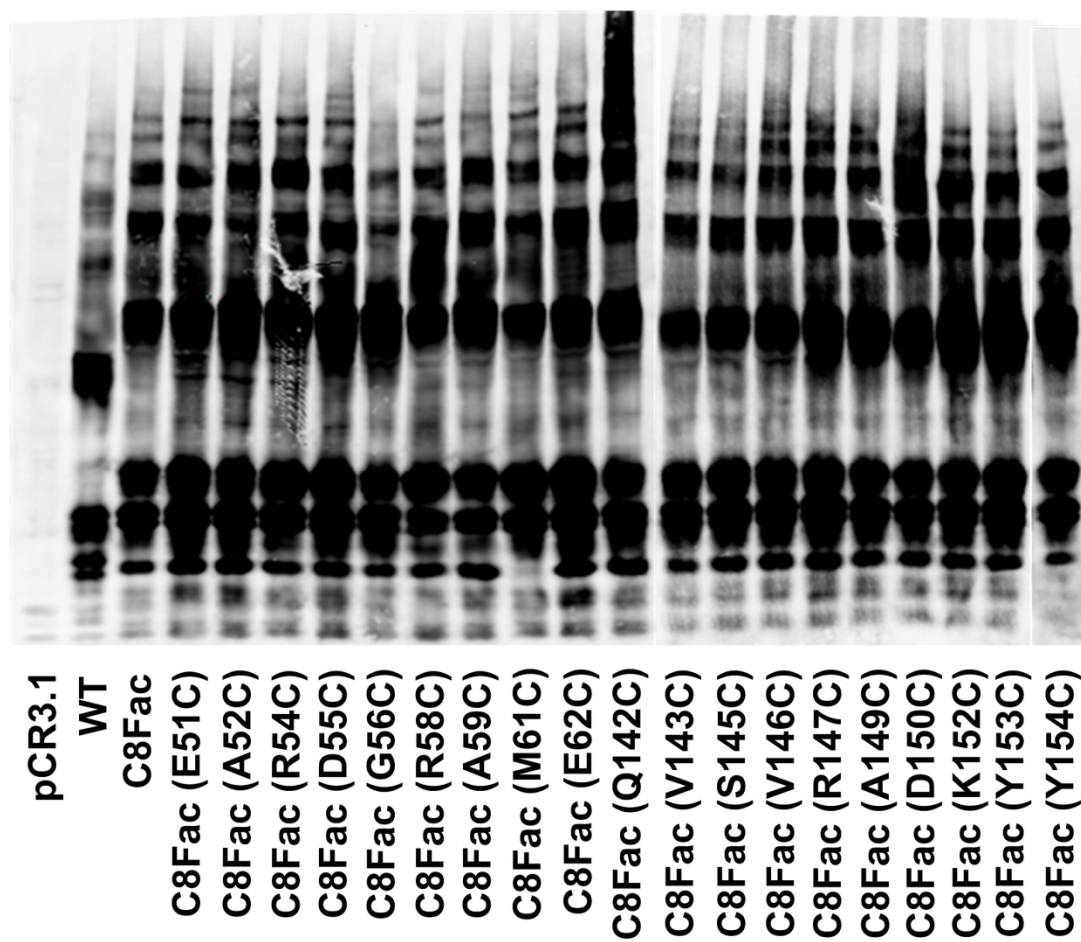
Factor Xa



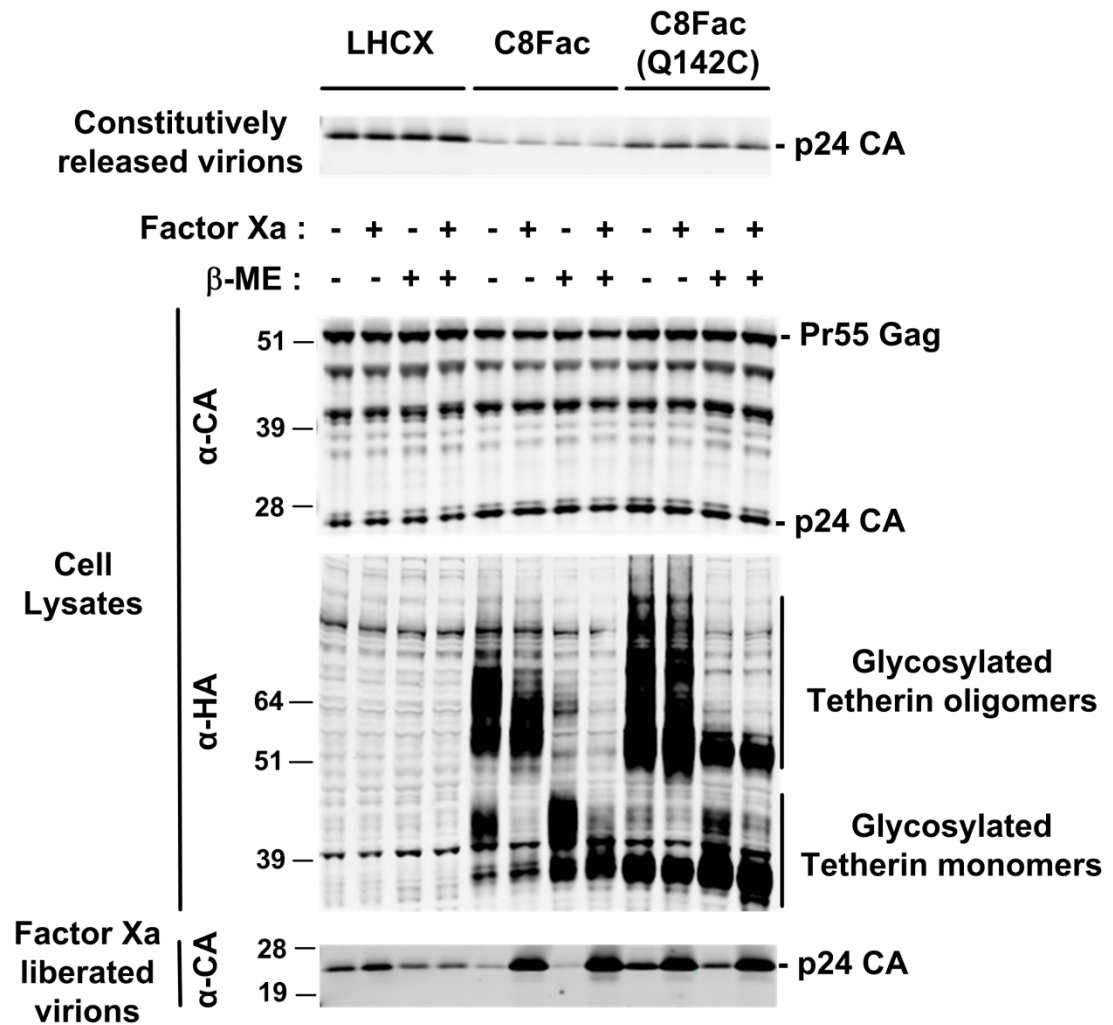
β -ME

Any liberated virions?

B



C



compared with control C8Fac cells (Figure 3.14A). The underlying rationale was that if β -ME triggered the release of virions that were trapped by the cysteine mutants, but not the C8Fac protein, then we could infer that tetherin molecules configured as higher order multimers contribute to virion entrapment.

Initially, we expressed each mutant protein to screen for candidates that would crosslink via inter dimer disulfide bridges. As a control, we also included the parental non-crosslinkable C8Fac protein. We observed that one mutant, Q142C, had the propensity to form higher order multimers (Figure 3.14B).

Next, we generated 293T cells that stably expressed the Q142C mutant and performed an extended version of the Factor Xa stripping assay (Figure 3.14C). Briefly, the cells were washed twice with Factor Xa buffer after harvesting Factor Xa-liberated virions, treated with 0.1% β -ME for 5 minutes, and any liberated virions were harvested. Note that this concentration of β -ME is low enough that it is not expected to cause cell lysis (Binley et al., 2000).

As before, infected tetherin-deficient cells constitutively released substantially high levels of particulate capsid into the culture supernatant, while virion yield from cells expressing the C8Fac protein was markedly diminished (Figure 3.14C). In contrast, the C8Fac (Q142C) protein was markedly impaired for antiviral activity, suggesting that perhaps higher order multimer formation might be detrimental for the antiviral activity of tetherin (Figure 3.14C). The expression levels of HIV-1 Gag expression in cell lysates were uniform, indicating that levels of infection were uniform across all samples (Figure 3.14C). The C8Fac (Q142C)

protein formed higher order multimers in cell lysates (Figure 3.14C). Importantly, β -ME treatment did not cause cell lysis and resulted in collapse of C8Fac dimers and C8Fac (Q142C) oligomers into the monomeric species, indicating that the concentration of β -ME used in the assay was optimal for reduction of disulphide bridges (Figure 3.14C). A sizable fraction (~30%) of the C8Fac (Q142C) protein in cell lysates was found to be resistant to the action of β -ME, and presumably reflects aggregation of mis-folded protein (Figure 3.14C).

As expected, low levels of particulate capsid protein were released from tetherin-negative cells (Figure 3.14C). In contrast, even lower levels of capsid were released when C8Fac cells were incubated in buffer alone, and Factor Xa treatment resulted in the liberation of a substantial amount of capsid (Figure 3.14C). Higher amounts of capsid were released into the supernatant when C8Fac (Q142C) cells were incubated during the 2h incubation in buffer alone, which reflects its modest antiviral activity (Figure 3.14C). Notably, there was no enhancement in the yield of extracellular virions when C8Fac (Q142C) cells were treated with β -ME (Figure 3.14C). These results suggest that higher order multimers of tetherin, if extant, do not contribute to HIV-1 restriction.

Unfortunately, repeated attempts to interrogate tetherin species in virions were not fruitful and the data that we obtained was often difficult to interpret, or was not always reproducible. Thus, our results did support the notion that tetherin molecules prefer to adopt the axial configuration during virion entrapment, but we

were forced to abandon this line of inquiry due to the ambiguity in the experimental results.

Long heterologous coiled coils as an alternative strategy to determine tetherin configuration

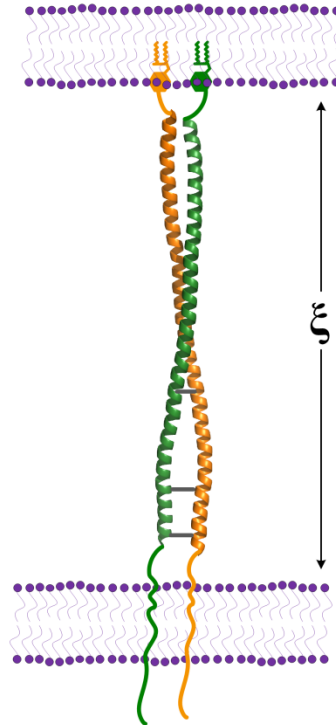
In an alternate approach, we generated chimeric tetherin proteins in which a portion of the tetherin ectodomain was substituted by heterologous longer coiled coils. We reasoned that such a manipulation would increase the separation between the host cell and viral membranes (ξ) if virions are trapped by axially configured tetherin molecules (Figure 3.15, top panel). In contrast, ξ would be unaffected if virions are trapped by dimers or higher order oligomers of tetherin molecules that are equatorially configured (Figure 3.15, bottom panel). Hence, we hypothesized that we could use electron microscopy (EM) to visualize whether any one of the configurations predominates during virion entrapment.

The coiled-coil domains in proteins such as tropomyosin, golgin-84, CASP, golgin-97, TATA modulatory factor 1 (TMF1), and early endosome antigen 1 (EEA1) are three- to ten-fold greater in length as compared to the ectodomain in WT tetherin. We appended these coiled-coil sequences C-terminal to the glycosylation sites and cysteine residues in tetherin, and N-terminal to its GPI anchor. Care was taken to fuse these coiled-coils in register with the remnants of the tetherin coiled-coil.

Figure 3.15. An alternate strategy using hybrid long coiled coils and electron microscopy to discriminate between axially and equatorially configured tetherin molecules. (A) Replacement of the coiled-coil domain of tetherin with a longer coiled-coil would be expected to increase the separation between the host cell and viral membranes (ξ) in the axial configuration (top panel). In contrast, ξ would be unaffected when virions are trapped by dimers or higher order oligomers of tetherin molecules that are equatorially configured (bottom panel). **(B)** 293T cells were cotransfected with wild-type (HIV-1 (WT)) or Vpu-deficient (HIV-1 Δ Vpu) proviral pNL4-3 plasmids along with varying amounts of wild type tetherin and a variant in which part of the ectodomain is replaced with the tropomyosin coiled-coil (Tropo). Infectious virion yield was measured by inoculating HeLa-TZM indicator cells with culture supernatant and is given as the logarithm to the base 10 of the relative light units (RLU). **(C)** Western blot analyses of transfected 293T cell lysates and virions corresponding to the above panel. All samples were probed with an anti-CA antibody. The numbers at the bottom represent measurement of CA protein levels in virion pellets (LI-COR).

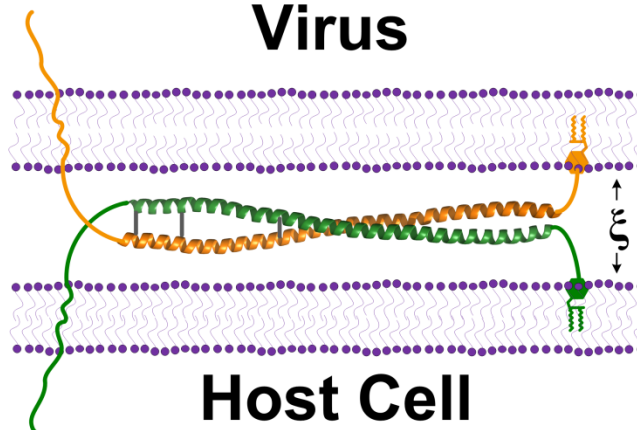
A

Virus



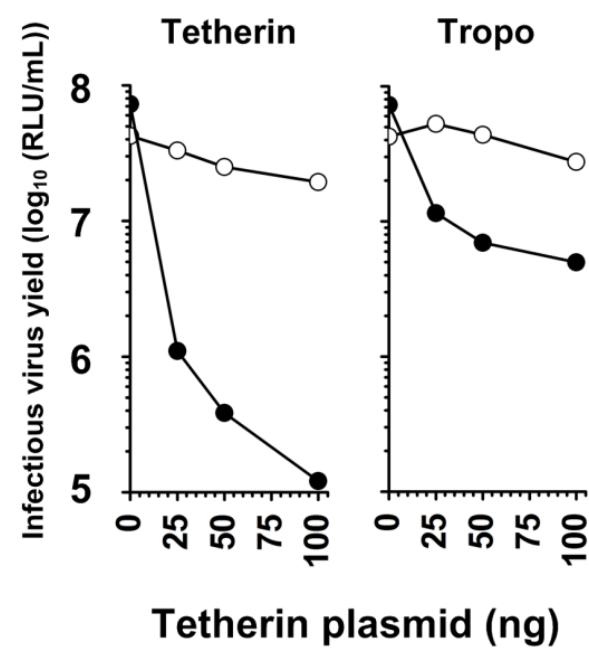
Host Cell

Virus

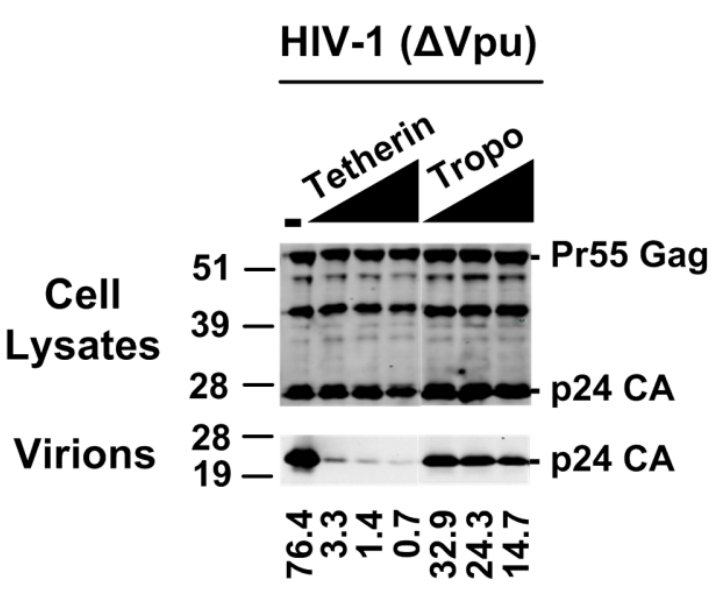


Host Cell

B



C



Analysis of antiviral activity of the panel of chimeric tetherin proteins revealed that the tetherin-tropomyosin protein (Tropo) was only modestly proficient at trapping virions as compared to Tetherin (Figures 3.15A and 3.15B). The other proteins, which were longer than Tropo, were poorly expressed (data not shown). We suspected that glycosylation of the long coiled coils, which are normally destined for the Golgi complex or endosomes, might be attenuating protein expression. Hence, we generated proteins in which we replaced potentially glycosylated asparagine residues to serine *en masse*. Although some of the proteins were expressed at slightly higher levels, all of them were impaired in their antiviral activity as compared to Tetherin (data not shown). We also attempted to improve the antiviral activity of the Tropomyosin-based proteins, by fusing the tropomyosin coiled-coil domain into the third, fourth, fifth, and sixth C-terminal spacers of C8Fac protein. Such proteins would mimic bacterial virulence factors wherein stretches of coiled-coil regions are separated by other domains or unstructured regions (Delahay and Frankel, 2002). However, analysis of the antiviral activity of these tropomyosin variant revealed that these proteins were also only modestly capable of inhibiting particulate capsid from being released into the supernatant (data not shown). Thus, any attempts to increase the length of the ectodomain came with the penalty of impaired antiviral activity. Most likely, the differences in length between the coiled-coil domains in the Tropo and Tetherin proteins would have been too small (~3-fold) to discern any differences in ξ using EM, and it might have been difficult to discriminate between axially and

equatorially configured tetherin molecules. Hence, we decided to abandon this line of investigation.

Summary

In the work discussed above, we employed biochemical assays and engineered tetherin proteins to demonstrate conclusively that virion tethers are composed of the tetherin protein itself, and to elucidate the configuration and topology that tetherin adopts during virion entrapment. We demonstrated that tetherin dimers adopt an “axial” configuration, in which pairs of transmembrane domains or pairs of glycosylphosphatidyl inositol anchors are inserted into assembling particles, while the remaining pair of membrane anchors remains embedded in the infected cell membrane. We used quantitative western blotting to determine that a few dozen tetherin dimers are used to trap each virion particle, and that there is ~3-5 fold preference for the insertion of glycosylphosphatidyl inositol anchors rather than transmembrane domains into tethered virions. We also used biochemical assays to demonstrate unequivocally that the ESCRT pathway is dispensable for HIV-1 restriction. In concert, these results demonstrated that axially configured tetherin homodimers, acting independently of the ESCRT pathway, are directly responsible for trapping virions at the cell surface.

Chapter IV Evolutionary origins of tetherin

In this chapter, we employed computational and biochemical analyses to trace the evolutionary origins of tetherin. How this restriction factor emerged in mammalian genomes has been utterly mysterious, primarily because there appear to be no candidate proteins of similar structure or sequence that might have given rise to tetherin. In this work, we build a case that tetherin arose via duplication of the neighboring PLVAP/PV1 gene. This project was conducted in collaboration with Daniel Blanco-Melo, a bioinformatics Ph.D. student in our laboratory, and the results presented here are being submitted as a research article. Our findings offer important insights into the remarkable capacity of our genomes to innovate novel functions from pre-existing genetic raw material using gene duplication.

Tetherin might have shared a common ancestor with PV1

One question that has captured the imagination of virologists is: how do antiviral factors evolve? Put another way, what are the mechanisms whereby our genomes innovate to produce novel functions? As described in the Introduction (Chapter 1), other restriction factors such as APOBEC3G and SAMHD1 have likely arisen via duplication of pre-existing genes whose regular cellular functions were exploited for antiviral activity by minor adaptations. In the case of TRIM5, it is possible that it arose via duplication and divergence of one of the ~100 family members of the TRIM family of proteins. In contrast, virtually nothing is known

about the ancestry of tetherin. In fact, similarity searches for possible ancestors of tetherin yield no candidate genes of similar sequence and structure. Moreover, tetherin is dispensable for viability, i.e. it does not perform a developmental or metabolic role, and is only expressed in most cell types upon stimulation with interferon. Thus, it is difficult to envisage a scenario wherein tetherin arose from a gene with a related cellular function. Rather, these observations are compatible with a simpler model- that tetherin emerged *de novo*, and purely as an antiviral gene.

In a seminal monograph, Susumu Ohno proposed that gene duplication creates new genetic “raw material” that is subjected to the action of evolutionary forces such as mutation, drift and selection to acquire novel functions (Ohno, 1970). Clearly, tetherin could not have arisen via retrotransposition, because it contains introns. Moreover, the other restriction factors probably emerged via gene duplication and divergence from closely related family members. Thus, we hypothesized that tetherin might have also evolved via gene duplication, and initiated a hunt for candidate genes that might have shared a common ancestor with tetherin. Because duplicated genes tend to lie adjacent to each other in the genome (Pan and Zhang, 2008), we inspected the genes that flank tetherin in human chromosome 19, i.e. MVB12A and PLVAP (hereafter referred to as PV1). We promptly discounted MVB12A because it is a component of the ESCRT-I complex and it has no predicted transmembrane domain (Morita et al., 2007). PV1 encodes for a type II integral membrane glycoprotein, whose expression is restricted to a subset of endothelial cells. It is an essential component of the

stomatal diaphragms of caveolae and of the diaphragms of fenestrae and transendothelial channels, and this seems to be its sole cellular function (Stan et al., 1999).

Remarkably, PV1 appeared to possess most of the topological features that are peculiar to tetherin, and are essential for its antiviral activity. Both proteins are type II integral membrane glycoproteins and comprise of a short N-terminal cytoplasmic tail, a single pass transmembrane domain, and an extracellular domain which is predominantly coiled-coil (Figure 4.1). Moreover, both proteins are glycosylated and possess cysteine residues that drive efficient parallel homodimer formation via disulfide bridges (Figure 4.1). Because PV1 and tetherin share such overt structural similarity and are located adjacent to each other, we surmised that tetherin might have shared a common ancestor with PV1, and that it emerged via gene duplication of that ancestor. Thereafter, the fortuitous acquisition of a sequence encoding a second membrane anchor (GPI anchor), might have contributed to the emergence of tetherin.

Phylogenetic analyses of PV1 and tetherin

We first sought to delineate the evolutionary histories of PV1 and tetherin. To that purpose, we used various platforms such as Ensembl, Uniprot and UCSC Genome Browser to conduct genome database searches for PV1 and tetherin genes across phylogeny. It should be noted that the tetherin protein in opossums and PV1 proteins in birds, reptiles and amphibians were not annotated and were

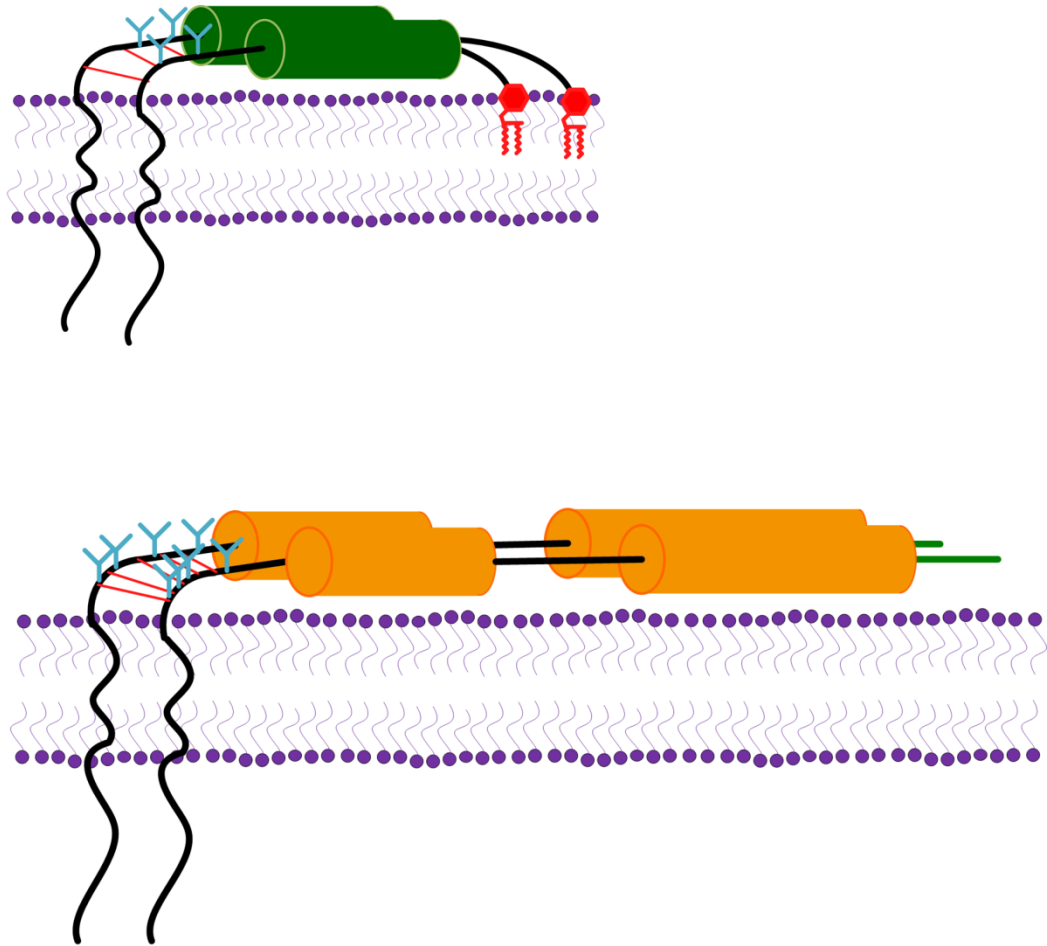


Figure 4.1. Structural homology between PV1 and tetherin. Schematic representation of the tertiary structures of tetherin (top panel) and PV1 (bottom panel) proteins depicting their shared architectural features. These include N-terminal cytosolic tails, transmembrane domains and extracellular coiled-coil domains. Both proteins are extensively glycosylated (indicated as blue Y symbols) and disulfide bridges (indicated by red lines) drive parallel homodimer formation. The ectodomain of PV1 is three times as long as that of tetherin, and comprises of two coiled-coil domains interrupted by an unstructured region. The only differences between the two proteins are a C-terminal GPI modification in tetherin (indicated as red symbols) and a proline-rich domain in PV1 (indicated by green lines). Note that crystal structures of the tetherin ectodomain are available, but none have been solved for PV1.

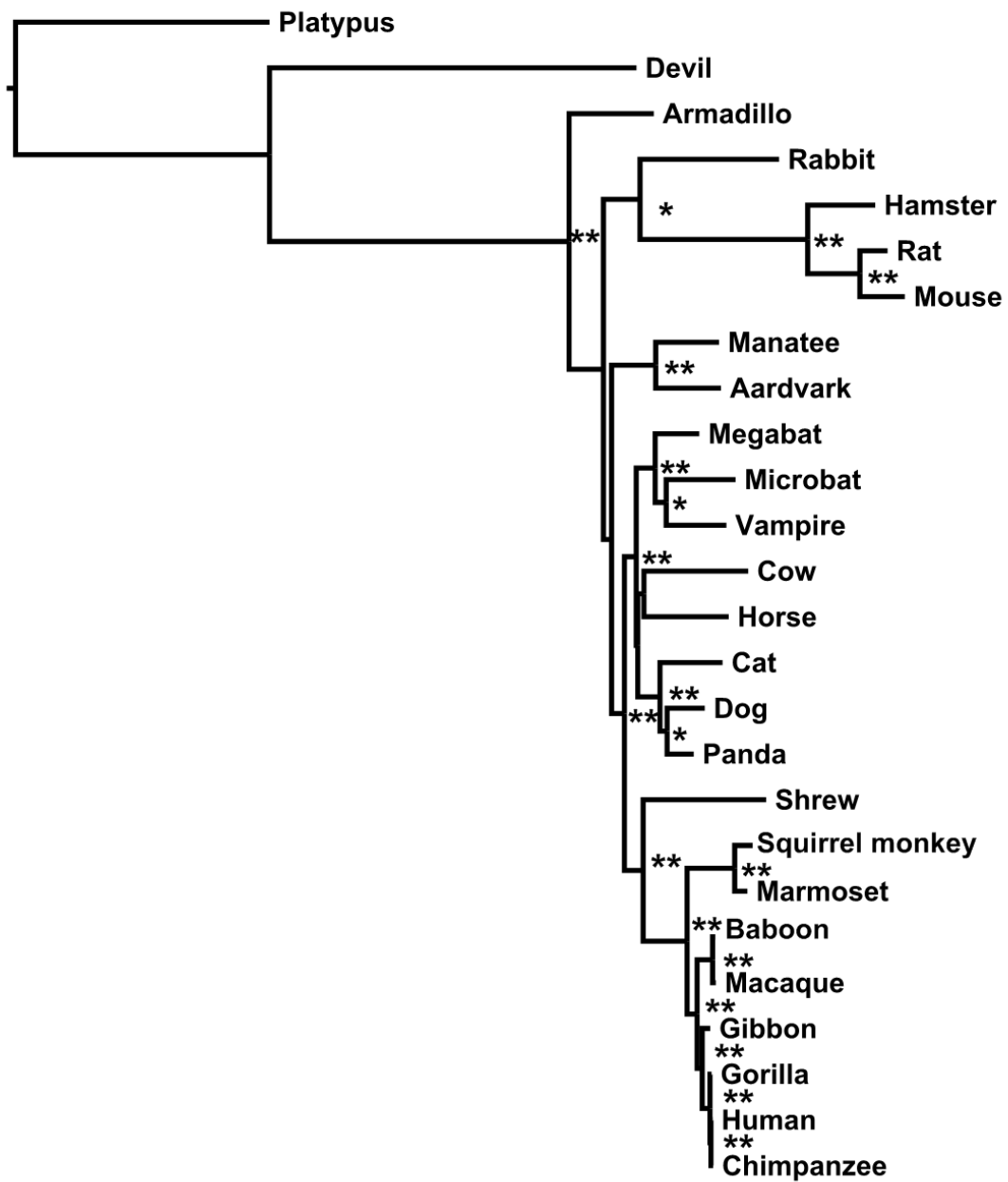
identified using orthologous protein queries from related species in BLAT searches. Our analyses revealed that PV1 is present in the genomes of all mammals (Figure 4.2A) with homologs in the genomes of birds, frogs and fish; indicating that it is ~400 MY old (Broughton et al., 2013). Tetherin, on the other hand, is only present in therian mammals and is absent in egg-laying monotremes and other animal species (Figure 4.2B), indicating that it is ~147 MY old (Bininda-Emonds et al., 2007). We were intrigued to notice that tetherin has duplicated relatively recently in bovids (such as cows) and microbats, leading to two and four copies of the gene respectively (Figure 4.2B).

The retrieved cDNA sequences for PV1 and tetherin were used to build multiple alignments using MUSCLE (Edgar, 2004) with manual refinement. These multiple alignments were used to construct phylogenetic trees using maximum likelihood methods (Stamatakis, 2006) (Figures 4.2A and 4.2B). The PV1 alignment displays high sequence conservation across mammals, which suggests that it has evolved under purifying selection (Figure 4.2A). On the other hand, it is well known that tetherin has evolved under positive selection in primate species (McNatt et al., 2009) and the longer branch lengths in its phylogenetic tree illustrates this unusual divergence across species (Figure 4.2B).

To clarify the types of selection pressures that these two genes have been subjected to during mammalian evolution, we performed codon-based tests of positive selection on both genes using CODEML (Yang, 1997) under different nested pairs of models (M0 and M3, M1a and M2a, M7 and M8) (Table 4.1). A

Figure 4.2. Distribution of PV1 and tetherin across mammals. (A) Phylogenetic tree of an alignment of PV1 coding sequences in therian mammals. **(B)** Phylogenetic tree of an alignment of the TM and coiled-coil domains of tetherin (codons 22 to 133 of human tetherin) in therian mammals. Both phylogenetic trees were estimated using a maximum likelihood approach (RaxML). Refer to methods for parameters. One asterisk (*) represents nodes that are supported in greater than or equal to 80% of the bootstrap replicates. Two asterisks (**) represents nodes that are supported in greater than or equal to 90% of the bootstrap replicates. Disclaimer: Daniel Blanco-Melo generated the data for this figure.

A



0.2

B

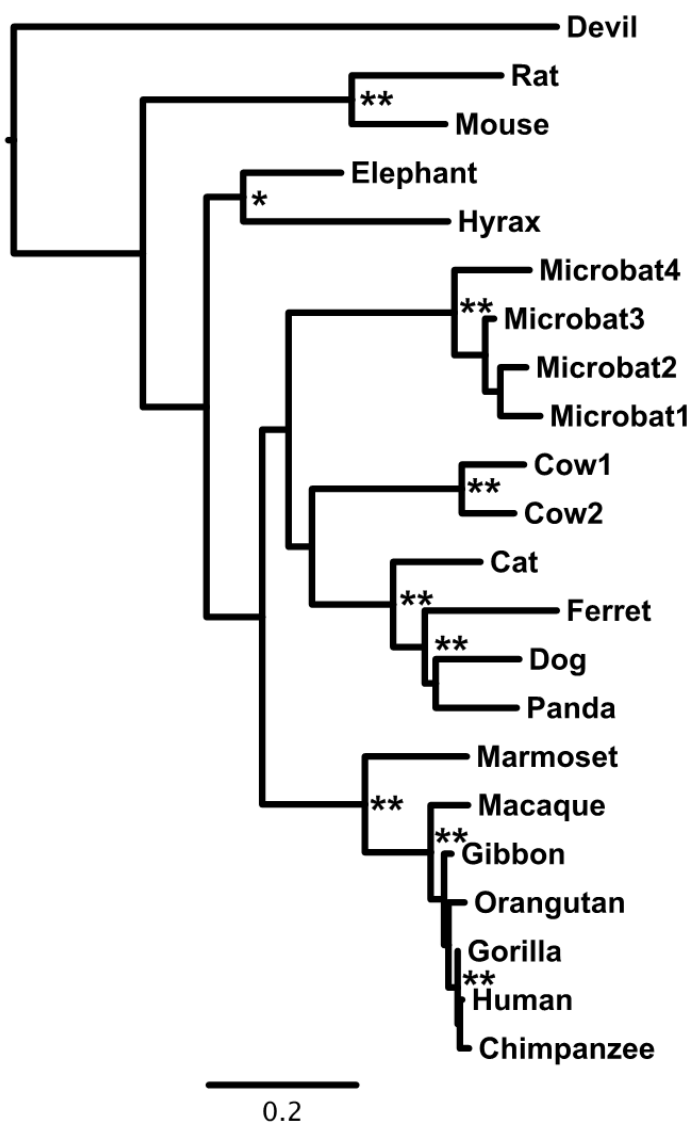


Table 4.1. Likelihood ratio test on nested models of variable ω ratios among sites for PV1 and Tetherin genes. Likelihood ratio tests on nested models of variable ω ratios among sites were performed to determine positions with dN/dS values > 1 (positive selection). Neutral models of selection (M0, M1, M7) were compared to models that allow variation on dN/dS among sites (M3) or selection models (M2, M8). P-values were calculated using a chi square distribution. DOF: degrees of freedom. PSS: number of positive selection sites (Naïve Empirical Bayes) with posterior probabilities > 0.95. NS: Non-significant.

Gene	Data Set	-2 ln (λ)	DOF	P-values	PSS
Tetherin	M0 vs. M3	152.675032	4	P < 0.001	4
	M1 vs. M2	13.171016	2	0.0014	3
	M7 vs. M8	19.830186	2	P < 0.001	4
PV1	M0 vs. M3	852.432408	4	P < 0.001	0
	M1 vs. M2	0.000366	2	0.9998 (NS)	0
	M7 vs. M8	8.029462	2	0.0180 (NS)	0

ratio of non-synonymous substitutions/non-synonymous sites (dN) versus synonymous substitutions/synonymous sites (dS), i.e. dN/dS , greater than 1 indicates that the sequence is diverging away from the ancestral state, and is evolving under positive selection. On the other hand, a dN/dS ratio lesser than 1 indicates that natural selection has conserved the ancestral state and that the sequence is evolving under purifying selection. Our analyses support previous findings that tetherin has evolved under positive selection across primates, and extend its time span to all therian mammals (M1 vs. M2: p-value < 0.002; M7 vs. M8: p-value <<0.001) (Table 4.1). We also showed that PV1 has evolved under purifying selection in therian mammals ($dN/dS < 1$). Interestingly, the M0 vs. M3 likelihood ratio test suggests that different portions of the PV1 sequence have been shaped by different selection pressures (all with $dN/dS < 1$). It is difficult to speculate the nature of these evolutionary forces, given that PV1 performs a housekeeping function. From these analyses, we conclude that tetherin probably originated between the times that monotremes and marsupials diverged away from mammals (~166-147 MYA). We speculate that the opposing selection pressures (i.e. positive versus purifying selection) might have been responsible for the neofunctionalization of one of the PV1 paralogs to tetherin, and its subsequent divergence from PV1.

Evidence that the antiviral activity of tetherin arose early during its evolution

It is possible that tetherin was subjected to viral selection pressures soon after its birth. Thus, we wondered if its ability to trap enveloped virions was ancient or had arisen recently in its evolutionary history. To that end, we assessed the antiviral activity of the tetherin protein in Tasmanian devils, which is the most divergent protein from the human variant (Figure 4.3A). Sequence comparison tools revealed that the identity between the two proteins is 31% and the similarity is 55% (Figure 4.3A).

We analyzed antiviral activity by transfecting 293T cells with either a HIV-1 proviral plasmid (HIV-1(WT)) or its Vpu-deficient counterpart (HIV-1(Δ Vpu)), along with various amounts of plasmids encoding human and devil tetherin proteins. As expected, human tetherin potently inhibited the release of HIV-1 (Δ Vpu) in a dose-dependent manner, while only marginally affecting the release of HIV-1 (WT) (Figures 4.3B and 4.3C). The devil tetherin protein potently inhibited the release of extracellular HIV-1 particles, and its antiviral activity was virtually indistinguishable from that of human tetherin (Figures 4.3B and 4.3C). Inhibition occurred without detrimental effects on Gag protein expression (Figure 4.3C). Predictably, devil tetherin was not antagonized by HIV-1 Vpu (Figure 4.3B), which has evolved specifically to antagonize human tetherin (Neil et al., 2006; Van Damme et al., 2008). Overall, it appears that tetherin proteins as widely divergent as those found in marsupials, possess antiviral activity against enveloped viruses. These results suggest that the ability to trap virions was acquired relatively early during the evolutionary history of tetherin.

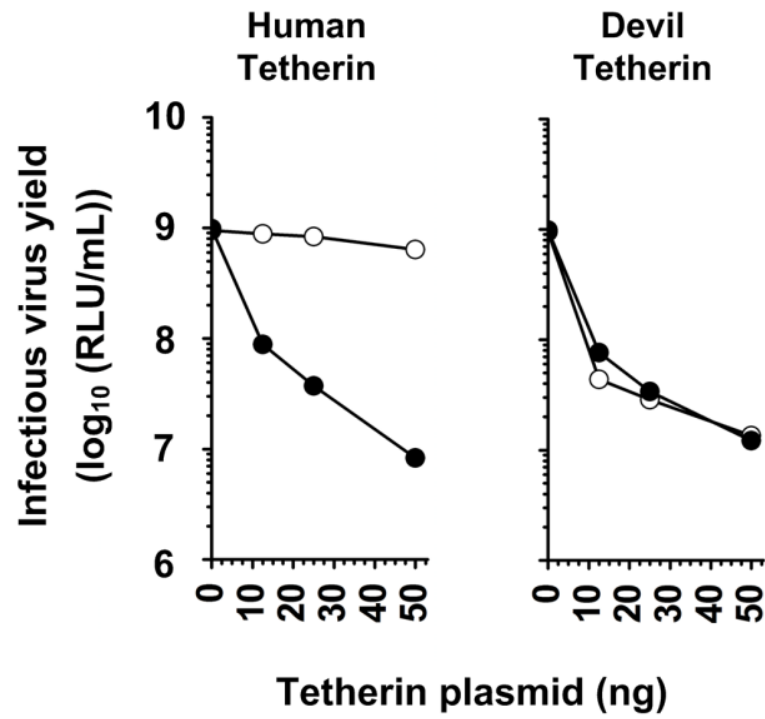
Figure 4.3. Tetherin acquired antiviral activity early during its evolution. (A)

A sequence alignment of human and Tasmanian Devil tetherin proteins generated using the Emboss Water Pairwise Sequence Alignment tool. The conserved residues (indicated as vertical lines) are shaded in blue for convenience. A colon (:) indicates conservation between strongly similar residues, while a period (.) indicates conservation between weakly similar residues. **(B)** 293T cells were cotransfected with WT (empty circles) or Vpu-deficient (filled circles) proviral pNL4-3 plasmids along with varying amounts of human or Tasmanian Devil tetherin proteins. Infectious virion yield was measured by inoculating HeLa-TZM indicator cells with culture supernatant and is given as the logarithm to the base 10 of the relative light units (RLU). **(C)** Western blot analyses of transfected 293T cell lysates and virions corresponding to the Vpu-deficient samples in **(B)**. All samples were probed with an anti-CA antibody.

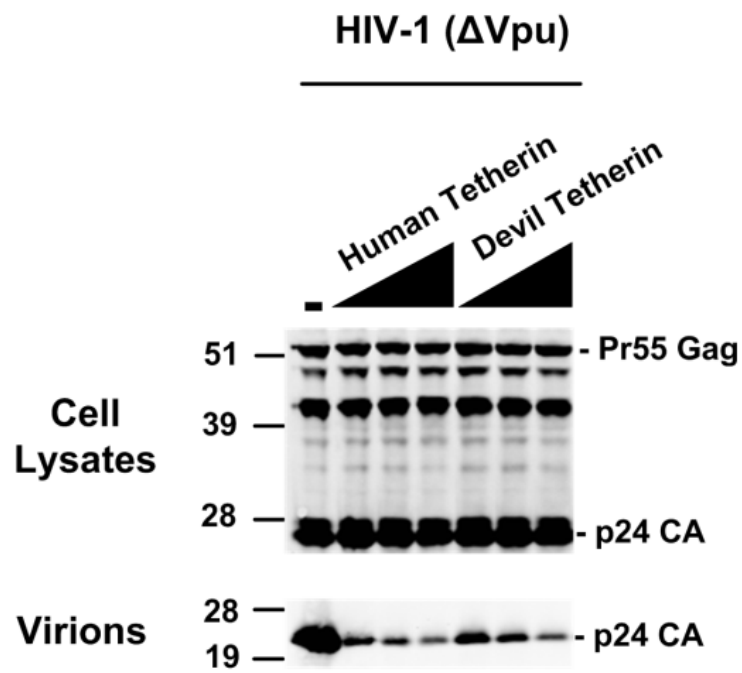
A

Protein	Position	Sequence	Position
Human Tetherin	28	ILVLLIIIV-ILGVPLIIIFTIKANSEACRDGLRAVMECRNVTHLLQQELTE	76
Devil Tetherin	9	VVVLAIVTFILLVPLVVF AIRANSETC---LKS-FELQNKKQLLEEKLRQ	54
Human Tetherin	77	AQKGFQDVEAQAATCNHTVMALMASLDAEKAQGQK-----KVEELEGEIT	121
Devil Tetherin	55	SKESLREWETQWWSCSNISKDL--KKDLEELTSQKNLMLSQQLQELKGENQ	102
Human Tetherin	122	TLNHKLQDASAEVERLRRENQVLSVRIADKKYYPSSQDSSAAAPQL-LI	170
Devil Tetherin	103	DIAEKLKRAEALQ-LERE-----KKNP-EKTNSALPGPQISKL	139
Human Tetherin	171	VLLGL	175
Devil Tetherin	140	LVLGL	144

B



C



PV1 and tetherin share no sequence homology

In principle, the gold standard to prove unequivocally that two genes share a common ancestor is to detect significant sequence homology between them at either the nucleotide or the amino acid levels. However, current global and local sequence alignment methods fail to detect any significant sequence similarity between PV1 and tetherin. Among PV1 sequences, the similarity between introns and other presumed neutrally evolving sites diminishes from primates to rodents and almost disappears from primates to marsupials and monotremes (Figure 4.4). Note that the non-eutherian mammals (i.e. monotremes and marsupials) diverged from eutherian mammals ~101 MYA (Bininda-Emonds et al., 2007; Margulies et al., 2005; Nikolaev et al., 2007). A key question is- should we expect to observe sequence homology, given the time at which PV1 and tetherin putatively diverged? It might be difficult to observe sequence homology between tetherin and PV1 due to opposite selection forces on the two genes, resulting in the accelerated evolution and divergence of the tetherin sequence away from PV1, and the amount of time involved since the duplication. Thus, it is possible that any sequence homology has been lost with the proviso that structural similarity still persists, to preserve its antiviral activity.

In order to test this possibility, we reconstructed ancestral PV1 sequences and simulated their evolution *in silico* under tetherin-like conditions, to ultimately assess the sequence similarity between the simulated sequences and contemporary PV1 sequences. We reconstructed ancestral PV1 sequences for the common ancestor of all mammals (166.2 MYA) and for the common ancestor

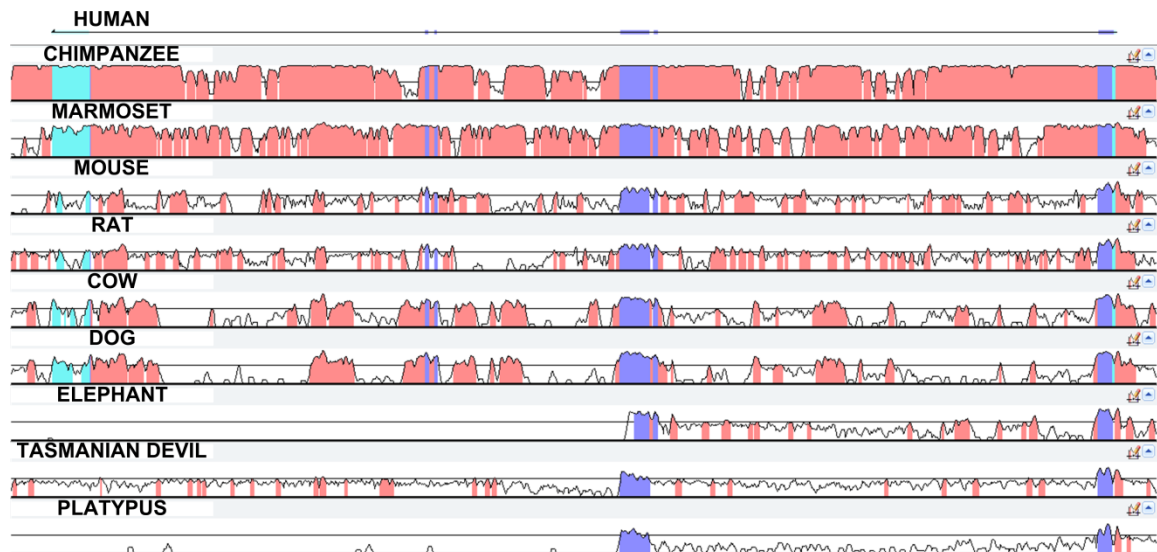


Figure 4.4. No significant similarity in introns and other presumed neutrally evolving sequences across widely divergent PV1 genes. A global sequence alignment of PV1 genes from primates to monotremes was generated using the VISTA visualization module for comparative genomics. The graph shows the percentage of conservation between the two organisms at any given coordinate. The pink regions correspond to noncoding sequences, the dark blue regions are exons, and the light-blue regions are the untranslated regions. The human PV1 gene serves as the reference sequence for the alignment.

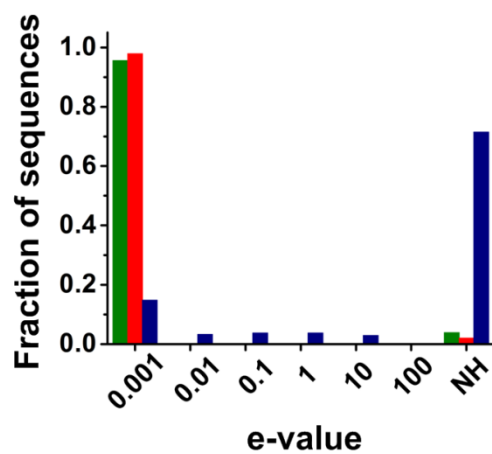
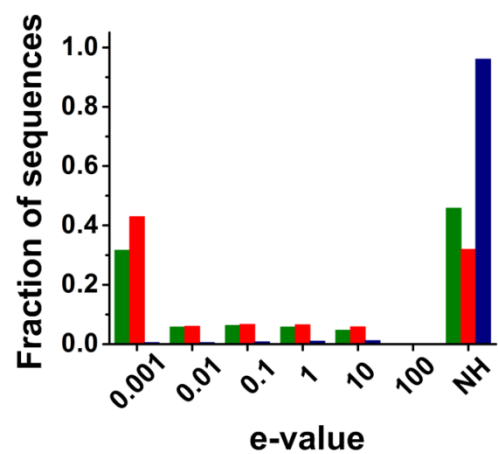
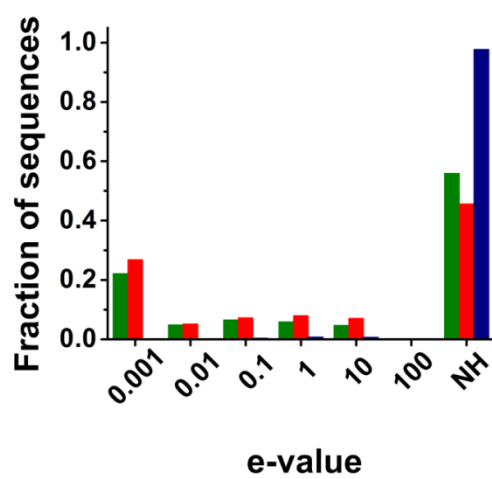
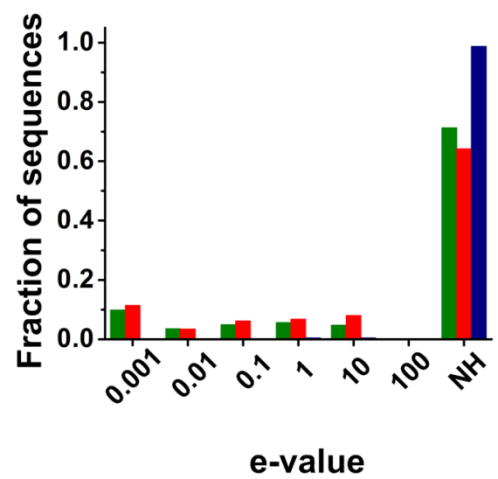
of therian mammals (147.7 MYA) using maximum likelihood methods (Yang, 1997). We allowed the mammalian ancestral PV1 sequences to evolve at typical mammalian neutral substitution rates (i.e. $dN/dS = 1$), for a time span of 18.5 MY (i.e. the time span wherein the putative duplication might have occurred). We used the EvolveAGene 3 software (Hall, 2008), with no selection against indels, to produce 100 fully coding simulated sequences. These sequences were used as input for the Seq-Gen software (Rambaut and Grassly, 1997) to simulate evolution under tetherin-like conditions for the rest of the mammalian tree. Parameters for Seq-Gen were estimated by ModelTest (Posada and Crandall, 1998) using the Bayesian Information Criterion (BIC) likelihood test. For each neutral substitution rate, 100 replicates for each neutrally evolved PV1 sequence were produced, and the simulated 10,000 mouse, dog and human sequences were compared to contemporary mouse, dog and human PV1 respectively, using tblastn. Additionally, Seq-Gen was used to evolve therian ancestral PV1 under tetherin-like parameters with 10,000 replicates. The simulated mouse, dog and human sequences were compared to mouse, dog and human PV1 proteins respectively using BLAST. Note that a commonly used measure of sequence similarity is the expectation value (e-value). Essentially, an e-value is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size (Koonin and Galperin, 2003). For example, an E value of 1 assigned to a hit can be interpreted as meaning that in a database of the current size one might expect to see 1 match with a similar

score simply by chance. Thus, the closer the e-value is to zero, the more significant is the match between query and hit (Koonin and Galperin, 2003).

Overall, the majority of the BLAST hits had ~30 percent identity; however the quality of the hits varied significantly depending on the neutral substitution rate and the PV1 sequence used as query (Figure 4.5). Without 18.5 MY of neutral evolution, BLAST was able to find significant high scoring hits with nearly all of the 10,000 simulated sequences using human and dog PV1 protein sequences as queries (Figure 4.5A). In contrast, using mouse PV1 as a query, BLAST was able to find significant hits only with 28% of the sequences (2,853 of 10,000), with the majority of them having e-values ≤ 0.001 (Figure 4.5A). This scenario was intensified if the simulated sequences were allowed to evolve under neutral selection prior to the tetherin-like conditions. Significant BLAST hits with mouse PV1 were found only in a small fraction of sequences, from 2.8% using a neutral substitution rate of 1.5×10^{-9} substitutions/ site/year, to 0.8% using a neutral substitution rate of 5×10^{-9} substitutions/site/year (Figures 4.5B-4.5D). Using dog and human PV1 as queries, the majority of the BLAST hits were found to have an e-value ≤ 0.01 irrespective of the neutral substitution rate used (Figures 4.5B-4.5D). Notably, there is always a subset of simulated sequences where BLAST fails to find sequence homology to contemporary PV1 sequences. The frequency of the “non-homologous” sequences varies from 45% and 31%, using 1.5×10^{-9} substitutions/site/year, to 71% and 64%, using 5×10^{-9} substitutions/site/year, in dog and human comparisons, respectively (Figures 4.5B-4.5D). Additionally, for those sequences where BLAST was able to detect sequence homology, a

Figure 4.5. *In silico* evolution of tetherin from ancestral PV1 sequences.

Frequency histograms of the significance of tBLASTn hits comparing contemporary PV1 protein sequences and in silico simulated “tetherin” sequences. The ancestral mammalian PV1 sequence was evolved under distinct neutral substitution rates for a period of 18.5 MY, i.e. the time interval in which tetherin is projected to have emerged (100 replicates): **(A)** 0, **(B)** 1.5×10^{-9} , **(C)** 3×10^{-9} and **(D)** 5×10^{-9} substitutions/site/year. Each sequence was then evolved under parameters that best fit the tetherin alignment for the remaining mammalian lineages (100 replicates). Simulated mouse, human and dog sequences were compared using tBLASTn to contemporary mouse (blue), human (green) and dog (red) PV1 protein sequences. X-axis intervals: 0.001 (e-value = 0.001), 0.01 (0.001 < e-value = 0.01), 0.1 (0.01 < e-value = 0.1), 1 (0.1 < e-value = 1), 10 (1 < e-value = 10), 100 (10 < e-value = 100) and NH (No Hits, fraction of simulated sequences with no blast hits). **Disclaimer:** Daniel Blanco-Melo generated the data for this figure.

A**B****C****D**

substantial fraction corresponds to non-significant poorly scoring hits. The large proportion of sequences lacking homology with mouse PV1 within any of the simulated data sets can be explained by its high degree of divergence and the high neutral substitution rate of mice (4.5×10^{-9} substitutions/site/year). Thus, it is clear that the introduction of neutral evolution immediately after the hypothetical PV1 duplication was the main source of sequence divergence. This observation is also supported by the lack of sequence homology between ancestral PV1 sequences and ancestral tetherin sequences (data not shown). We conclude that it is possible to evolve sequences from an ancestral PV1 sequence that bear no significant sequence homology to contemporary PV1, after simulating the evolutionary processes that might have contributed to the emergence of tetherin. Thus, PV1 and tetherin might share common ancestry, even though they share no significant sequence homology.

PV1 can be endowed with antiviral activity upon acquisition of a second anchor

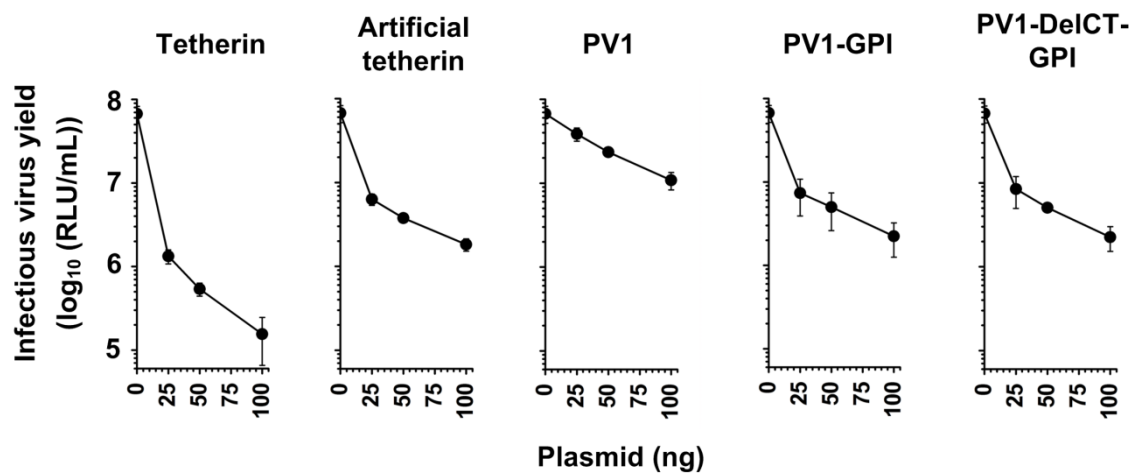
Both membrane anchors of tetherin are indispensable for trapping virions at the cell surface (Perez-Caballero et al., 2009). Because the only glaring difference between the overall architectures of PV1 and tetherin is the second anchor in tetherin, we reasoned that the relatively trivial manipulation of appending the C-terminus of PV1 with the tetherin GPI anchor would be sufficient to endow it with the ability to trap virions. The underlying rationale is that if two proteins share a

common structure, then the proteins might be expected to perform the same function.

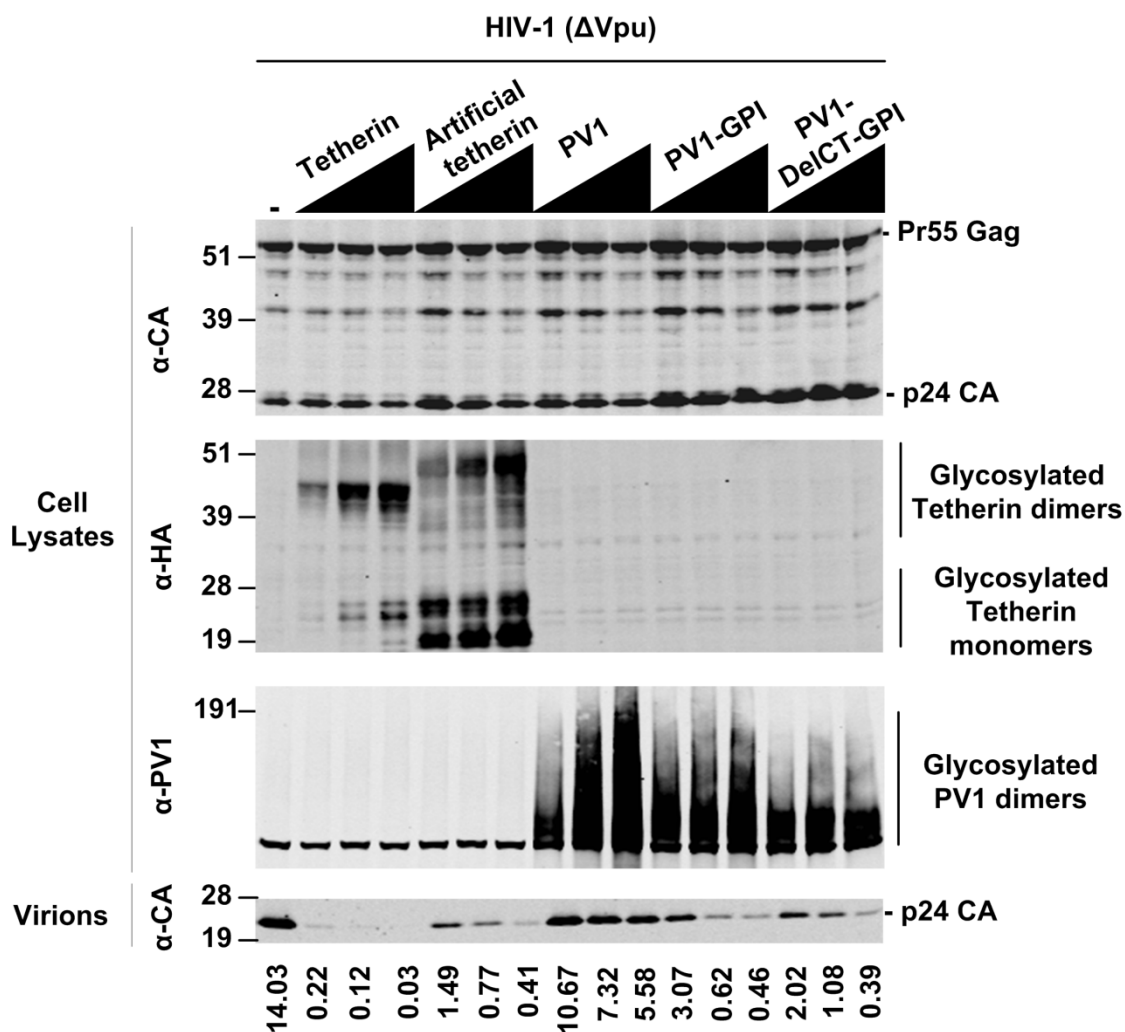
Analysis of the antiviral activity of the panel of PV1 proteins revealed that the PV1-GPI protein is at least as potent as artificial tetherin, which is an entirely synthetic protein that bears no sequence homology to human tetherin, but preserves its overall architecture (Perez-Caballero et al., 2009) (Figures 4.6A and 4.6B). Moreover, the PV1-GPI protein is only moderately impaired as compared to human tetherin (Figures 4.6A and 4.6B). Note that the endogenous levels of PV1 are insufficient for trapping virions (Figure 4.6B). Interestingly, the PV1 protein itself has modest antiviral activity (Figures 4.6A and 4.6B), perhaps due to its propensity to dimerize and organize itself into ring-like structures via C-terminal tail-tail interactions (Stan, 2005). We also wondered if we could further improve the antiviral activity of the chimeric PV1-GPI protein by truncating the N-terminal cytoplasmic tail. Such a protein (hereafter referred to as PV1-DelCT-GPI) cannot be internalized by the cellular endocytic machinery, and is expected to freely diffuse into sites of particle assembly. However, truncation of the cytoplasmic tail had no effect on antiviral activity (PV1-DelCT-GPI; Figures 4.6A and 4.6B). Note that the PV1 and tetherin proteins are heterogeneously glycosylated, and because the cells were lysed in non-reducing buffer, all proteins migrated primarily as a smear of dimeric species (Figure 4.6B). We conclude that PV1 performs the biological function of tetherin when it is engineered to encode a C-terminal GPI anchor.

Figure 4.6. Antiviral activity of the panel of modified PV1 proteins. **(A)** 293T cells were cotransfected with Vpu-deficient proviral pNL4-3 plasmid (HIV-1 Δ Vpu) along with varying amounts of the indicated tetherin and modified PV1 proteins. Infectious virion yield was measured by inoculating HeLa-TZM indicator cells with culture supernatant and is given as the logarithm to the base 10 of the relative light units (RLU). **(B)** Western blot analyses of transfected 293T cell lysates and virions corresponding to the above panel. The cell lysates were probed with anti-CA, anti-HA, and anti-PV1 antibodies, and the virions were probed with anti-CA antibody. The numbers at the bottom represent measurement of CA protein levels in virions (LI-COR).

A



B



Genomic distribution of PV1/tetherin-like proteins

The hypothesis that PV1 and tetherin might share a common ancestor stems in part from the observation that they share a relatively unusual overall architecture. To quantify the abundance of this architecture in animal genomes, we searched for human and mouse proteins that share structural features with PV1. We downloaded all 105,281 human and 54,447 mouse annotated protein sequences from Ensembl (Release 71) (Flicek et al., 2013) using BioMart. Proteins were then selected using the following criteria: (i) presence of one transmembrane domain (TMD) using tmhmm 2.0 (probability > 0.5) (Krogh et al., 2001), and (ii) coiled-coil domains of various lengths C-terminal to the TMD using COILS (Lupas et al., 1991) (probability > 0.5). We identified 211 human and 175 mouse PV1/tetherin proteins with a minimal coiled-coil domain of 21 amino acid residues and subsequently determined which of those genes lay adjacent to each other in the genome, and bore significant homology to each other.

It is noteworthy that 30% of duplicated genes (i.e. those genes with significant sequence homology) are located adjacent to each other and are in the same orientation (Pan and Zhang, 2008). Interestingly, 7 out of 10 PV1/tetherin-like gene pairs that are adjacent to each other in the human genome and that are in the same orientation, share significant homology, and thus likely arose via gene duplication. The exceptions are CLEC4M and EVI5L, BCAP29 and SLC26A4, and PV1 and tetherin. Notably, PV1 and tetherin is the only pair that conforms to a more stringent criterion, i.e. the coiled-coil domains in both proteins are greater than 28 amino acid residues. In contrast, the only PV1/tetherin-like genes that

are adjacent to each other in the human genome, but that are in the reverse orientation (EVC and EVC2), share no significant homology. This is intuitive because adjacent genes in the reverse orientation are not expected to have evolved via gene duplication. In the mouse genome, of a total of 6 PV1/tetherin-like gene pairs that lie adjacent to each other and that are in the same orientation, PV1 and tetherin are the only genes that share no sequence homology. To summarize, an architecture N-TMD-Coiled-coil-C is quite rare in the human and mouse genomes, and accounts for only ~0.3% of all annotated proteins. It is rarer still to find adjacent genes that bear this architecture. Moreover, the majority of these genes that are in the same orientation, share significant homology, and hence likely arose via gene duplication. These results indicate that it is quite possible that tetherin arose from PV1, although the two genes share no significant sequence homology.

Some, but not all, PV1/Tetherin-like proteins can be endowed with antiviral activity

To test if PV1/tetherin-like proteins can be engineered to trap virions, we selected candidates that have at least one cysteine residue in their ectodomain, and whose coiled-coil domains are similar in length or exceed that of human and mouse tetherin proteins. Next, using the Uniprot database (Uniprot, 2014), we pruned this list further using two criteria. First, the proteins should reside at the cell surface. Second, they should form homodimers efficiently. Two candidate

proteins (i.e. CD72 and CLEC1A) conformed to the above constraints and were selected for structure-function analyses as earlier.

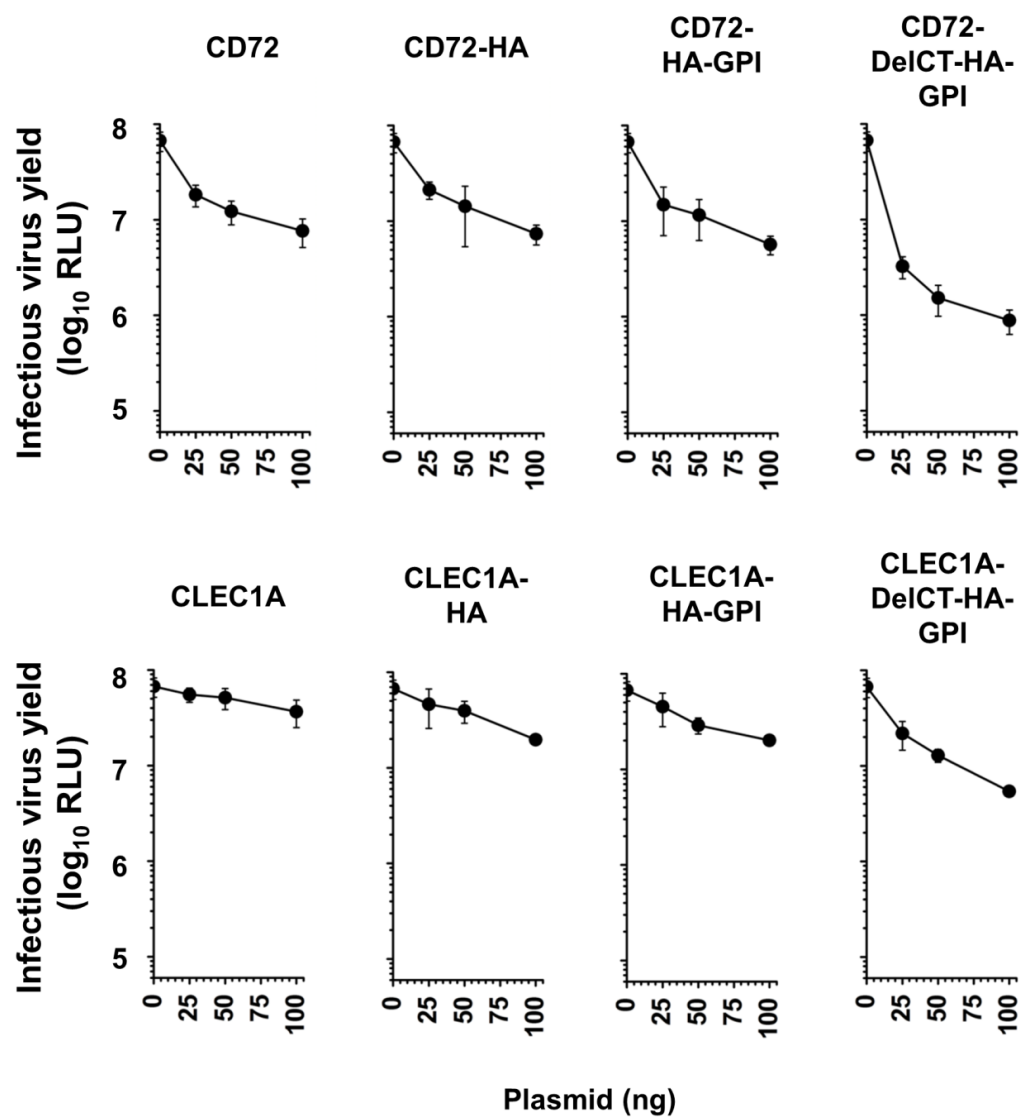
Interestingly, the CD72 protein had a modest intrinsic capacity to inhibit the release of extracellular HIV-1 particles, whereas CLEC1A was almost completely inactive (Figures 4.7A and 4.7B). Unlike PV1, the addition of a second membrane anchor did not stimulate the antiviral activity of these proteins (Figures 4.7A and 4.7B). In contrast, truncation of the N-terminal cytoplasmic tail substantially improved antiviral activity, and a CD72 protein with both manipulations was as potent as artificial tetherin at trapping virions (CD72-DelCT-HA-GPI, Figures 4.7A and 4.7B). Taken together, it appears that straightforward alterations endow, some, but not all, PV1/tetherin-like proteins with the intrinsic ability to trap virions.

Model for the genesis of tetherin via duplication of an ancestral PV1 gene

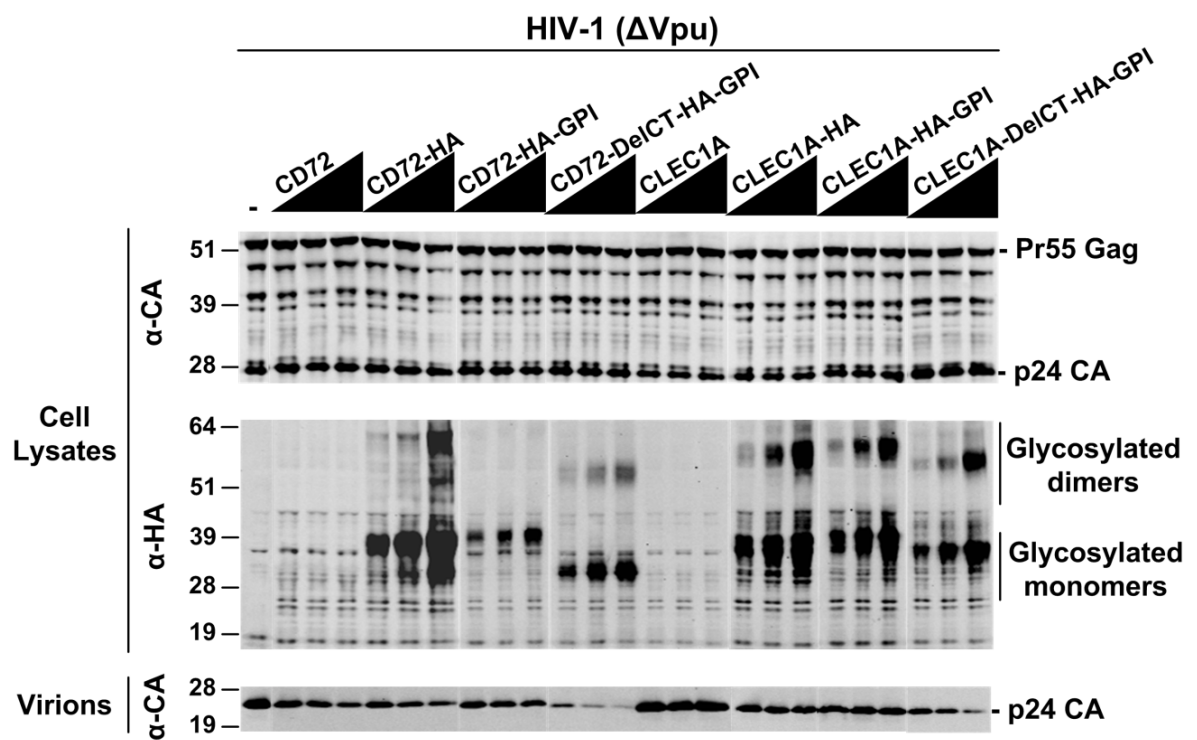
To reconstruct the events that lead to the genesis of tetherin, we analyzed the organization of genes in the PV1 locus across the phylogenetic tree. In eutherians, the PV1 and tetherin genes lie adjacent to each other, and their neighbours form a perfect synteny block (Figure 4.8). In contrast, PV1 and tetherin are separated by ~31 MB in marsupials such as the opossum, and tetherin lies immediately adjacent to CILP2 and YJEFN3 (Figure 4.8). Note that the latter two genes are separated from tetherin in eutherians (Figure 4.8). Our efforts to reconstruct the PV1 locus in birds and monotremes (where tetherin is

Figure 4.7. Antiviral activity of the panel of PV1/tetherin-like proteins. (A) 293T cells were cotransfected with Vpu-deficient proviral pNL4-3 plasmid (HIV-1 Δ Vpu) along with varying amounts of the modified CD72 and CLEC1A proteins. Infectious virion yield was measured by inoculating HeLa-TZM indicator cells with culture supernatant and is given as the logarithm to the base 10 of the relative light units (RLU). **(B)** Western blot analyses of transfected 293T cell lysates and virions corresponding to the above panel. The cell lysates were probed with anti-CA and anti-HA antibodies. Virions were probed with anti-CA antibody.

A



B



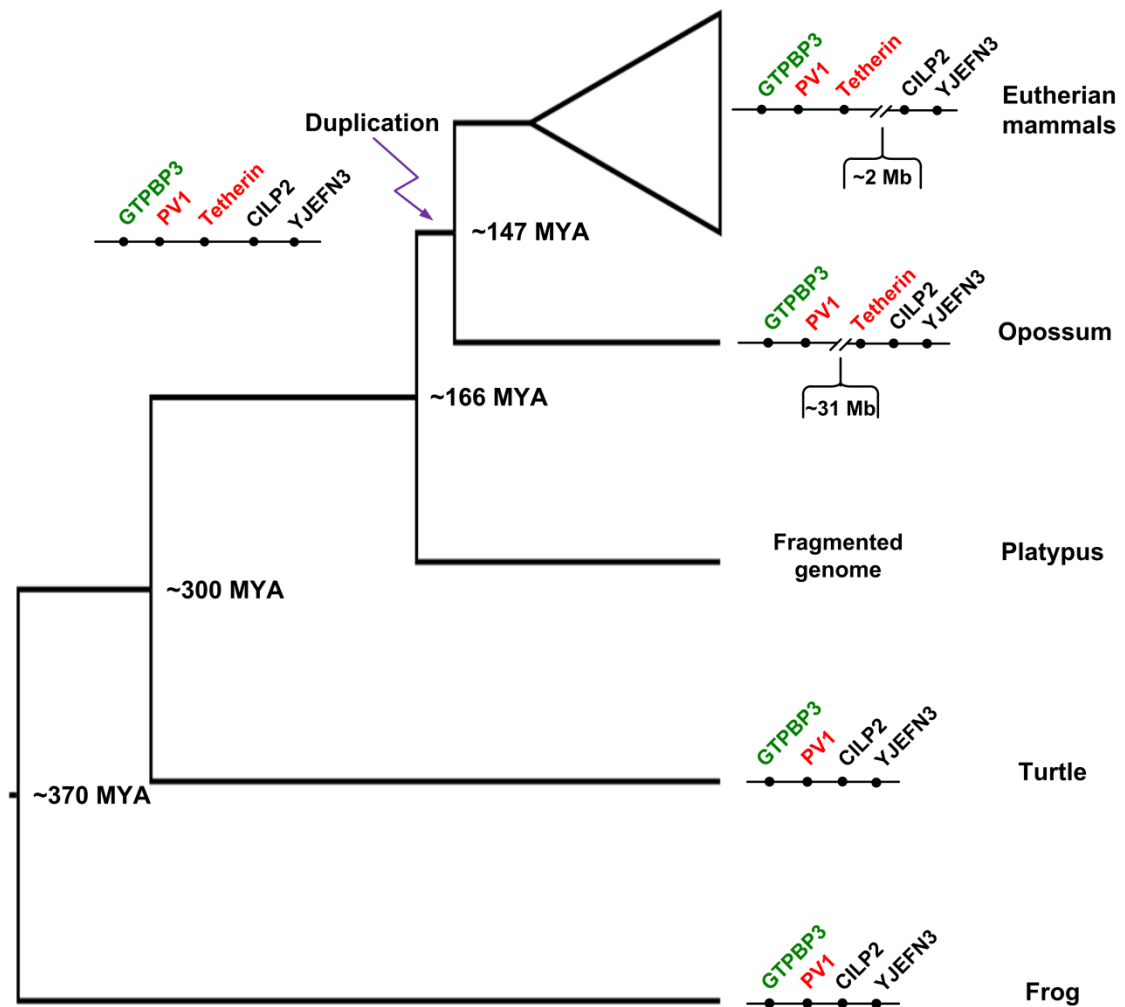


Figure 4.8. Model for the genesis of tetherin. Inspection of the genes that flank PV1 in amphibians (frog) and reptiles (turtle) yields a syntenic gene block that is presumably also maintained in monotremes (platypus). Tetherin proteins are only found in marsupials and eutherian mammals. The duplication of PV1 is estimated to have taken place between the times that monotremes and marsupials diverged from mammals (~166-147 MYA). Tetherin evolved under neutral selection for a short while, acquired a GPI anchor, became neofunctionalized and fixed. Its antiviral activity was then shaped by interactions with past viruses. Clade-specific insertions interrupted the ancestral gene configuration in therian mammals, leading to the separation of PV1 and tetherin in marsupials such as opossums. Importantly, tetherin and PV1 continue to lie adjacent to each other in the rest of the mammals, although they are separated from CILP2 and YJEFN3.

absent) were impaired because their genomes are either poorly annotated or have not been assembled completely. However, we observed that PV1 is contiguous with CILP2 and YJEFN3 in amphibians (frog) and reptiles (turtle) (Figure 4.8). Importantly, GTPBP3 lies immediately adjacent to PV1 in all animal species (Figure 4.8). Thus, we speculate that the ancestral configuration of the PV1 locus might have been GTPBP3, PV1, CILP2, YJEFN3 (Figure 4.8). One could envisage a scenario wherein a duplication of PV1 led to the emergence of tetherin at some point between the times that the monotreme and marsupial lineages diverged from the mammalian lineage (i.e. ~166-147 MYA) (Figure 4.8). Thereafter, lineage-specific insertions and deletions could have resulted in tetherin either segregating with CILP2 and YJEFN3 in marsupials or with GTPBP3 and PV1 in eutherians (Figure 4.8).

Summary

The evolutionary origins of tetherin are obscure. In this work, we employed computational and biochemical approaches to build a case that tetherin arose via duplication of the neighboring PV1 gene. The PV1 and tetherin genes are located adjacent to each other in eutherian mammals and encode proteins with a shared and relatively unusual overall architecture. Phylogenetic analyses provided evidence that tetherin is ~147 MY old and that it has evolved under positive selection. Conversely, PV1 is ~400 MY old and has evolved under purifying selection in therian mammals. Using biochemical assays, we demonstrated that

PV1 can be endowed with antiviral activity against HIV-1 when it is engineered to encode a C-terminal GPI anchor. Although we did not detect any sequence homology between PV1 and tetherin, *in silico* evolution experiments indicated that it is possible to evolve sequences from an ancestral PV1 sequence that bear no significant homology to contemporary PV1, after simulating the evolutionary processes that contributed to the emergence of tetherin. We found that genes encoding proteins with PV1/tetherin-like architecture are quite rare in the human and mouse genomes, and that the majority of these genes that are adjacent to each other likely arose via gene duplication. Moreover, some, but not all, PV1/tetherin-like proteins have the intrinsic ability to trap virions, when appended with a GPI anchor. Finally, we analyzed the organization of genes in the PV1 locus from amphibians to eutherian mammals and identified an ancestral synteny block that supports a model in which tetherin arose from a PV1 duplication that occurred between the times that monotremes and marsupials diverged from other mammals (~166-147 MYA). Cumulatively, these findings suggest that tetherin arose by gene duplication of PV1 and highlight the remarkable capacity of our genomes to innovate antiviral factors from pre-existing genetic raw material.

Chapter V Discussion

It is only recently that we have come to appreciate that there exists an arsenal of proteins that constitutes the first line of resistance against viral infections (Bieniasz, 2004). These defenses are either constitutively active or are mobilized upon viral infection, and directly inhibit viral replication using intriguing and unanticipated mechanisms of action. As antiviral host genes continue to be identified, we have also come to appreciate how their histories are shaped by pathogens. In this body of work, we strived to dissect the molecular mechanisms that underpin the antiviral activity of one such protein, tetherin. In parallel, we also aimed to chart the evolutionary course of this protein, and to arrive at a molecular explanation as to how it might have acquired its antiviral activity.

We began our investigation by formulating a biochemical approach to probe tetherin molecules that have infiltrated virions at the cell surface, with the goal of elucidating the configurations adopted by tetherin during virion entrapment. This approach was based on two previous findings. First, a non-specific protease, subtilisin A, could be used to liberate tethered particles from the infected cell's surface (Neil et al., 2006; Neil et al., 2007). Second, the primary sequence of tetherin can be drastically altered whilst retaining biological activity (Perez-Caballero et al., 2009). Thus, we employed the site specific protease Factor Xa to liberate virions trapped by tetherin molecules that were engineered to include its cleavage site. This manipulation gave the approach tight specificity and enabled the unequivocal demonstration that the tetherin protein itself is an essential component of virion tethers. Moreover, the use of a site specific

protease to release tethered virions from cell surfaces enabled the preservation of epitope tags inserted into the tetherin ectodomain, allowing us to infer the organization of tetherin molecules in virion tethers. We could use a double epitope-tagged version of tetherin, as well as single epitope-tagged versions to analyze the incorporation of both N- and C-terminal proteolytic fragments into virions, and thereby determine tetherin configuration. Additionally, we constructed a protein standard and performed quantitative western blotting to estimate the numbers of tetherin dimers in each orientation that are associated with trapped virions.

Because virions were efficiently liberated by Factor Xa treatment of N5Fac or C8Fac expressing cells, our data effectively exclude the “equatorial” configuration shown in Figure 3.1B, as cleavage of the tetherin peptide backbone in this context would leave intact the majority of the bonds anchoring the virion to the cell surface. Moreover, the fact that tetherin fragments found in virions liberated by Factor Xa were exclusively disulphide linked homodimers also constitutes strong evidence disfavoring this model. While our data do not completely discount the possibility that tetherin multimers adopt the equatorial configuration, with virions becoming trapped via hypothetical non-covalent dimer-dimer interactions (Figure 3.1C), this scenario appears unlikely for two reasons. First, such a configuration would not be expected to result in virion release upon Factor Xa cleavage, because dimer-dimer interactions would not be expected to be perturbed, particularly since the Factor Xa cleavage site is placed within a foreign spacer sequence whose insertion does not itself perturb tetherin function.

Second, the scenario envisaged in Figure 3.1C would result in precisely equal numbers of tetherin N- and C termini being placed in tethered virions. We found that there were modestly, but clearly, more tetherin C-termini than N-termini in virions, arguing that tetherin N- and C-termini partition separately into virion and cell membranes. In summary, our experiments indicated that tetherin homodimers adopt an axial configuration in their functional state, with a preference for the insertion of their GPI anchored C-termini into virions during their entrapment at the surface of infected cells. Quantitative analysis indicated that an average of 80 to 400 tetherin dimers (depending on how many CA molecules are assumed to be present in each virion) associated with each tethered particle. Our findings do not discount the possibility that higher order tetherin multimers, e.g. tetramers, might contribute to tethering, but if such complexes do exist, then they must involve non-covalent interactions between axially configured tetherin molecules and be arranged in such a way that all N-termini are in one membrane (be it virion envelope or cell membrane) and all C-termini are in the opposing membrane.

To fortify our claim that axially configured tetherin homodimers are responsible for trapping virions, we adopted two approaches to further investigate the role for the configurations depicted in Figures 3.1B and 3.1C. First, we attempted to generate a longer chimeric tetherin molecule, because such a protein might have allowed us to visualize the increase in separation between cell and viral membranes when virions are trapped by axially configured as opposed to equatorially configured molecules. However, we were unable to design a longer

protein that would also recapitulate the antiviral activity of tetherin. One possible reason for this is that the length of the ectodomain might have been optimized by evolutionary forces for virion entrapment, and that artificially lengthening it might compromise antiviral activity. In our second approach, we compared the yield of β -mercaptoethanol-liberated virions from cell lines expressing a cysteine mutant that had the propensity to form higher order multimers and a non-crosslinkable mutant. Because there was no further release of virions, it does suggest that higher order multimers of tetherin do not contribute to the retention of virions. However, our inability to probe virions for the identity of the associated tetherin species led us to abandon this line of questioning.

Previous studies have not resolved the configuration adopted by tetherin as it engages in virion entrapment. For example, conflicting results have been obtained in studies where the release of virions was attempted by cleavage of the tetherin GPI anchor using phosphatidyl-inositol-specific phospholipase C (PI-PLC). In one study, the efficiency of virion release induced by PI-PLC treatment was poor (20% compared to subtilisin) (Lehmann et al., 2011), while other studies indicated that PI-PLC treatment fails to liberate any virions (Andrew et al., 2011; Fitzpatrick et al., 2010). Second, the failure of reducing agents to release virions would tend to suggest that the equatorial model shown in Figure 1B is incorrect (Fitzpatrick et al., 2010). However, this argument is somewhat confounded by the fact that tetherin molecules are twisted around each other in a dimer, and so breaking the disulphide bonds in an already-formed tether would not necessarily be expected to cause virion release.

One caveat of our assay is that some tetherin dimers might infiltrate particles and yet be uninvolved in restriction. Thus, it is possible that the number of tetherin molecules that we measured to be associated with a virion might be greater than the number of molecules actually involved in virion entrapment. Indeed, previous studies have shown that low levels of complete tetherin molecules can be found in the small number of virions that are released from tetherin-positive cells (Fitzpatrick et al., 2010). However, to be uninvolved in restriction would require that both tetherin N- and C-termini were embedded in virions. If the numbers of tetherin dimers that were inserted into virions in this way was in excess of the numbers of tetherin dimers involved in tethering, with N- and C-termini partitioned separately into virion and cell membranes, then there would be little or no difference in the number of tetherin N- and C-termini found in virions. The fact that we do indeed observe a 3-to 5-fold excess C-termini in tethered virions, argues strongly that most of the tetherin molecules (at least 65–80%) that are tethered-virion associated, have their N- and C-termini separately partitioned into virion and cell membranes. Thus, most tetherin molecules must be in the axial configuration with only their C-termini embedded in virions.

Our estimates of the number of tetherin molecules that are associated with tethered virions are several-fold higher than those obtained using super-resolution microscopy approaches (i.e. 4–7 dimers per virion) (Lehmann et al., 2011). At least three factors could account for this discrepancy. First, the microscopy studies use a tetherin-mEosFP fusion protein, that includes a bulky 230 amino acid (~26 kDa) protein at its N-terminus, appended to the otherwise

short (21 amino acid) native tetherin cytoplasmic tail. This could very easily reduce the numbers of tetherin molecules that associate with virions. Second, the estimates made in the microscopy studies correspond to groups of tetherin molecules present at the same location as clusters of Gag molecules that may not represent completely assembled virions. Thus, microscopy studies cannot determine whether the imaged tetherin molecules are in the act of restriction. Conversely, our estimates are based on *bona fide* tethered virions that are recovered from cells by specific cleavage of the tether. Finally, the cell lines that we used to derive our estimates modestly overexpressed tetherin (1.5- to 3- fold) as compared to HeLa cells, which might have slightly elevated the numbers of tetherin molecules that were associated with virions. In previous studies (Lehmann et al., 2011), transfected HeLa cells were used, and the levels of tetherin mEosFP relative to preexisting endogenous tetherin, or the total (endogenous plus exogenous) levels of tetherin expression were not determined, which could lead to underestimates or overestimates of tetherin association with tethered virions.

Given that virions are trapped not only at the cell surface, but are also linked to each other, it should be expected that both tetherin N-and C-termini would be found in virions. Most likely, the appearance of virions tethered to each other results from the assembly of a virion at a location on the plasma membrane already occupied by a tethered particle. This being so, our finding of a 3- to 5-fold preference for the insertion of C-termini rather than N-termini into virion membranes may represent an underestimate of the true preference. If this is the

case, then one might expect that the apparent preference for the insertion of C-termini into virions would become diluted over time as virions accumulate at the cell surface and the likelihood of a virion assembling at a site already occupied by a tethered virion increases. However, we did not observe such a trend, and thus it remains unclear whether the 3- to 5-fold preference for C-terminus insertion into virions is an accurate number, or an underestimate resulting from virion accumulation.

The biophysical mechanism underpinning the apparent preference for the insertion of GPI-anchored C-termini over TM domain-anchored N-termini into virions is unclear at present. Although it is not the predominant scenario, the tetherin N-terminal domain is clearly capable of being incorporated into virions. Indeed, a tetherin molecule lacking the GPI anchor is efficiently incorporated into released virions (Perez-Caballero et al., 2009). Moreover, it is the N-terminus that is targeted by Vpu to block tetherin incorporation into virions (McNatt et al., 2009; Perez-Caballero et al., 2009). Perhaps the tetherin N-terminal domain acts as a sensor of membrane curvature, driving localization to assembly sites, but the GPI anchor diffuses more freely into virion membranes. Consistent with this idea, recent work indeed indicates that tetherin colocalizes better with HIV-1 Gag proteins that cause membrane curvature than those which do not (Grover et al., 2013). Incidentally, this ability is not reliant on the ESCRT pathway, as illustrated by our finding that tetherin is proficient at trapping virions that assemble independent of the ESCRT pathway. Using conventional microscopy, a previous study noted that the ability of tetherin to be recruited to late domain mutants of

Gag is severely impaired (Grover et al., 2013). Certainly, the antiviral activity of tetherin is compromised when it is localized incorrectly (McNatt et al., 2013). However, assembly of a late domain mutant is arrested before completion, whereas tetherin traps mature, nascent particles that have budded from the infected cell membrane. Thus, the claims made by previous studies must be treated with circumspection because the use of late domain mutants to test for tetherin function is confounded by the fact that late domain mutants arrest assembly at an earlier stage than when tetherin traps virions.

There is potential biological utility in preferentially inserting GPI anchored tetherin C-termini rather than N-termini into virions. In such a scenario, the tetherin N-terminus remains available to the cytoplasm of the infected cell, from where it may execute important functions. For instance, virions trapped at the cell surface are internalized and degraded in lysosomes (Harila et al., 2006; Neil et al., 2006). Moreover, human tetherin appears capable of initiating signaling cascades, particularly when it is engaged in tethering, and is certainly in pole position to act as a virion sensor (Cocka and Bates, 2012; Galao et al., 2012; Tokarev et al., 2012). As such, the need to interact with the endocytic machinery and/or initiate signaling might favor a scenario in which tetherin dimers are oriented with their N-termini in the infected cell and their C-termini in the virion membrane. Thus, the mechanistic principles that we have elucidated are also consistent with the auxiliary functions of tetherin such as endocytosis and signaling.

In addition to these efforts, we also endeavored to trace the ancestry of tetherin. The evidence that we gathered built a provocative case for its emergence via

gene duplication of PV1, followed by neofunctionalization and selection. A number of circumstantial lines of evidence supported this hypothesis. First, PV1 and tetherin share a relatively unusual overall architecture. Second, phylogenetic analyses demonstrate that tetherin is only present in therian mammals, whilst PV1 is far more ancient. Third, appending the C-terminus of PV1 with a second membrane anchor confers antiviral activity against HIV-1. Fourth, both proteins are located adjacent to each other in eutherian chromosomes, indicative of gene duplication. Additionally, reconstruction of the PV1 locus across species yields a putative ancestral configuration that supports a model for the events that led to the genesis of tetherin.

We were unable to find a tetherin gene in the monotreme genome, which engendered the reasonable hypothesis, that tetherin emerged after monotremes diverged away from therian mammals (i.e. ~166-147 MYA). PV1, on the other hand, is ~400 MY old and our analyses confirmed that it has evolved under purifying selection in therian mammals (Figure 4.2A and Table 4.1). In essence, PV1 and tetherin seem to follow a paradigm of gene duplication wherein one of the paralogs is free to diverge and acquires a novel function (Figure 5.1). We posit that the duplication of PV1 yielded two paralogs; one continued to perform the ancestral function of PV1 in endothelial cells, whilst the other became redundant and diverged under neutral selection (Figure 5.1). We speculate that this paralog might have acquired a novel antiviral function by fortuitously gaining a GPI anchor via mutation and was thereafter optimized by a variety of selection pressures, likely of viral origin (Figure 5.1). Subsequently, it became fixed,

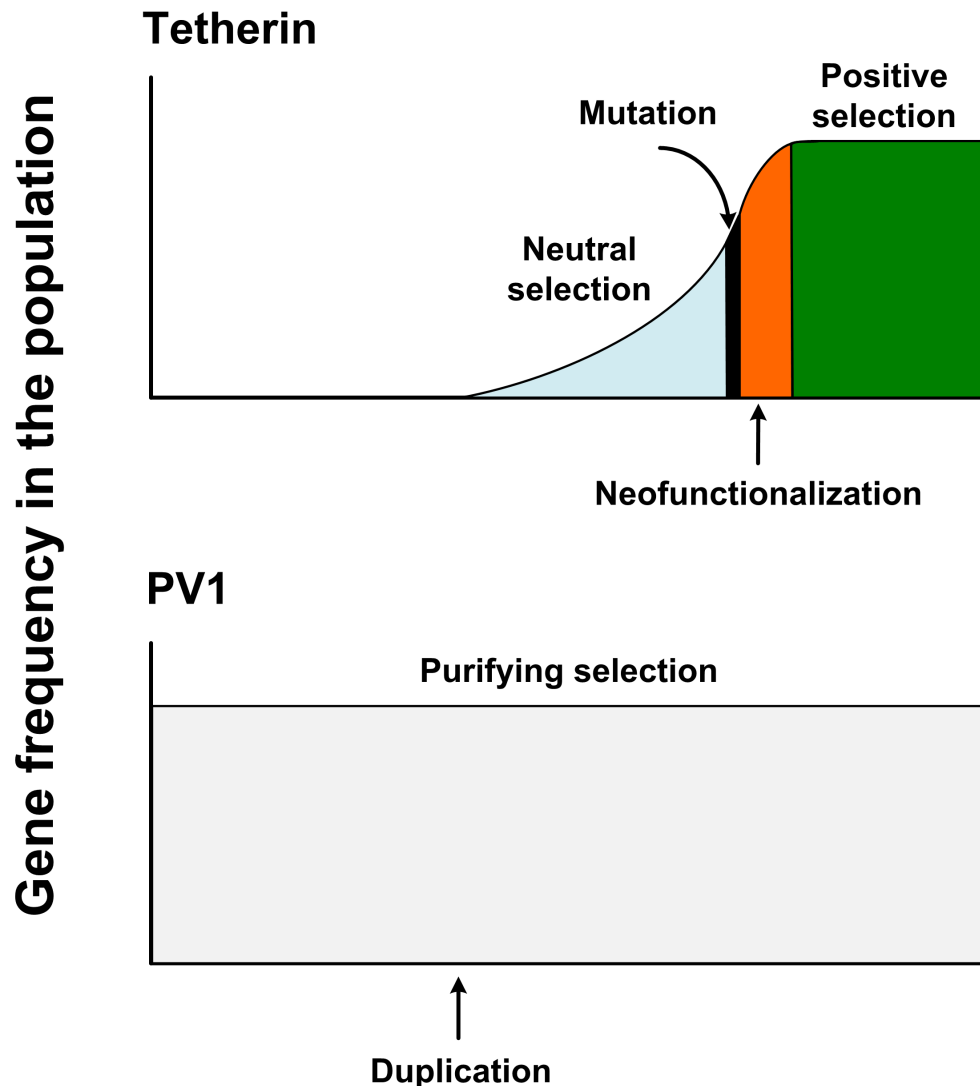


Figure 5.1. Evolutionary processes that might have contributed to the genesis of tetherin. PV1 has evolved under purifying selection in mammals, and upon gene duplication, one of the paralogs continued to perform its cellular function. The other paralog probably evolved under neutral selection and acquired mutations that led to the acquisition of a GPI anchor, thus leading to the birth of tetherin. Subsequently, tetherin acquired antiviral activity (i.e. it was neofunctionalized) and it evolved in competition with viruses soon after its emergence. Eventually, it was fixed in the population and evolved under positive selection in therian mammals.

leading to the emergence of tetherin (Figure 5.1). As a consequence of its genetic conflict with enveloped viruses, tetherin then evolved under positive selection (Figure 5.1). The striking observation that the tetherin protein in Tasmanian devils potently traps HIV-1 virions (Figures 4.3B and 4.3C) bolsters the view that tetherin evolved in competition with viral pathogens soon after its birth.

It is reasonable to expect sequence homology if two proteins are related. In spite of our best efforts, we could not detect any significant homology at the nucleotide or amino acid levels by employing current sequence alignment methods (Figure 4.4). We concede that this observation weakens our proposition that tetherin might have shared an ancestor with PV1. Nevertheless, it is encouraging that we were able to evolve sequences that bore no significant homology with contemporary PV1 sequences in our in silico evolution experiments (Figure 4.5). It is important to note that the events that occurred between the times when the monotremes and marsupials diverged from the rest of the mammals were responsible for rapid sequence divergence of early tetherin. We conducted our analysis under neutral evolution, which seemed to be the best approximation of those events. We observed that more than 50% of the simulated data bore no homology to dog PV1 or human PV1 using neutral substitution rates greater than 1.5×10^{-9} (Figures 4.5B-4.5D). Furthermore, when we considered non-significant hits, the number of sequences that bore no homology was quite high even when we used low substitution rates and compared within species that evolve slowly (i.e. they have low neutral substitution rates) (Figures 4.5B-4.5D). For instance,

~45% of the human simulated sequences bore no significant homology to human PV1, using a neutral substitution rate of 1.5×10^{-9} substitutions/site/year (Figure 4.5B). Because we were unable to accurately simulate the process of neofunctionalization, these percentages likely represent underestimations.

Accumulating evidence supports the notion that the biological activity of tetherin can be attributed to its unique dual-anchored topology. It is noteworthy that proteins with a single transmembrane domain N-terminal to coiled-coil domains comprise only ~0.3% of the annotated human proteome. An even smaller fraction of these proteins are heterogeneously glycosylated and carry at least one cysteine residue, features that are well-established to be critical for the antiviral activity of tetherin. Therefore, it is not too far-fetched to speculate that adjacent gene products might be related if they share such a rare architecture, and if so, might even be able to perform the same function. Remarkably, our functional analyses indeed revealed that PV1 seems to have a modest ability to trap extracellular HIV-1 virions, and that it is possible to improve this activity when it is engineered to mimic the dual-anchored architecture of tetherin (Figures 4.6A and 4.6B). Note that a unifying property that is essential for both the cellular function of PV1 and for the antiviral activity of tetherin is the capacity to dimerize efficiently. Thus, PV1 might already have an intrinsic antiviral potential that could easily have been appropriated for adaptation to tetherin. In this regard, we found that some, but not all, PV1/tetherin-like proteins also share the intrinsic ability to trap virions, and that this activity is dramatically improved when their cytoplasmic tails are truncated (Figures 4.7A and 4.7B).

The identification of the ancestral configuration of the PV1 locus in reptiles and amphibians reinforces our premise that tetherin is an ancient paralog of PV1. We concede that it remains formally possible that tetherin arose elsewhere and was inserted as a single gene into the PV1 ancestral configuration (Figure 4.8). However, the likelihood of such an event is minimal because (i) the probability of tetherin being inserted adjacent to another gene with such a rare architecture is quite low (~200 genes/20,000 human genes), and, (ii) the majority of the PV1-like gene pairs likely arose via duplication. Even more unlikely is the scenario that tetherin was inserted precisely next to CILP2 in marsupials and next to PV1 in eutherians, after originating elsewhere. Further annotation and assembly of the platypus genome could potentially support our model.

Viral infections impose a potent selection pressure on their hosts and shape their antiviral strategies. Tetherin continues to co-evolve with modern pathogens, and in fact, bovids and microbats have two and four copies of tetherin respectively (Figure 2B). We speculate that the pathogenic burden in these species imposed the need for a recent expansion of the tetherin locus. In fact, it might very well be the case that each paralog is antagonized by a subset of viruses, but can inhibit the rest.

In conclusion, the emergence of tetherin presumably conferred a considerable selective advantage to its host in the face of ancient retroviral infections, leading to its rapid fixation in mammals. The acquisition of a GPI anchor might have empowered natural selection to devise an elegant means of potentially inhibiting a hitherto untargeted step in the life cycle of enveloped viruses, i.e. assembly and

release. More generally, these findings underscore the incredible capacity of our genomes to innovate novel functionalities via gene duplication.

Chapter VI Epilogue

Ode to Tetherin

“My name is tetherin, and my fortunes are on an upward spiral,
For I am an effector of intrinsic immunity; indeed, I am antiviral.
I am the real deal; no charlatan, no actor,
And there are those who would even call me a restriction factor.
So when a virus is sensed, and the time is ripe,
I am induced by interferon in almost any cell type.
I have been fine-tuned by evolution to shackle viruses in their prime.
Discovered by Neil, Guatelli and Bieniasz; been around for a longer time.”

“My amino acid sequence can be wild-type or chimeric,
But in order to be potent, I have to be dimeric.
I have a fascinating topology, and can be designed from scratch,
Just preserve my overall architecture; that virus is no match.
And when you substitute every motif with a heterologous domain,
Behold! Sans homology, my powers do not wane!”

“With an anchor in the cell, and yet another in the virion,
Axially configured, I trap them by the million.
I have a predilection that is not entirely insane,
I’d rather insert my GPI anchor into the viral membrane!
This facilitates endocytosis of the rogues that I hold,
Or, alternatively, I function as a signaling scaffold.”

“Almost invincible, there is nothing that I cannot thwart,
Except for Vpu, who is an antagonizing sort!
Our battles are grim; signatures of positive selection bear witness,
My nemesis neutralizes me to enhance transmission fitness.
All in all, our struggles always live up to their billing,
My life in genetic conflict- endlessly fulfilling!”

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