Interaction between the ovine Bst-2 paralogs and sheep Betaretroviruses

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Abstract

There is a delicate evolutionary balance between viruses and their hosts. The host has evolved the intrinsic, innate and adaptive immunity to fight viral infections. However, viruses have acquired several counteracting measures to evade host defences. Ovine Betaretroviruses, including the exogenous and pathogenic Jaagsiekte sheep retrovirus (JSRV) and the highly related endogenous enJSRVs are a unique model system to investigate virus-host interaction over long evolutionary periods. Sheep have co-opted some defective enJSRV loci to (i) counteract infection by exogenous viruses and likely (ii) to cope with the continuous retroviral invasion of their genome. In addition, various genes of the innate and intrinsic immunity of the host have evolved to block viral replication. The work presented in this thesis focuses on the ovine bone marrow stromal cell antigen 2 (Bst-2)/ tetherin, a recently identified cellular restriction factor with a broad antiviral activity, and its interaction with sheep Betaretroviruses. In sheep, the BST-2 gene is duplicated into two paralogs termed oBST-2A and -2B. Studies presented in this thesis show that oBST-2B possesses several biological properties distinct from the paralog oBST-2A and from all the other BST-2 orthologs. oBST-2A prevents the release of JSRV/enJSRV viral particles by 'tethering' them at the cell membrane similarly to what observed by human BST-2. On the other hand, oBST-2B, does not reach the cell membrane but remains within the Golgi stacks and the trans-Golgi network. Several lines of evidence obtained in this thesis suggest that oBST-2B reduces significantly Env incorporation into viral particles. Therefore, oBST-2B possesses a unique antiviral activity that complements the classical tethering restriction provided by oBST-2A.

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List of Accompanying Material

A copy of the following article is included within the Appendix of this thesis:

Arnaud, F. Black, S. G., **Murphy, L.**, Griffiths, D. J., Neil, S. J., Spencer, T. E. & Palmarini, M. (2010) Interplay between ovine bone marrow stromal cell antigen 2/tetherin and endogenous retroviruses. *Journal of Virology*, 84: 4415-25.

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Declaration

I declare that all of the work submitted herewith has been carried out by myself.
Collaborative work is acknowledged where present.

Lita Murphy
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Abbreviations

ALV	Avian Leukosis Virus
AP2	Activator Protein 2
APOBEC	Apolipoprotein B mRNA-editing enzyme
ASLV	Avian Sarcoma Leukaemia virus
BAC	Bronchioloalveolar adenocarcinoma
BST-2	Bone stromal cell antigen 2
CARD	Caspase activation and recruitment domain
CA	Capsid
C/EBP	CCAAT-Box/enhancer binding protein
CMV	Cytomegalovirus immediate early promoter
DC	Dendritic cells
DLS	Dynamic Light Scattering
ER	Endoplasmic reticulum
ENV	Envelope
ENTV	Enzootic nasal tumor virus
FBS	Foetal bovine serum
GAG	Group specific antigen
GFP	Green Fluorescent Protein
GPI	Glycophosphatidylinositol
HIV	human Immunodeficiency Virus
HTLV	Human T cell leukaemia
Hyal-2	Hyaluronidase 2
IFN	Interferon
IL-6	Interleukin
IN	Integrase
ISG	IFN stimulated genes
ISRE	IFN responsive element
JSRV	Jaagsiekte sheep retrovirus
JLR	JSRV late response
LTR	Long terminal repeat
MA	Matrix

MDA5	Melanoma differentiation associated gene 5
MLV	Murine Leukaemia Virus
MoMLV	Moloney Murine Leukemia Virus
MMTV	Mouse mammory tumour virus
M-PMV	Mason Pfizer monkey virus
MTOC	Microtubule organizing centre
NC	Nucleocapsid
NLS	Nuclear localisation signal
NTA	Nanoparticle Tracking Analysis
OPA	Ovine pulmonary adenocarcinoma
PAMPs	Pathogen associated molecular patterns
PBS	Primer binding site
pDC	Plasmacytoid dendritic cells
PCR	Polymerase chain reaction
Pol	Polymerase
PRRs	Pattern recognition receptors
PRO	Protease
RD	Repressor domain
RIG	Retinoic- acid inducible protein
RLR	(RIG-I) like receptors
RNA	Ribonucleic acid
RSV	Rous Sarcoma Virus
RT	Reverse transcriptase
RTC	Reverse transcriptase complex
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRV	Simian Retrovirus
SU	Surface domain
TBS-TWEEN	Tris Buffer Saline/Tween
TGN	Trans Golgi Network
TLR	Toll receptor receptor
TM	Transmembrane
TRIM	Tripartite motif protein
U3	Unique 3
U5	Unique 5
VLP	Viral like particle
VR1/2/3	Variable regions

v/v	volume/volume

Chapter 1 - Introduction

1.1 Overview of retroviruses

1.1.1 Taxonomy

Retroviruses are positive-sense single-stranded RNA viruses, which infect a wide range of vertebrates (Coffin, Hughes et al. 1997). The defining feature common to all retroviruses is their ability to reverse-transcribe their RNA genome into double stranded DNA ("provirus") that is then inserted into the host genome (Vogt 1997). The family *Retroviridae* encompasses a large number of diverse retroviruses divided into two sub-families (Orthoretrovirinae and Spumaretrovirinae) (Fauquet, Mayo et al. 2010). The majority of retroviruses are included in the Orthoretrovirinae, which is the further divided into six genera (Alpharetroviruses, Betaretroviruses, Gammaretroviruses, Deltaretroviruses, Epsilonretroviruses, Lentiviruses) based upon genome divergence (defined by reverse transcriptase sequence similarity and genome complexity), virion site of assembly, and morphology, as detailed in Figure 1 (Coffin, Hughes et al. 1997) (Vogt 1997).

Retroviruses were also classified as one of four groups (A-D) based on the appearance of virion morphology by electron microscopy. Type A retroviral particles are used to describe the intracisternal particles of rodent endogenous retroviruses. These particles are non-enveloped virions with a structure similar to that of immature virions with an electron-lucent centre (Vogt 1997).

In contrast, type B, C and D group retroviruses demonstrate morphology similar to that of mature particles, with differences observed within their core region. Type B and type D retroviruses, such as the Betaretrovirus Jaagsiekte Sheep Retrovirus (JSRV) and Mason-Pfizer Monkey Virus (M-PMV), assemble in the cytoplasm and are distinguishable by their round, eccentrically positioned or bar shaped cores respectively (Payne, Verwoerd et al. 1983; Sfakianos, LaCasse et al. 2003; Murcia, Arnaud et al. 2007). Type C retroviruses, such as the Lentiviruses or Gammaretroviruses, assemble at the plasma membrane and possess a central spherical inner core (Yeger, Kalnins et al. 1978; Coffin, Hughes et al. 1997; Swanstrom and Wills 1997).

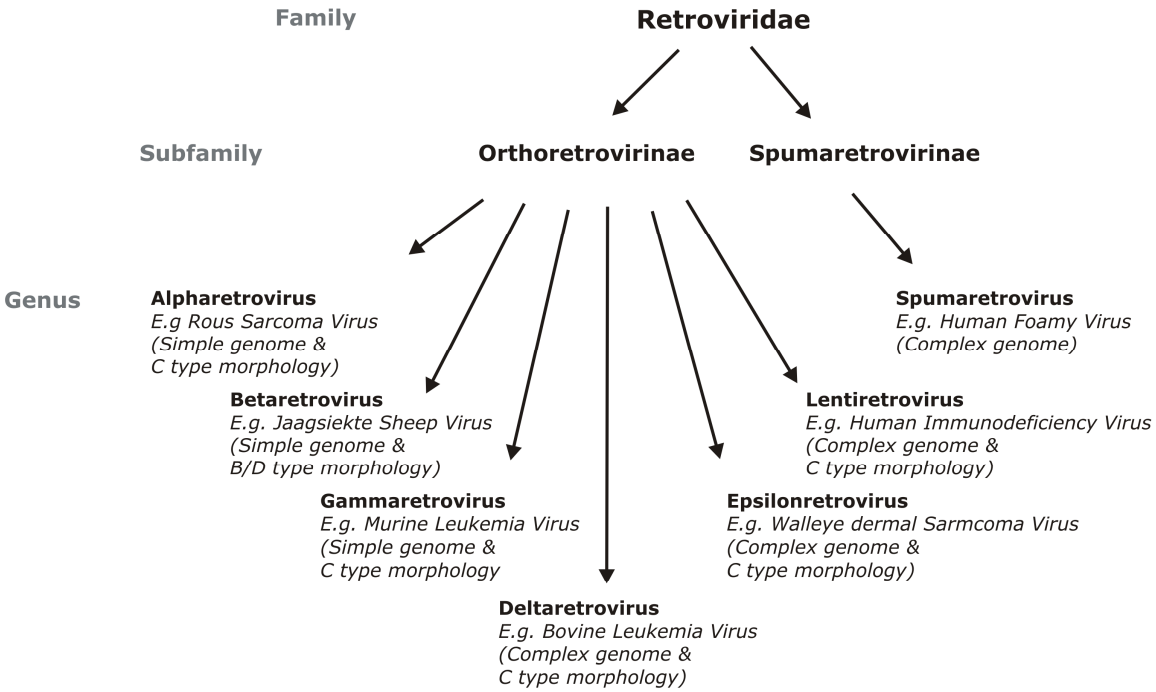


Figure 1. Overview of the taxonomy of the Retroviridae

1.1.2 Genomic Structure

The retroviral genome consists of two identical copies of single stranded positive sense RNA molecules of approximately 7-10 kb in length. The two molecules of RNA are held together by hydrogen bonds and are similar to the cellular messenger RNA as they possess a cap structure at the 5' end and are polyadenylated at the 3' end (Beemon, Duesberg et al. 1974; Vogt 1997).

All retroviruses possess the same genomic arrangement with at least the canonical *gag*, *pro, pol* and *env* genes flanked by the R ("repeated") and U5 ("unique region at the 5' end) region at the 5' end and by a U3- R region at the 3' end (see 1.2.2.2 Reverse Transcription for details) (Wang, Galehouse et al. 1976; Wang, Duesberg et al. 1976; Wang, Duesberg et al. 1976; Vogt 1997). Upon entry the RNA genome is reverse transcribed into double-stranded DNA following entry into the host cell (Baltimore 1970; Temin and Mizutani 1970). The end of the RNA viral genome is duplicated during reverse transcription into two LTRs ("long terminal repeats") arranged invariable as U3 – R – U5. The DNA copy of the viral genome (termed "provirus") is stably integrated into the host chromosomal DNA (Hughes, Shank et al. 1978). The site for transcription initiation, known as the primer binding site (PBS), is found immediately after the

5’ LTR, whereas the site for polyadenylation is found within the U5-R region of the 3’ LTR (Vogt 1997).

Retroviruses can be also defined as either ‘simple’ or ‘complex’ viruses based upon their genomic structure. Simple retroviruses rely upon the minimal number of genes sufficient for viral replication (*gag, pro, pol* and *env*). Jaagsiekte Sheep retrovirus (JSRV) and the endogenous enJSRVs, the viruses studied in this thesis, are “simple” Betaretroviruses (Palmarini and Fan 2003). However, complex retroviruses, such as the HIV, encode a number of additional “accessory” and “regulatory” genes which play roles in supporting replication and evading host immune responses, as summarised in Figure 2 (see 1.5 Host vs. Virus) (Coffin, Hughes et al. 1997)

1.1.3 Viral Proteins

Retroviruses possess a lipidic envelope that surrounds the viral particle and incorporates the Env glycoproteins (Swanstrom and Wills 1997). Env mediates retroviral infection and contributes to virion structure and stability. The Env glycoprotein is translated from the env gene as a single polyprotein. Env forms a trimer in the ER (Chan, Fass et al. 1997) before passing into the Golgi for further post-translational modifications such as glycosylation. Env eventually enters the Trans Golgi Network (TGN) where it is cleaved (processed) into the transmembrane (TM) and the surface (SU) domains (Henderson, Sowder et al. 1984; Coffin, Hughes et al. 1997; Swanstrom and Wills 1997). SU is the external, hydrophilic region of Env that mediates the interaction with the host receptor/s, whereas TM is the membrane spanning portion of Env that anchors the SU to the lipid membrane. This cleavage is essential both for the arrangement and stabilisation of virions as well as the functional aspect of retroviral entry. For most retroviruses Env must be transported to the surface where it is incorporate into virions as they bud from the cell. Mutational analysis of MLV, which assembles at the plasma membrane suggests that cleavage plays an important role in the intracellular trafficking of the protein. Uncleaved Env will not traffic to the cell surface and is retained within the Golgi. Therefore it is not incorporated into viral particles which in turn reduce the infectivity of VLPs assembled void of Env (Apte and Sanders 2010). The necessity of correct

cleavage has been shown for other retroviruses including HIV (Bosch and Pawlita 1990) as well as Betaretrovirus MMTV (Goodman, Kain et al. 1993).

Env is critical in mediating viral entry and it is under strong immune pressure from the host as it is targeted by neutralizing antibodies. Consequently, the Env glycoproteins are highly divergent between different retroviruses and contain the most variable regions within different strains of the same virus. TM also undergoes conformational changes in order to assist the fusion of the cellular receptor and viral proteins during cell entry. Env is arranged as an oligomeric complex on the cell surface and in this way is thought to increase Env stability (Coffin, Hughes et al. 1997).

All of the structural proteins required to drive the process of virion assembly and encapsidate the viral genomic RNA, are derived from the gag gene product. Gag is regarded as the determinant of virion size and morphology (Ako-Adjei, Johnson et al. 2005). Gag is cleaved by the viral protease (PR, encoded by *pro*) during maturation to form the matrix (MA), the major capsid protein (CA) and the nucleocapsid (NC) (Swanstrom and Wills 1997). All of the proteins derived from Gag provide similar structural functions among retroviruses although their sequence varies considerably between different genus. The major homology region (MHR) within CA is, like the name suggests, the region with the greater degree of homology between retroviruses (Strambio-de-Castillia and Hunter 1992).

The pol gene encodes both the reverse transcriptase (RT) and the integrase (IN) required for reverse transcription of the gRNA and integration of the proviral DNA into the host genome, respectively (Swanstrom and Wills 1997).

1.1.4 Virion structure

The structure of the virion after exit from the host cell is distinctly different from the structure of the virion during assembly. This difference is likely due to the different requirements of these two processes. Whereas the assembly process is largely focused upon gathering together all of the required components of the virion, the infectious virus released from the cell must be

competent to infect, reverse transcribe and finally integrate into a new host cell (see 1.2, Overview of the retroviral cycle). The difference in structure has been highlighted by HIV where the immature virions have an incomplete spherical shape with the N terminal of MA arranged adjacent to the virion surface. The inner layers comprise of the CA, NC and genomic RNA. Following budding, cleavage of viral proteins by the viral encoded protease allows NC and the genomic RNA to condense into the “mature” morphology observed in the extracellular virions by electron microscopy (de Marco, Muller et al. 2010) (Figure 2B).

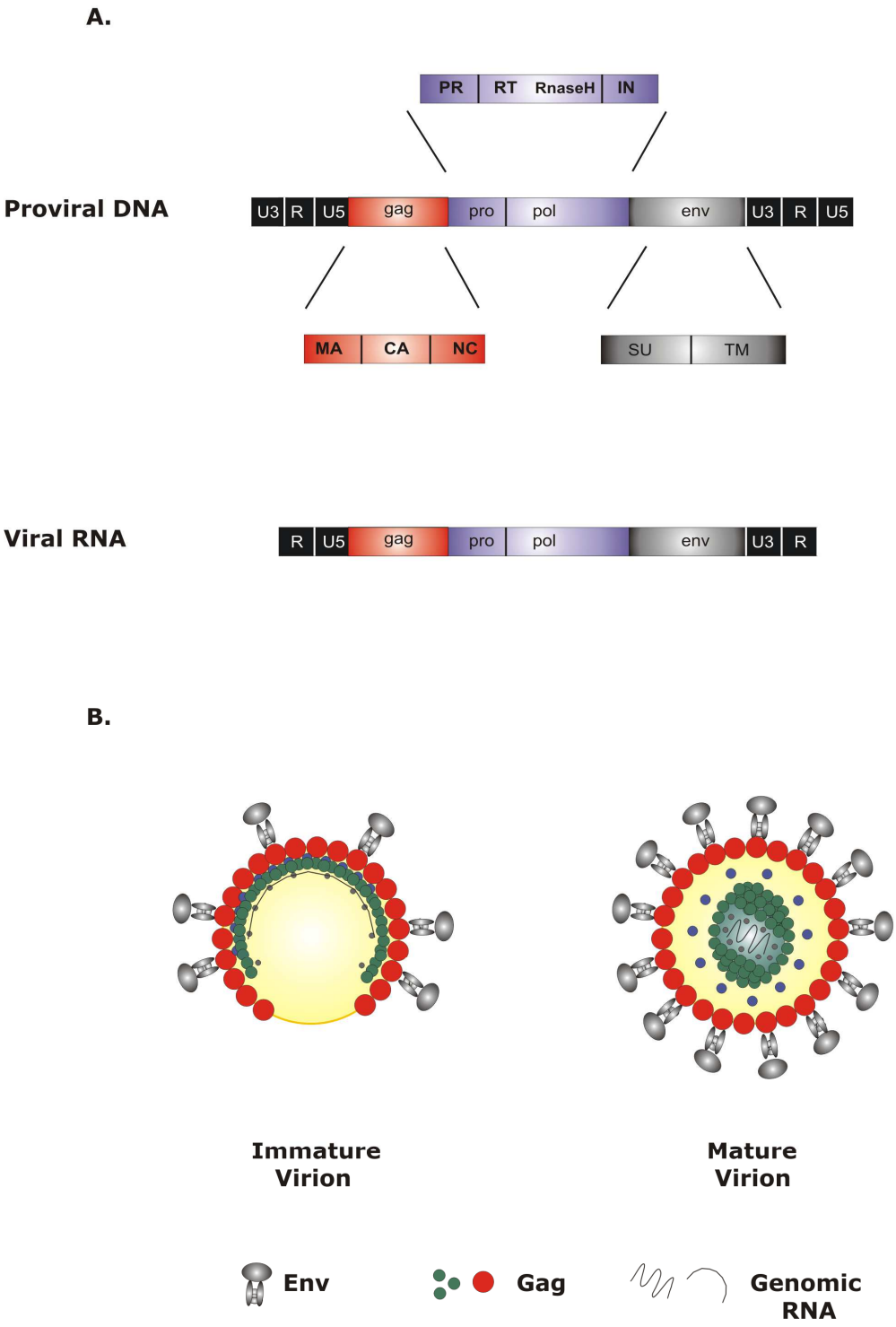


Figure 2. Schematic diagram of the retroviral genomic organisation and virion morphology. The genomic organisation as both proviral DNA and viral RNA is shown (adapted from (Coffin, Hughes et al. 1997)). In addition, the distinction between immature and mature virions morphology is shown based upon HIV as an example (B, adapted from (de Marco, Muller et al. 2010)). This schematic diagram does not take into account the arrangement of the virion core that contributes to the A-D morphological classification.

1.2 Overview of the retroviral cycle

The major steps of retroviral replication are common among all retroviruses. However, each retroviral genera, including the Betaretroviruses, possess unique aspects to their replication cycle (Vogt 1997; Vogt 1997).

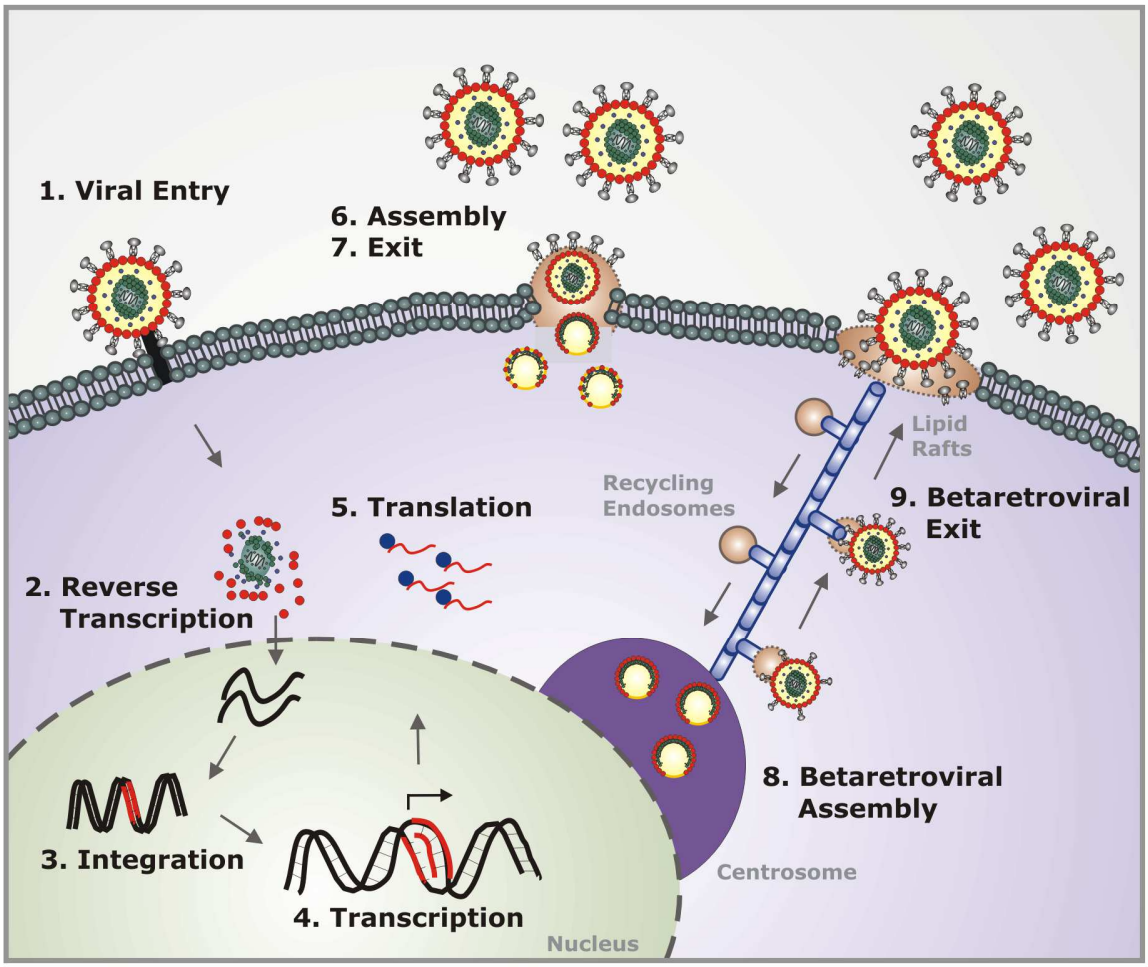


Figure 3. An illustration of the key steps of the retroviral replication cycle.
Viral entry (1), reverse transcription (2), integration (3), transcription (4), translation (5), viral assembly (6) and exit (7). Note that, in contrast to the majority of retroviruses (which assemble at the cell membrane), Betaretroviruses assemble at the perinuclear region prior to exit (8 & 9). Note the exact stage at which Env incorporation occurs for Betaretroviruses is currently unknown.

1.2.1 Viral Entry

Viral entry, the first phase of the retroviral lifecycle, is mediated by a direct interaction between the retroviral Env glycoprotein and the cellular receptor/s on the host cell (Hunter 1997). Virus-cell receptor interaction is a key point in the viral replication cycle and one of the primary determinants of host and tissue/cell tropism. Each retrovirus utilises a specific cellular receptor that may function independently or with the aid of a co-receptor (Hunter 1997).

1.2.1.1 Receptor recognition and entry

Virions must directly engage a specific host cellular receptor to gain access into the cell and initiate the replication cycle.

The initial virion “attachment” or “adsorption” to the host plasma membrane is independent of the receptor and is mediated by surface molecules such as lectins and heparan sulphate proteoglycans (Roderiquez, Oravecz et al. 1995; Pizzato, Marlow et al. 1999). Following attachment of the virions to the cell membrane, there is evidence suggesting that virions ‘surf’ the membrane and filopodia in order to reach and interact with the target receptor that will ultimately allow proper “penetration” into the cell (Lehmann, Sherer et al. 2005). The virion movement across the cell surface stimulates the underlying intracellular actin and initiates the oligomerisation of receptors which in turn primes the membrane for virus entry (Ewers, Smith et al. 2005; Lehmann, Sherer et al. 2005)

The specificity of the receptor and viral Env interaction largely determines the cellular tropism of the virus. For example, ecotropic Murine Leukaemia Virus (MLV) only infects mouse cells while xenotropic MLV infects non mouse cells, whereas amphotropic viruses can infect both (Boeke and Stoye 1997).

It is usual for the host cell receptor to be downregulated from the cell surface upon virus entry and replication within the cell. This mechanism prevents apoptosis of the cell following superinfection and inhibits the entry of newly assembled and released virions into the cell (Hunter 1997). For example, HIV-1 utilises its Env and the viral accessory proteins Vpu and Nef to downregulate the

target receptor (CD4) from the host cell surface by a mechanism not yet fully understood (Wildum, Schindler et al. 2006).

In many ways, environmental cues are necessary for the viral envelope fusion with the cell membrane and entry of the virus into the cell. In some cases virus entry may be driven simply by receptor recognition alone at the cell surface, as has been shown for HIV (Maddon, McDougal et al. 1988). Alternatively, a virus may initially be internalised by endocytosis and, following exposure to the low internal pH, be released from the endosome into the cell, as is the case for both MMTV and JSRV (Redmond, Peters et al. 1984; Bertrand, Cote et al. 2008). However, different entry pathways are not always mutually exclusive. For example, a combination of both pH-dependent and pH-independent entry has been observed for HIV, although in this case only the direct entry route appears to result in a productive infection (Nisole and Saib 2004). Some viruses require additional co-factors to allow successful entry, for example HIV-1 engages both the receptor CD4 and in addition co-receptors CCR5 and/ or CXCR4, as reviewed by (Doms 2000). Alternative co-receptors such as CCR3 and FPRL1 have also been associated with HIV-1 infection (Nedellec, Coetzer et al. 2009).

1.2.2 Reverse transcription and Integration

The synthesis of proviral DNA and its integration into the host genome are key events in the retroviral replication cycle. Following entry, the incoming virion uncoats and utilises the microtubule network for particle progression towards the nucleus (Goff 2007). Reverse transcription occurs after entry and before provirus integration in a complex known as the “reverse transcriptase complex”, Following reverse transcription, the “preintegration complex” (PIC) which contains the proviral DNA ready for integration enters the nucleus and mediates successful integration within the host genome (Brown 1997), as illustrated in Figure 4.

1.2.2.1 Reverse transcriptase complex (RTC)

The primary role of the RTC is the generation of proviral DNA. It consists of the retroviral RNA genome and co-packaged virion proteins necessary for reverse transcription, including reverse transcriptase and RNase H. A tRNA (carried within the virion core) initiates reverse transcription by binding to the primer binding site (PBS) of the 5'LTR. The RTC is a continually evolving nucleoprotein complex that varies in composition and activity as it progresses from the cell periphery to the nucleus and will also carry components of the downstream PIC into which it develops, such as integrase proteins (Telesnitsky and Goff 1997).

Following successful viral entry of the virion, the capsid is gradually disassembled as the formation of the RTC is initiated. The speed and extent of the viral particle uncoating process is unique to specific retroviruses, but the capsid protein may not be completely removed during this process, as there is evidence of its presence further downstream at the nuclear import level (Risco, Menendez-Arias et al. 1995). There has been some debate as to whether reverse transcription is initiated prior to, or after, the uncoating process has begun and cDNA can be detected within newly produced viral particles of HIV and Moloney murine leukaemia virus (MoMLV) (Trono 1992). In addition, it has been observed that viral particles with HIV Capsid mutants are deficient in reverse transcriptase activity (Forshey, von Schwedler et al. 2002). Therefore, it is likely that the Capsid has a role in the reverse transcription process. An alternative explanation is that uncoating occurs directly at the nuclear pore prior to import (Arhel, Souquere-Besse et al. 2007). Extensive RT activity in mature viral particles is thought to be prevented by the presence of the nucleocapsid (NC), confirming the control it exerts over the timing of reverse transcription (Telesnitsky and Goff 1997).

The RTC cannot simply diffuse from the cellular periphery to the nucleus. The "crowded" nature of the cytoplasm heavily restricts intracellular movements and provides many obstacles that hinder RTC progression (Luby-Phelps 2000). The RTC therefore utilises the host microtubule network (McDonald, Vodicka et al. 2002; Goff 2007) and, in the case of HIV, the Matrix protein is important for

recruitment onto the host cytoskeleton for transport to occur (Allain, Lapadat-Tapolsky et al. 1994; Bukrinskaya, Brichacek et al. 1998).

1.2.2.2 Reverse transcription (RT)

Reverse transcription is the unique feature of the retroviral replication cycle and involves the conversion of the viral RNA genome into the double-stranded DNA provirus. Reverse transcription is a multi-step process mediated by the viral encoded enzyme reverse transcriptase, an RNA dependent DNA polymerase, and RNase H (Fig. 4) (Telesnitsky and Goff 1997).

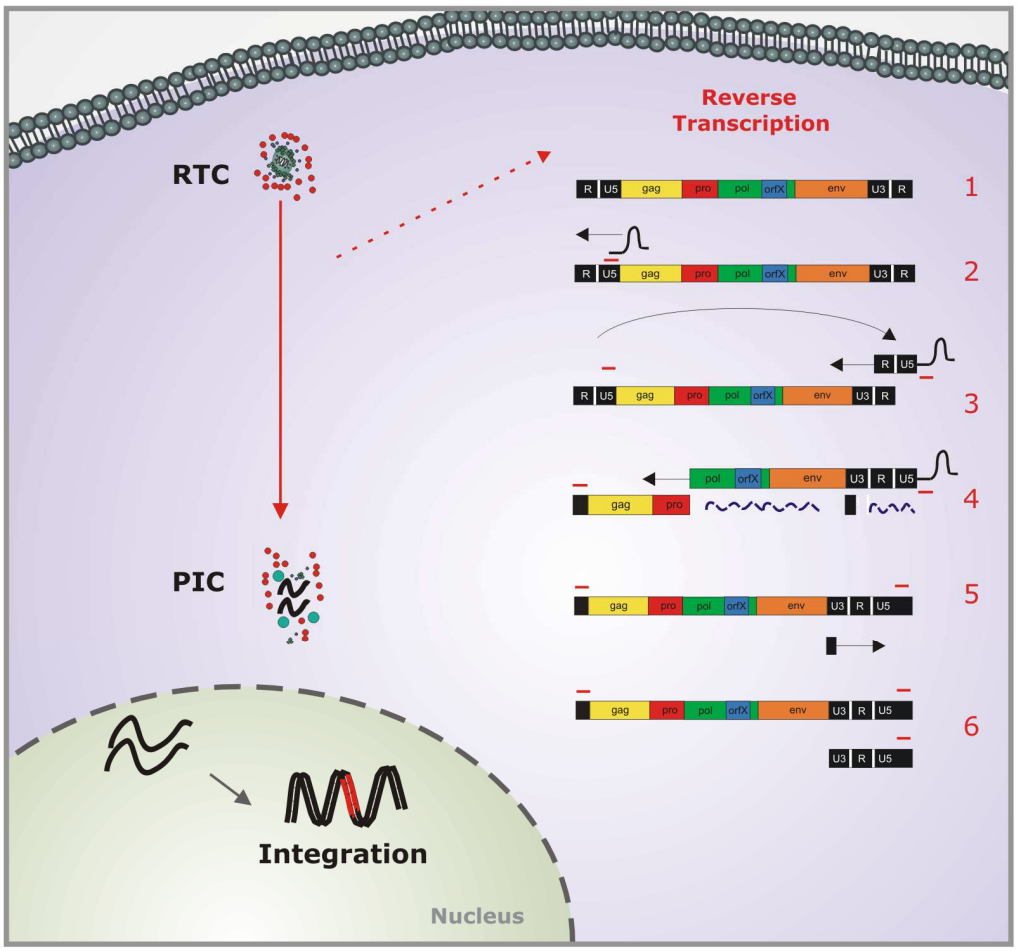


Figure 4. Illustration highlighting the major steps of the process of reverse transcription.

The reverse transcription complex generates proviral DNA for insertion into the host genome from a starting viral gRNA template (1). The first action involves the production of a short minus strand

DNA (-ssDNA) (2). This –ssDNA undergoes first strand transfer shifting from the 5' end to the 3' end (3). Elongation of the –ssDNA continues to generate the minus strand. This is accompanied by the degradation of the viral gRNA template by RNaseH (4). A short polypurine tract is non degradable by RNaseH and this primes synthesis of the corresponding plus strand (5 and 6).

The first action (Figure 4, step 2) involves the production of the short minus strand DNA (–ssDNA). Reverse transcription is initiated by the recognition of the primer binding site (PBS) at the end of the U5 region contained within the flanking 5' region of the genome by an approximately eighteen nucleotide stretch of a tRNA. Binding of the tRNA to the PBS results in elongation from the PBS to the 5' cap. Each viral genus utilises a distinct pattern of tRNAs to initiate RT. For example, M-PMV uses tRNALys1-2 (Sonigo, Barker et al. 1986) whereas MMTV and HIV exclusively utilise tRNA Lys3 as a primer for reverse transcription (Peters and Glover 1980; Ratner, Fisher et al. 1987). Production of HIV mutants with an altered PBS site that utilises a selection of other tRNAs results in the emergence of reverse mutants that use the preferred tRNA Lys3 (Ni and Morrow 2007). However, MLV is more versatile and can use alternative tRNAs for priming reverse transcription although the maximum efficiency of the process is reached when using its preferred tRNA Pro (Palmer, McPherson et al. 2005).

The –ssDNA undergoes first strand transfer (Figure 4 step 3), shifting from the 5' end to the 3' end which serves as a template for further transcription (in an intra or intermolecular fashion). The R region of the –ssDNA anneals with that of the 3' end and elongation of the –ssDNA in the direction of the 5'LTR continues, generating the minus strand DNA. RNase H activity is essential for successful first strand transfer as it degrades the RNA from the -ssDNA:RNA complex and mutations within RNaseH have been shown to inhibit the process of reverse transcription (Figure 4 step 4) (Telesnitsky and Goff 1997). The RNA genome contains a short polypurine tract (PPT) resistant to degradation by RNaseH from which the +ssDNA is generated (Figure 4 step 5 & 6). Annealing of the PBS of the +ssDNA to that of the 5' end of the minus strand DNA generates the second strand transferred where after the plus strand of DNA is synthesised and proviral DNA has been fully generated. During the process of reverse transcription both the 5' and 3'LTRs of the proviral DNA generated are identical containing U3-R-U5 arrangement, in contrast to the viral mRNA where the 5' and 3'LTRs only contain the R-U5 and U3-R portions respectively (Telesnitsky and Goff 1997).

Whilst the process of reverse transcription is uniform the structure of the enzyme reverse transcriptase varies between retroviruses. For example, the Betaretrovirus MMTV has a reverse transcriptase (RT) protein which is a functional monomer (Taube, Loya et al. 1998) whilst the lentivirus HIV-1 RT is a dimer. Reverse transcription *per se* is an error prone process as the reverse transcriptase has no proof reading activity and therefore contributing to viral genetic diversity (Moya 1999). Far from being detrimental, the lack of proof reading activity by RT allows retroviruses the flexibility required to rapidly evolve and meet the requirements of their environment and also to evade host immune responses (see section 1.5 Host vs Virus).

1.2.2.3 The Preintegration complex (PIC) and nuclear import

During the progression from the plasma membrane to the nucleus along the microtubule network, the properties of the RTC are modified allowing it to mature into the PIC. This is reflected in the change of viral particle density following infection (Fassati and Goff 1999; Fassati and Goff 2001). The PIC is defined as a complex containing proviral DNA ready for integration. The ends of the cDNA generated within the RTC are closely associated with integrase (IN) (Wei, Mizuuchi et al. 1998) which drives integration. The PIC must first cross the nuclear membrane to gain access to the genome. Generally, most retroviruses including Betaretroviruses require the host cells to be actively dividing (i.e. passing through M phase with the nuclear membrane disassembled) in order to successfully enter the nucleus and integrate. However, HIV and other lentiviruses are capable of infecting non dividing cells due to the presence of a nuclear localisation signal (NLS) (Brown 1997).

1.2.2.4 Integration

Following nuclear import it is essential that the proviral DNA is integrated into the host genome. Successful retroviral infection requires that the provirus is permanently integrated in the host cell genome, otherwise the DNA is unstable and decays within the nucleus. Stable integration of proviral DNA allows the virus to mimic host DNA thus allowing it to utilise the cell machinery for gene

expression. The physical process of integration involves DNA cleavage and ligation in two distinct stages. First, the 3' end processing of proviral DNA to be integrated occurs with the removal of two nucleotides from the 3' end by integrase, thus exposing the hydroxyl group that will join to the host DNA. The second step is called 'DNA strand transfer' whereby the exposed proviral 3' end hydroxyl group joins the 5' end of the host target genome and is repaired by cellular enzymes (Brown 1997).

Generally, on a structural basis retroviruses may have a preference for the major groove of DNA rather than the minor groove that is curved along the nucleosomes. The major groove is more accessible, providing less steric inhibition from DNA binding proteins. Retroviral integrations can in many ways be considered a random event. However, there is evidence suggesting that different retroviruses tend to integrate in different areas of the genome (Mitchell, Beitzel et al. 2004). The site of integration by JSRV or enJSRVs within the genome has not yet been defined.

A recent study of integration target site selection by a "reconstituted" human endogenous retrovirus (HERV)-K has shown that new integration sites were predominately found in active gene dense areas slightly enriched in transcription sites (Lee and Bieniasz 2007). This is in contrast to established HERV-K elements which are found mainly outside active gene rich areas. These data suggest that HERV-K integration that occurred in areas that negatively altered gene function were counter-selected during evolution.

1.2.3 Transcription and viral mRNA processing and export

1.2.3.1 Expression

The successful integration of proviral DNA is essential as it provides a template for transcription (Rabson and Graves 1997). Non-integrated DNA produced during the process of reverse transcription can account for as much as 99% of intracellular viral DNA at the early stages of infection but it makes no contribution to viral expression. Unintegrated DNA can be found as 1-LTR, 2-LTR and linear DNA forms. In turn, viral transcription is influenced by the site of

proviral integration. Generally, successful and stable integration occurs in transcriptionally silent regions causing minimal disruption to the host. Therefore, successful translation often does not just depend upon the recruitment of transcription factors to the U3 of the 5'LTR, but in the depletion of those which are repressive and restricting access (Rabson and Graves 1997).

The initiation site of transcription is defined within the 5'LTR at the beginning of the central short direct repeat "R" region. The U3, which precedes R, regulates transcription and contain the promoter and enhancer regions. The promoter contains the initiating TATA binding element and a range of positive binding regulatory sequences, while upstream the enhancer region contains positive acting transcription factor binding regulatory elements and negative regulatory elements. These positive binding regulatory transcription factors will often be related to the viral tropism (Rabson and Graves 1997). Cell type and differentiation status dictates the enhancing factors available to the provirus and obviously impacts its transcription levels. For example, the HIV LTR recruits the lymphocyte enhancer factor (LEF) to its promoter region which is commonly present in immature B cells and both immature and mature T cells (Waterman and Jones 1990; Waterman, Fischer et al. 1991). This also has the added advantage of assisting with promoter accessibility as it alters the promoter conformation (Giese, Cox et al. 1992). Related viruses might be expressed in different cells/tissues due to differences in their LTRs. For example, the exogenous JSRV transcription is promoted by the lung specific hepatocyte nuclear factor 3ß (HNF-3β) (Palmarini, Datta et al. 2000; McGee-Estrada and Fan 2006; McGee-Estrada and Fan 2007) while enJSRVs are driven by hormone responsive elements (Palmarini, Gray et al. 2001). Similarly, MMTV has functional hormone responsive elements within its promoter region (Cato, Henderson et al. 1987). Retroviruses exploit the host RNA polymerase II to drive viral mRNA production.

1.2.3.2 Viral mRNA processing

The provirus exploits the host cell machinery for expression, viral mRNA processing, export and translation. All retroviral mRNAs are produced initially as

full length transcripts that are initially processed in order to have a 5' 7-methyl-guanosine cap (which assist with translation initiation) combined with cleavage and polyadenylation of the 3' end within the R-U5 region, giving all transcripts identical 3' ends and stability during transcription.

All retroviruses use a full length viral mRNA (organised in 5'cap-R-U5-gag-pol-env-u3-R-polyA) for the translation of both Gag and Pol proteins and for the genome of the viral progeny. For some retroviruses like HIV-2, it has been shown that the template used during translation can also be packaged (Rabson and Graves 1997; Bolinger and Boris-Lawrie 2009).

1.2.3.3 Splicing

Splicing is an important general intracellular function. Env is instead translated from a single spliced mRNA while some accessory proteins of complex retroviruses derive from multiple spliced mRNAs (Rabson and Graves 1997). Through splicing, complex genomes can produce regulatory proteins that facilitate transcription to maximise successful viral particle production. For example HIV encodes Tat, which *trans* activates the viral LTRs and help to recruit various cellular proteins such as histone acetylase transferase which acetylates histones therefore promoting access by transcription factors to the viral LTRs (Benkirane, Chun et al. 1998).

Simple retroviruses, which only produce one splice variant for the production of Env, utilise standard splicing donor and acceptor sites. Complex retroviruses produce multiple transcripts (e.g. HIV has been described to produce in excess of 40 splice mRNA variants) (Rabson and Graves 1997).

1.2.3.4 Nuclear Export

The full length unspliced mRNA must be exported from the nucleus in order to be available for translation and/or to be packaged in the virion genomic RNA. It is important that the correct proportions of both spliced and unspliced populations are maintained in order to optimise viral particle production. Complex retroviruses encode additional proteins to assist with this process. For example, the transport of full length HIV viral mRNA from the nucleus is mediated by the regulatory protein Rev (Yi, Bogerd et al. 2002). Rev is

synthesized in the cytoplasm and transported back to the nucleus for action by its nuclear localisation signal (Truant and Cullen 1999). Within the nucleus, Rev acts by binding RRE (Rev responsive element), a cis acting sequence within env. Rev-RRE binding masks the NLS and reveals the nuclear export signal (NES) (Henderson and Percipalle 1997). Rev binds the cellular export factor Crm1 (Izaurralde, Kutay et al. 1997; Bogerd, Echarri et al. 1998). Once successful export of RNA has occurred, Crm1 is dissociated from the Rev-RRE complex allowing Rev to interact with Importin B and cycle back into the nucleus. Rev-like viral proteins are also found encoded for other retroviruses such as mouse mammary tumour virus (MMTV) (Mertz, Simper et al. 2005) and human endogenous retrovirus-K (HERV-K) (Ruggieri, Maldener et al. 2009). However, the simple retrovirus M-PMV encodes no Rev-like protein and utilises a cis acting RNA element (the constitutive transport element, CTE) which interacts with the cellular nuclear export factor Tap1/NFX1 to promote nuclear export (Braun, Rohrbach et al. 1999). Interestingly, JSRV is a simple retrovirus but the signal peptide of the glycoprotein Env (Rej/SP) functions as a Rev-like protein and promotes full length viral RNA export by interacting with a cis acting responsive element within the 3' end of the genome (Caporale, Arnaud et al. 2009; Nitta, Hofacre et al. 2009, Hofacre, 2009 #25).

1.2.4 Viral assembly, budding and virion maturation

1.2.4.1 Viral Proteins and Virion assembly

The Env glycoproteins are synthesized specifically on the ER polysomes before being modified and delivered via the secretory pathway to the plasma membrane. In contrast, Gag and Gag-Pol protein precursors are produced within the cytosol on free polysomes (Swanstrom and Wills 1997). It is Gag which supports virion assembly/release and the various subdomains of Gag serve different functions during this process. The nucleocapsid (NC) domain of Gag is particularly important during the initial process of encapsidation of the viral RNA genome (Berkowitz, Ohagen et al. 1995). Recently, the presence of retroviral Gag proteins has been detected in some cases within the nucleus and this

supports the idea that Gag has a key role in viral genomic RNA export (Parent 2011).

Viral gRNA has a packaging signal which drives the encapsidation process and likely gives viral RNA the selection specificity required in the presence of a large volume of cellular mRNAs (Adam and Miller 1988). The NC domain of Gag is capable of non-specifically binding RNA and DNA, however, it relies upon the encapsidation sequence to specifically associate with the viral gRNA. The viral gRNA is dimerized at the 5' end providing an important evolutionary factor as this can aid the diversification of the genome (Onafuwa-Nuga and Telesnitsky 2009). Furthermore, it is the contact between the NC and the viral gRNA, which leads to the formation of the virion inner core. The spontaneous assembly of Gag when contacted by a non-specific single stranded nucleic acid highlights its importance in driving virion assembly (Campbell and Vogt 1995). Mutations in both the NC and CA region demonstrate their importance during virion formation and release (Hansen, Jelinek et al. 1990, Dorfman, 1993 #510; Dorfman, Luban et al. 1993). Mutations in CA affect virion formation (Ganser-Pornillos, von Schwedler et al. 2004) and it is the oligomerisation and assembly of the CA which encloses the gRNA genome associated with NC. Those virions which are formed initially are immature in structure, spherical and defined by a distinctive layer under the lipid envelope (Kurth 2010) Retroviral assembly and budding is thought to occur predominantly at the plasma membrane (Yeger, Kalnins et al. 1978) although there is also evidence of assembly within the cytosol. Interestingly, Betaretroviruses are unique among retroviruses as their assembly occurs within the cytoplasm in the vicinity of the mitochondrial organizing centre (MTOC) independently of plasma membrane association (Payne, Verwoerd et al. 1983).

1.2.4.2 Viral Budding

The matrix (MA) is considered important for Gag membrane targeting during assembly and budding. In particular, it is the post-translational modification of MA by myristoylation (the attachment of a myristoyl group to the N terminal of the Gag protein) which influences membrane association of Gag (Facke, Janetzko et al. 1993; Ono, Orenstein et al. 2000). This in turn affects Env

distribution as Gag – Env interaction is an important factor for the presence of Env within the lipid rafts of the plasma membrane and subsequent incorporation into the assembling virions as they exit the cell (Bhattacharya, Repik et al. 2006).

Mutational analysis of Gag revealed that there exists specific position-independent domains in Gag, the late (L) domains, which are common to many retroviruses and plays crucial roles in recruiting host factors during viral exit (Parent, Bennett et al. 1995). Several different L domains have been indentified for various retroviruses. For example, the P(T/S)AP L domain is present in both HIV-1 (Gottlinger, Dorfman et al. 1991) and M-PMV (Gottwein, Bodem et al. 2003) while the PPxY domain is found in Rous Sarcoma Virus (RSV) (Parent, Bennett et al. 1995) and M-PMV (Yasuda and Hunter 1998). Other late domains have been also identified within retroviruses (Morita and Sundquist 2004). Late domains allow assembling virions to engage with host cofactors in order to reach the cell membrane for viral exit. For example the P(T/S)AP domain recruits TSG101 (Garrus, von Schwedler et al. 2001) and similarly PPxY allows interaction with TSG101 and AIP1 both of which are part of the endosomal ESCRTs (endosomal sorting complex required for transport) machinery that normally function at the surface of the endosome to induce the budding of vesicles into the multivescular bodies (MVB). During infection, retroviruses redirect the ESCRT machinery to the cell membrane in order to bud out of the cell (Morita and Sundquist 2004). During exit from the cell, retroviruses also interact with cellular proteins that induce a curvature of the plasma membrane observed during exit (Strack, Calistri et al. 2003; Morita and Sundquist 2004).

The virions which assemble at the plasma membrane for budding are still immature in morphology. The processing of Gag into MA, CA and NC domains and Gag-Pol cleavage by the viral protease occurs after viral budding from the cell and allows the morphological and functional maturation of the virion (see 1.1.4 Figure 2B). Multiple interactions between Gag, the plasma membrane and gRNA are required for correct virion maturation (Datta, Heinrich et al. 2010).

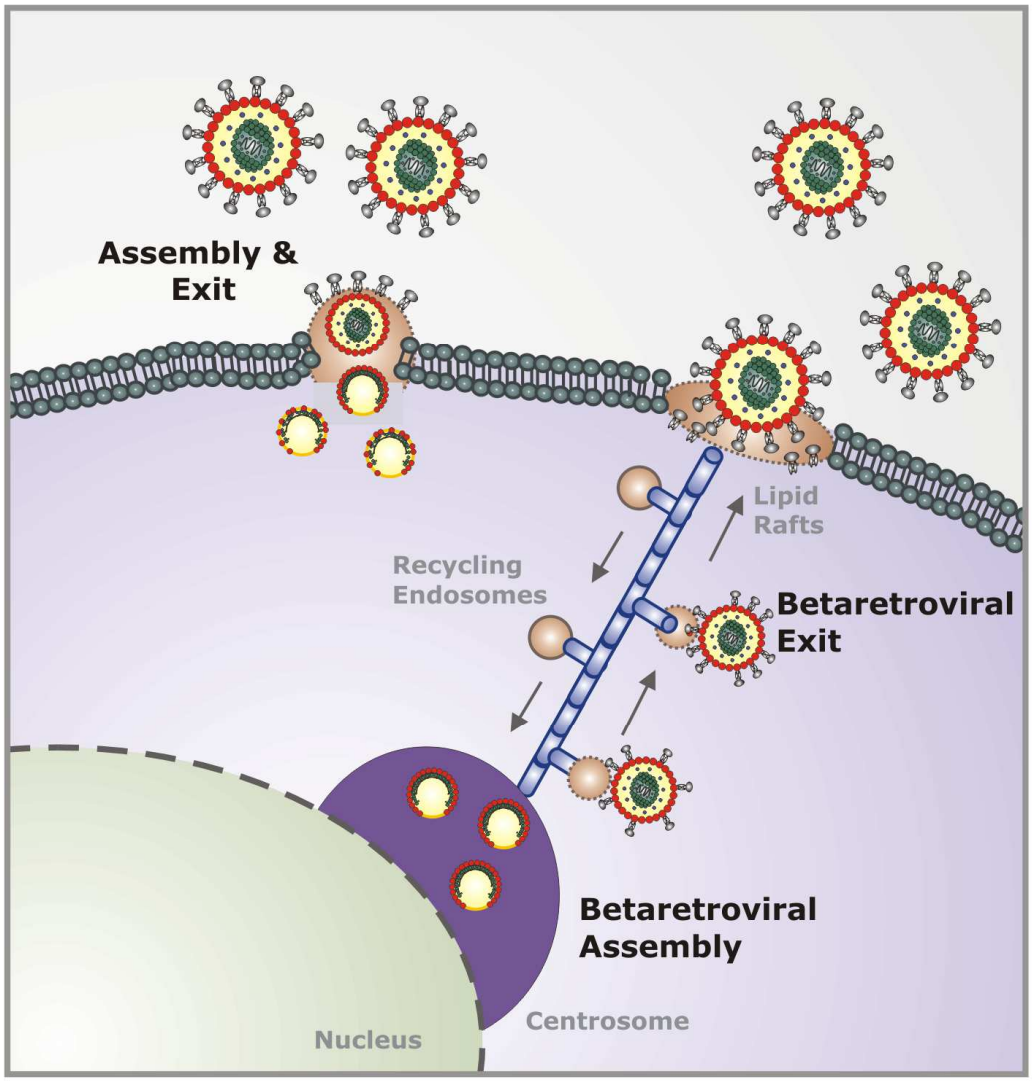


Figure 5. Schematic diagram highlighting the two major routes of virion assembly. For the majority of retroviruses viral particle assembly occurs at the plasma membrane and virions acquire Env as they bud from lipid rafts. Betaretroviruses are unique as they appear to assemble at the centrosomal region prior to collecting the envelope as they exit via lipid rafts.

1.2.4.3 Cell-to-cell vs. cell-free transmission

Once successful replication has taken place, retroviruses must exit the cell in which infection is established and enter another uninfected cell in order to spread infection within the host. In theory, cell free virus spreading provides the widest potential range for propagation to ensure survival. Virus exits the infected cell and is free to diffuse within the host infecting both close and distant tissues and cells. However, cell free transmission of virus encounters physical and kinetic challenges during the process of viral entry (see viral entry). Steric hindrance is associated with cell-free infection. Similarly, the negative charge density at the cell surface affects the accessibility of binding sites to

invading virions. Indeed, the addition of polybrene is required *in vitro* to increase the likelihood of successful infections (as much as ten fold for retroviruses) by reducing electrostatic repulsion between the incoming virions and the host cell membrane (Coffin, Hughes et al. 1997). Virions also face an increasing repertoire of cell host factors which target unique features of the replication cycle both during exit and entry. In response to this, retroviruses and other enveloped viruses have developed a strategy to pass from an infected to an uninfected cell by cell-to-cell transmission. This mode of transmission has the advantage to avoid the physical barriers imposed by the host and the immune system, increasing viral kinetics and efficiency but it has the disadvantage of "travelling" only a short distance between infected and uninfected neighbouring cells. Documented retrovirus cell to cell transmission occurs via the plasma membrane, filopodial bridges, nanotubules and virological synapses (reviewed in (Sattentau 2008)). Plasma membrane based fusion may result in the formation of syncytia observed for both Human T cell leukaemia virus 1 (HTLV-1) and HIV-1 although the ability of efficient cell to cell spread by this method is still debated. Filopodia, are projections of actin from both infected and uninfected cells which support the movement of virus from an infected to an uninfected cell. This movement is driven by the aggregation of viral Env and the corresponding host cell receptor (Lehmann, Sherer et al. 2005; Sherer, Lehmann et al. 2007), HIV-1 transport along the exterior of T cell nanotubules has also been observed. Nanotubules can be multiple actin membrane based projections linking infected and uninfected cells forming after the formation of the virological synapse. Virions migrate along the exterior of the tubule before the interacting with the cellular receptor and entering the cell. Virological synapses, similar to immunological synapses, are another example of how cell-to-cell transmission of retroviruses and have been observed for HIV-1 and HTLV transmission between immune cells. (HTLV-1) infected T cells produce very few cell-free virions (Derse, Hill et al. 2001) in vivo and the virus is spread directly between lymphocytes via virological synapses (Jolly and Sattentau 2004). The budding of HTLV-1 virions and their entry into surrounding uninfected cells occurs at cell contact zones enriched in microtubules and adhesion factors (Igakura, Stinchcombe et al. 2003).

The virological synapses are supermolecular complexes that are formed at the site of contact between an infected cell and a target cell expressing the viral receptor. The virological synapses require a functional actin cytoskeleton and the expression of viral receptor/s in the target cells (summarised by (Sattentau 2008)).

1.3 Exogenous retroviruses

1.3.1 Overview of exogenous retroviruses and disease

Retroviruses were first associated with the development of disease over a century ago when an infectious, filterable agent was found to cause chicken leukosis and sarcoma. The viral aetiology for these diseases was identified by Ellermann in 1908 and Rous in 1911 and the viruses they described were named avian leukosis virus (ALV) and Rous sarcoma virus (RSV), respectively (Vogt 1997). Later, retroviruses were discovered to be the cause of cancers in many other animal species including mice, cats, sheep, cattle and humans. Retroviruses are differentiated in “exogenous” and “endogenous” viruses. Exogenous retroviruses are transmitted horizontally from an infected host to an uninfected host like any other virus (Rosenberg and Jolicoeur 1997).

Oncogenic retroviruses can be divided into different groups based on the mechanism of transformation they follow: acutely transducing, cis-acting and trans-acting retroviruses (Rosenberg and Jolicoeur 1997; Kurth 2010).

Acutely transducing retroviruses, carry a host derived oncogene, for example, the Alpharetrovirus RSV carries the host derived oncogene *src* (Duesberg and Vogt 1970; Martin 2004). Acutely transducing retroviruses are usually replication defective and they need a “helper” virus for replication. They are capable of rapidly transforming an infected cell, once they integrate their genome (carrying an oncogene) within the infected cell.

Cis-acting retroviruses lack an oncogene and do not directly transform cells in culture. Instead, transformation occurs during a period of latency as a result of mutagenesis of the host genome by proviral insertion (a process also known as

"insertional activation") (Rosenberg and Jolicoeur 1997). An example of a cis acting retroviruses is MMTV which induces mammary tumours in mice by insertional mutagenesis (Theodorou, Kimm et al. 2007). Tumours induced by insertional activation are monoclonal or oligoclonal. Different tumours induced by the same retroviruses acting by insertional activation are characterized by the presence of common integration sites (Rosenberg and Jolicoeur 1997; Cousens, Bishop et al. 2004; Philbey, Cousens et al. 2006).

Trans-acting retroviruses are those which induce transformation by a viral gene product. The Betaretrovirus of interest for this thesis, JSRV, has a trans-activating Env and this mechanism will be discussed in detail below. Retroviruses have also been linked with a wider range of diseases, including neurological (Ances and Ellis 2007), autoimmune (Ansari, Pattanapanyasat et al. 2007) and more notably immunodeficiency diseases (Moanna, Dunham et al. 2005) which affect a range of species including humans.

1.3.2 Ovine pulmonary adenocarcinoma (OPA)

Jaagsiekte sheep retrovirus (JSRV) is unique among retroviruses as it is the only virus to cause a naturally occurring lung cancer, Ovine pulmonary adenocarcinoma (OPA) (Palmarini, Fan et al. 1997; Palmarini, Sharp et al. 1999; Palmarini and Fan 2001).

OPA is a fatal disease and involves the development of lung adenocarcinoma as a result of infection and transformation of type II pneumatocytes within the bronchial and alveolar epithelium (Palmarini, Dewar et al. 1995; Palmarini, Fan et al. 1997; Murgia, Caporale et al. 2011; Yu, Linnerth-Petrik et al. 2011). JSRV is an "exogenous" retrovirus and is classically transmitted horizontally from infected to uninfected sheep. More recently, an important natural transmission route assisting the spread of OPA has been identified from mothers to their young via colostrum, although the respiratory secretions of affected animals remain the main cause of the contagium (Voigt, Kramer et al. 2007; Grego, De Meneghi et al. 2008) OPA is a veterinary disease that poses significant economic problems to several sheep-rearing countries. However, it also provides a large animal model for human lung cancer as it bears resemblance to human

bronchioloalveolar adenocarcinoma (BAC), an unusual form of human adenocarcinoma (Platt, Kraipowich et al. 2002) thus making it an interesting model for investigation.

OPA is clinically identified through progressive acute respiratory distress of the host due to the development of multifocal tumours. In addition, secondary pulmonary bacterial infections are also common in affected animals. The pulmonary cells from which the tumours originate are type II pneumocytes (Palmarini, Dewar et al. 1995; Palmarini, Fan et al. 1997). More recently, it has been shown that JSRV infects a population of proliferating type II pneumocytes which, whilst abundant in young lambs, are relatively rare in adult sheep (Murgia, Caporale et al. 2011).

Histologically, OPA generally manifests itself as multifocal tumours which extend from the lung surface and may be grouped into two distinct patterns classified as either “typical” or “atypical” forms (Garcia-Goti, Gonzalez et al. 2000). The secondary bacterial infections which occur frequently in OPA can distort the pathology presented. Sheep affected by OPA generally produce an excess of pulmonary surfactant which is an essential functional and protective component of the respiratory tract (De las Heras, Gonzalez et al. 2003; Johnson and Fan 2011).

In vivo, JSRV is found to be expressed preferentially in the tumour cells of affected animals. Interestingly, in reporter assays *in vitro*, the JSRV LTRs (containing the viral promoter and enhancers) are expressed preferentially in type II pneumatocytes and in Clara cell derived MLE-15 and mtCCl-2 cell lines (Palmarini, Datta et al. 2000; McGee-Estrada, Palmarini et al. 2005).

A major issue at present is the lack of a simple laboratory test to diagnose JSRV infection. Diagnosis is difficult at the preclinical stages due to the lack of an antibody response from the host against JSRV. This lack of immune response is explained in part by the expression of enJSRVs during ontogeny that likely tolerize sheep against JSRV (Ortin, Minguijon et al. 1998). Early detection prior to OPA development is possible by highly sensitive nested PCR assays which are not a cost effective diagnostic tool (Palmarini, Holland et al. 1996; Holland, Palmarini et al. 1999; Gonzalez, Garcia-Goti et al. 2001; De Las Heras, Ortin et

al. 2005). In general, only 2-5% of the animals within an affected flock develop OPA. This suggests that JSRV may be expressed at low level in the majority of infected hosts (Caporale, Centorame et al. 2005) and low concentrations of viral DNA within blood during this latent period are linked with difficulty in accurate diagnosis (Lewis, Brulisauer et al.). OPA is experimentally reproducible in lambs by intratracheal inoculation using the JSRV21 molecular clone (or by using virus purified from lung secretions of an affected animal). The rate of disease induction changes depending on the age of the infected animal. Lambs are most susceptible to experimental infection and may develop tumours in as little as ten days (Fan, Palmarini et al. 2003).

1.3.3 JSRV taxonomy and genome

JSRV is a member of the Betaretrovirus genus within the family of the *Retroviridae*, subfamily, Orthoretroviranae. JSRV has the classic genomic organization of a simple retrovirus with *gag, pro, pol*, and *env* genes flanked at either side by the 5' and 3' long terminal repeats (LTRs) (Palmarini and Fan 2003). Within the JSRV genome there is an accessory open reading frame, *orf-X*, to which no function has yet been assigned. All strains of JSRV studied have maintained this open reading frame intact suggesting that it may have an essential role for virus replication, although by mutational analysis it has been determined that Orf-X is not required for the process of transformation (Rosati, Pittau et al. 2000; Maeda, Palmarini et al. 2001).

JSRV can infect a wide variety of cells in vitro and in vivo (Palmarini, Dewar et al. 1995; Palmarini, Holland et al. 1996; Holland, Palmarini et al. 1999; Palmarini, Sharp et al. 1999). However, in OPA-affected animals, viral antigens have been detected mainly if not almost exclusively in the tumour cells (Palmarini, Dewar et al. 1995; Murgia, Caporale et al. 2011). Within the JSRV U3 region there are enhancer-binding motifs for lung-specific transcription factors, such as HNF-3β that drives the expression and transformation of the lung epithelial cells (McGee-Estrada, Palmarini et al. 2002). The U3 region also contains several sites that engage general transcription factors such as CEB/P, commonly required by most cell types (McGee-Estrada and Fan 2006; McGee-Estrada and Fan 2007).

1.3.4 JSRV oncogenesis

JSRV follows unique mechanisms to induce cell transformation. The JSRV Env is a dominant oncoprotein both in vitro and in vivo (Maeda, Palmarini et al. 2001; DeMartini, Platt et al. 2004; Wootton, Halbert et al. 2005; Caporale, Cousens et al. 2006). The JSRV envelope is a type 1 transmembrane protein approximately 620 amino acids (aa) in length. Within the cytoplasmic tail of the TM domain there is an important SH2 binding motif (YXXM) that has been found to be critical for Env-induced cell transformation (Caporale, Cousens et al. 2006; Hull and Fan 2006; Cousens, Maeda et al. 2007). The JSRV Env induces the activation of cellular signalling pathways such as the Ras-MEK-MAP and PI3-kinase-Akt-mTOR pathways, although the relative importance of these pathways for oncogenesis remains to be elucidated (Palmarini, Maeda et al. 2001; Liu, Duh et al. 2003; Liu, Lerman et al. 2003; Maeda, Inoshima et al. 2003; Zavala, Pretto et al. 2003; Liu and Miller 2005; Maeda, Fu et al. 2005; Liu and Miller 2007; Maeda and Fan 2008).

1.3.5 Hyal2 mediates viral entry of both JSRV and related enJSRVs

JSRV and the related enJSRVs utilize Hyaluronidase 2 (Hyal2) for cell entry (Rai, Duh et al. 2001). Hyaluronidases degrade hyaluronidin, a glycosaminoglycan critical to maintain the integrity of the extracellular matrix (Lepperdinger, Mullegger et al. 2001). Hyal2 is a glycophosphatidylinositol (GPI) anchored protein with a weak hyaluronidase activity and it is the only member of the hyaluronidase family that JSRV utilises for cell entry (Miller 2003), which suggests that the hyaluronidase activity per se is not a requirement for viral receptor function (Vigdorovich, Strong et al. 2005; Vigdorovich, Miller et al. 2007). JSRV is believed to enter the infected cells via a caveolar-dependent receptor-mediated endocytosis.

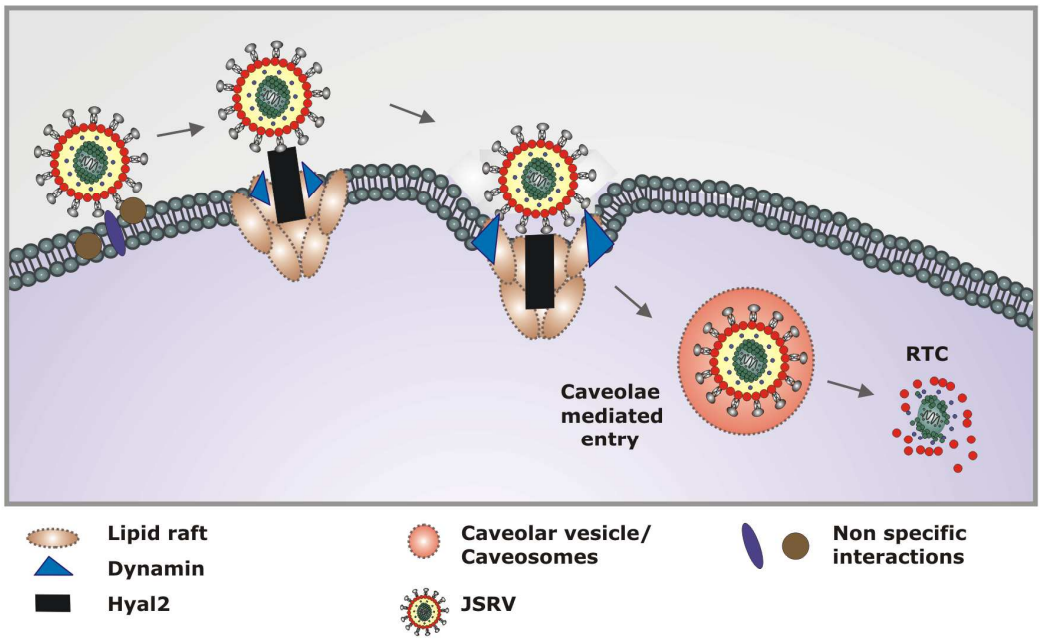


Figure 6. Illustration outlining the proposed caveolae-mediated viral entry pathway.

JSRV makes a non-specific interaction on the cell surface before surfing towards hotspots and entering via a caveolae mediated pathway (Bertrand, Cote et al. 2008). Caveolae are grooves on the plasma membrane associated with lipid rafts. The latter are regions of the plasma membrane high in sphingolipids and GPI anchored proteins. A prerequisite for caveolar-mediated entry of a virus is the cluster of lipid rafts on the cell plasma membrane. This may enhance infection by concentrating virions and their receptors at a site prior to entry. The attachment of JSRV to Hyal2 (a GPI and lipid raft associated protein) may occur with a pre-existing cluster of lipid rafts, or alternatively clustering may be induced following virion binding to non-specific receptors, as described previously. Caveloae domains are very flexible regions of the plasma membrane and this clustering of lipid rafts combined with virus attachment to the host receptor distorts the membrane shape leading to its curvature. This curvature promotes cleavage of the forming caveolar vesicle from the plasma membrane via Dynamin to complete the internalisation of the virus.

1.4 Endogenous retroviruses (ERVs)

1.4.1 Overview of endogenous retroviruses

Retroviruses must integrate their genomic DNA (“provirus”) into the host cell genome in order to carry out a successful replication cycle. Cell division increases the capacity to produce viral particles and indirectly maximises viral infection and virus “survival” within the host. In turn, survival during evolution is dependent on the ability of the virus to be successfully transmitted between infected and uninfected hosts. Retroviruses can be transmitted either horizontally or vertically. “Exogenous” retroviruses are transmitted horizontally like any other virus and include many common pathogens of humans and animals, such as human immunodeficiency virus (Chiu, Yaniv et al. 1985), Feline

leukemia virus (Hardy, Old et al. 1973), Jaagsiekte sheep retrovirus (Palmarini, Sharp et al. 1999) and several others. “Endogenous” retroviruses (ERVs) are instead transmitted from generation to generation like any other Mendelian gene (Boeke and Stoye 1997). Some ERVs are derived from the integration of ancient and now extinct exogenous retroviruses within the germline cells of the host, for example Human endogenous retrovirus (HML-2) (Lee and Bieniasz 2007). Others, such as enJSRV, are regarded as modern ERVs as they are found to be highly related to current exogenous retroviruses which are still infectious (Palmarini, Hallwirth et al. 2000; Palmarini, Mura et al. 2004; Arnaud, Caporale et al. 2007; Arnaud, Varela et al. 2008; Varela, Spencer et al. 2009; Black, Arnaud et al. 2010). Once present in the germline, the proviral DNA establishes itself as a permanent fixture within the host genome and is then vertically transmitted to future generations (Coffin, Hughes et al. 1997). ERVs are clearly an integral part of the mammalian genome as they represent approximately 8% of the human genome (Lander, Linton et al. 2001).

ERVs originate from germline integration. The germline is a population of highly demethlyated, undifferentiated and active cells which provides optimum conditions for successful retroviral integration with ample unrestricted integration sites and uninhibited expression patterns thus allowing the rapid expansion of integrated elements (Boeke and Stoye 1997). Due to the selection pressure the host exerts over non-self sequences, many ERVs are silenced as they accumulate mutations which render them replication incompetent. Generally, the majority of examples of replication competent genomes will be found to be recent integrations or transposition events. Several animal species, including mice and sheep, have active and intact ERVs capable of viral particle production while in higher species (primates) the majority are inactive (Kurth, Bannert 2010). It is fair to assume that the ERVs identified within host genomes represent a very small proportion of those which have challenged the germline during evolution. Proviruses that pose a deleterious effect to the host have been likely counter-selected during evolution. In many ways, ERVs provide clues as to the ancient and now extinct retroviruses which challenged the genome and the interplay between virus and host during co-evolution. Several attempts have been made to “resurrect” endogenous retroviral sequences in order to investigate their infectious properties (Lee and Bieniasz 2007). There are few

opportunities to study a genome that is instead currently under invasion by retroviruses and to date the koala and sheep represent good examples of these genomes (Tarlinton, Meers et al. 2006; Arnaud, Caporale et al. 2007). ERVs are not just “fossils” and redundant junk DNA (Bock and Stoye 2000) within the host genome, as several studies suggest that these elements play major roles in host physiology (see 1.4.5 Placental formation).

Interestingly, until recently retroviruses were considered the only viruses present in vertebrate genomes. However, analysis of a variety of vertebrate genomes has revealed that both RNA (Bornaviruses and Filoviruses) viruses (Horie, Honda et al. 2010), (Belyi, Levine et al. 2011) and DNA viruses (Densoviruses and Parvoviruses) have been endogenised during evolution (Belyi, Levine et al. 2010; Liu, Fu et al. 2011). Thus, the contribution to genomic diversity provided by viruses is far greater than previously recognised.

1.4.2 The discovery of endogenous retroviruses

ERVs were first highlighted during studies in the 1960s which focused on maintaining Avian Sarcoma Leukaemia virus (ASLV)-free egg producing chickens. Uninfected chickens consistently yielded positive results for a serologic assay targeting the viral Gag (group specific antigen) (Dougherty and Di Stefano 1966). Additionally, Gag and retroviral like particles were identified within healthy chicken tissues (Dougherty, Di Stefano et al. 1967). These data were further validated when it was shown that Gag could be inherited following a Mendelian cross between Gag+ and negative breeds of chickens (Payne and Chubb 1968). However, at that time it was suspected that these data could be either due to a latent retroviral infection or cross reactivity with a host gene. It was later observed that the fibroblasts of chick embryos were capable of releasing Rous Sarcoma Virus (RSV) in the absence of helper virus (Weiss 1967). Until this point, RSV required complementation by the envelope of RSV in order to release viral particles from cells in culture (Hanafusa, Miyamoto et al. 1970). However, it was later identified that virus was released spontaneously following ionizing radiation of both embryo and somatic cells (Weiss, Friis et al. 1971). The data at that time therefore revealed two fundamental properties of endogenous retroviruses: firstly that retroviral sequences were integrated within the

germline of the host and were inherited vertically by future generations, and secondly that, although they are predominately silent within the genome, they can be reactivated. The discovery of ERVs has been reviewed by Weiss (Weiss 2006).

1.4.3 Classification of ERVs

Transposable elements are mobile DNA segments (McClintock 1956) which utilise cellular machinery to support replication. They constitute a large fraction of the mammalian genome and represent up to approximately 50% of the human genome in comparison to the 3% of protein encoding genes identified to date, as described by (Coffin, Hughes et al. 1997). Transposable elements may be further categorized into Class I and II based upon structure and movement patterns (Finnegan 1989). Class I are DNA transposons which cut and paste themselves within the genome, while Class II are retroelements which are capable of copy and paste movements within the genome. Furthermore, retroelements consist of either LTR based DNA sequences (ERVs) or non-LTR based DNA sequences (LINES, SINES) (Boeke and Stoye 1997).

ERVs represent a subclass of transposable elements that are self sufficient and capable of efficiently copying and reinserting themselves into the genome. They are restricted to vertebrates and there are many examples. With the exception of deltaretroviruses, many endogenous retroviruses have closely related exogenous forms (Boeke and Stoye 1997). ERVs are further categorized into three classes. First those which resemble most exogenous gammaretrovirus like sequences, the second Betaretrovirus like sequences and the third and final those which share homology with spumaretrovirus-like elements. This classification is, however, now deemed insufficient with the addition of subgroups due to the discovery of endogenous lentiviral (Katzourakis, Tristem et al. 2007; Gifford, Katzourakis et al. 2008) and authenticated spumaretroviral sequences (Lagaye, Vexiau et al. 1992; Coffin, Hughes et al. 1997). The rapidly expanding abundance and knowledge of ERVs has lead to problems with nomenclature and classification, as highlighted in a recent review by (Blomberg, Benachenhou et al. 2009)

1.4.4 The effects of ERV expression on host function and genome plasticity

Endogenous retroviruses have overcome several barriers in order to achieve successful fixation in the host genome. Any given ERV must be able to infect reproductive tissues in order to facilitate the infection of germline cells. In addition, it must also be able to evade any host restriction mechanisms (see 1.5 Host vs Virus), successfully integrate and persist without any detriment to the host and, finally, transcend from an insertionally polymorphic trait to a fixed gene within the host species (Boeke and Stoye 1997).

Most ERVs are defective and transcriptionally repressed but in rare occasions they are functional, providing useful and essential functions to the host. Recombination between related ERV *loci* present in different chromosomal locations can also result in chromosomal rearrangements (Hughes and Coffin 2001). Thus, ERV integration can modulate gene function and provide more plasticity for the genome of the host species (Eiden 2008). Whenever functional, ERVs are capable of exerting significant transcriptional control over host genes residing either as integral elements of the promoter or as enhancers from distal intergenic regions. For example, the salivary amylase promoter region contains several structures derived from retrotransposons (one of which is an ERV) that exert transcriptional control resulting in tissue specific expression of this gene (Ting, Rosenberg et al. 1992). An example of long range function is provided by ERV-9 which regulates fetal γ and adult β globin genes from 40-70kB upstream. The ERV-9 LTR binds NF-Y and GATA2 and generates an LTR/RNA polymerase II complex modulating downstream globin promoters (Pi, Zhu et al. 2010). In addition, ERVs have been reported to regulate particular host splicing events, for example an ERV derived from a human teratocarcinoma cell line promotes human PLA2L gene expression in these cells (Feuchter-Murthy, Freeman et al. 1993). ERVs also have a prominent role in placental morphogensis (see 1.4.5 Placental formation) and host immunity (see 1.5 Host vs. Virus) which will be discussed in detail later.

1.4.5 Placental formation

Despite the significant physiological differences in the reproductive biology of mammals, the placentas of various animal species have been found to express ERVs and in some cases viral particles. Electron microscopy (EM) studies as early as the 1970s indicated the presence of viral-like particles within the reproductive tract of humans and animals (Kalter, Helmke et al. 1973; Kalter, Heberling et al. 1975). Further EM studies of suspected retroviral particles from placental tissue confirmed associated RT-activity with a density and size relative to that of C-type retroviruses (Lyden, Johnson et al. 1994). The placenta and the syncytiotrophoblast in particular, is the central point of exchange of nutrients between mother and child. Extensive HERV expression is detected within the human placenta (de Parseval and Heidmann 2005). In particular, expression of HERV-W Env protein Syncytin-1 is credited as the driving force behind the fusion event that leads to the formation of the syncytiotrophoblast during placental formation (Frendo, Vidaud et al. 2000; Frendo, Olivier et al. 2003). Indeed, knockout of a similar protein in mice (Syncytin-A) is embryo lethal. Histopathology examination of embryos revealed a lack of trophoblast fusion to the maternal uterine epithelium (Dupressoir, Vernochet et al. 2009). In humans, abnormal expression of Syncytin has been linked to preeclampsia (Lee, Keith et al. 2001) highlighting the important role of ERVs during pregnancy. ERVs can also exhibit immunosuppressive properties and interfere with the replication cycle of exogenous related retroviruses, as will be discussed later (see 1.5 Host vs Virus).

In summary, there is significant evidence which indicates that the continuous challenges posed by ERVs have shaped the evolution and development of several mammals in many different ways. Several ERV LTRs appear to exert control over host genes while viral proteins have been found to be involved in essential physiological processes.

1.4.6 Endogenous JSRVs

JSRV-related viruses also exist as inherent “endogenous” (enJSRV) viruses stably integrated and established within the host genome. When compared to their

exogenous counterpart, enJSRVs share 85-89% identity with JSRV along the entire genome (Arnaud, Varela et al. 2008). Major differences are found across the U3 region of the LTRs as well within three variable regions, two of which reside in gag (VR1/2) and in the other within env (Bai, Bishop et al. 1999; Palmarini, Hallwirth et al. 2000).

There are at least 27 enJSRV loci in the sheep genome which have been identified, isolated and cloned from a bacterial artificial chromosome library which originates from a single Texel ram (Figure 8) (Arnaud, Caporale et al. 2007). The 27 enJSRVs appear to be distributed across the sheep chromosomes (Carlson, Lyon et al. 2003; Armezzani, Arnaud et al. 2011) and first entered the genome approximately 5-7 million years ago. The invasion of the host genome by enJSRVs has continued throughout the evolution of the Caprinae (Fig. 7) as the youngest provirus is predicted to have entered the host genome less than 200 years ago. Thus, enJSRV colonization of the ovine genome remains an ongoing process. The 27 enJSRVs identified to date were likely acquired during evolution from multiple infection events of the germline by JSRV-related exogenous viruses or by reinfection of an existing active enJSRV locus. Most of the oldest enJSRV proviruses have acquired mutations and deletions that render them replication deficient. However, several enJSRVs loci possess an intact genome and are indeed able to express intact viral particles (Arnaud, Caporale et al. 2007).

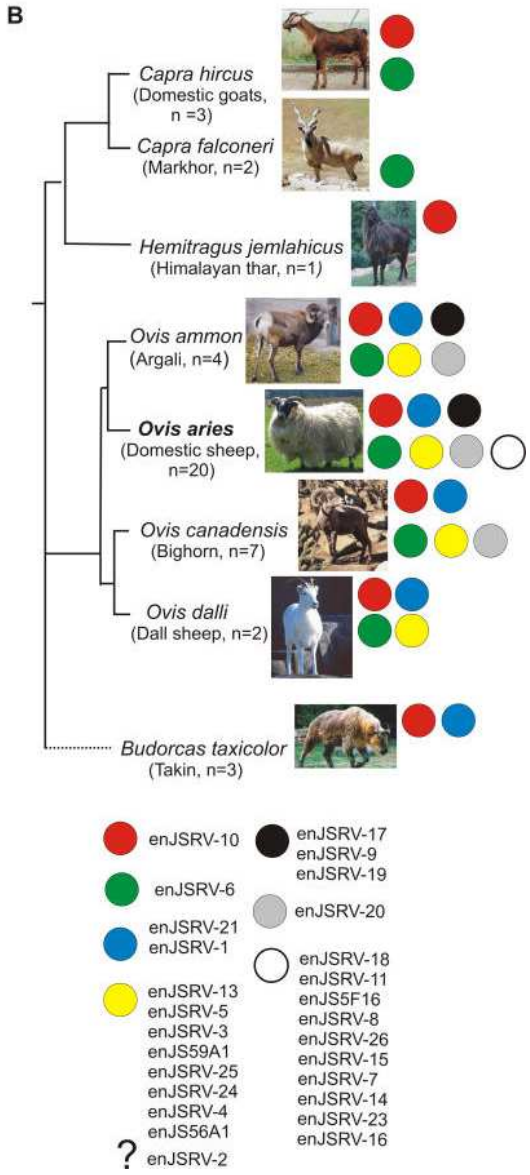


Figure 7 – Distribution of the 27 enJSRVs within the Caprinae.

The insertionally polymorphic nature of the more recent integration indicated in white within the Ovis aries. Reproduced with permission from PLoS Pathogens (Arnaud, Murcia et al. 2007).

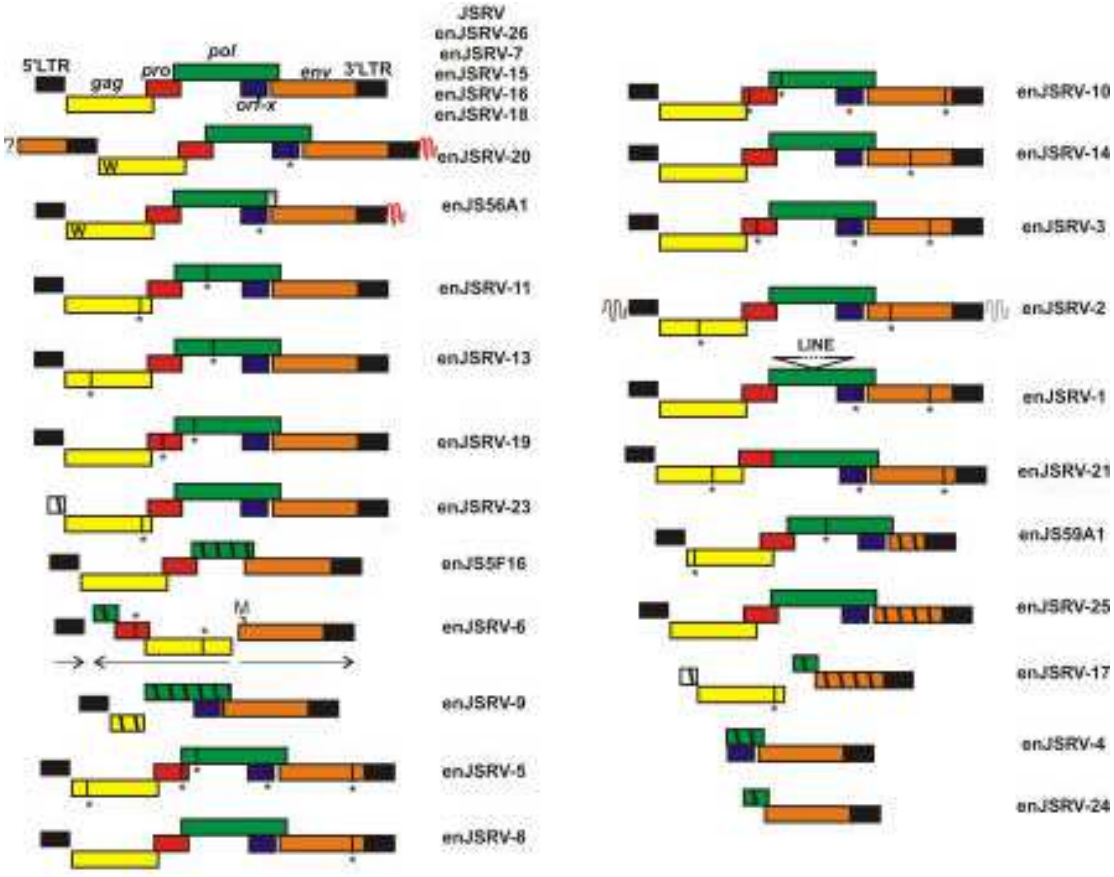


Figure 8- The 27 enJSRV loci isolated from the sheep genome.
Many of the enJSRVs have accumulated mutations and deletions across their genome which render them replication incompetent. Several more recent integrations have retained open reading frames and are capable of viral particle production. Reproduced with permission from PLoS Pathogens .(Arnaud, Murcia et al. 2007).

1.4.7 Evolutionary history of enJSRVs

The "age" of a provirus can initially be estimated by determining its presence in phylogenetically related species. If a provirus is present in exactly the same chromosomal location in two phylogenetically related animal species then it is safe to assume that the original infection occurred before the divergence during the evolution of the two animal species in question (Boeke and Stoye 1997). As mentioned above, the oldest of the enJSRV proviruses, such as enJSRV-10, integrated before the divergence of the Ovis and Capra genera (Arnaud, Caporale et al. 2007). Another way to determine the age of a proviral sequence is by correlating the divergence between the 5' and 3'LTRs, with the mutation rate of non-coding sequences in mammals. Retroviral LTRs are identical upon

integration of the proviral sequence. Therefore, the greater the level of divergence between LTRs the older the provirus will likely be. Although these calculations are subject to a wide margin of error due to homologous and heterologous recombination of the LTRs of different proviruses, they are nevertheless a useful indication of the age of any given provirus (Arnaud, Caporale et al. 2007).

The sheep genome contains several enJSRVs that are fixed within the domestic sheep population (i.e. every sheep contains these proviruses and their fixation within the genome occurred prior to the domestication of sheep) and others (enJSRV7, 8, 15, 16, 18, 26 and enJS5F16) that are insertionally polymorphic. In other words these proviruses are younger and present only in a proportion of the domestic sheep population. Five of the insertionally polymorphic enJSRV proviruses have, as expected, identical 5' and 3' LTRs sequences (Arnaud, Caporale et al. 2007).

Older enJSRV loci such as enJS59A1, enJSRV25 and enJSRV17 contain large deletions within the *env* gene. Both enJSRV6 and enJSRV2 have evidence of recombination events with other proviruses and contain internal sequence in the opposite direction to the general 5' to 3' sequence (Arnaud, Caporale et al. 2007). It is thought that enJSRV2 derives from a recombination event as the flanking genomic regions lack the 6bp duplication at the site of chromosomal entry common to all proviral insertions (Arnaud, Caporale et al. 2007). Many enJSRV proviruses also have premature termination codons that "litter" their genome (e.g. enJSRV10 contains a premature stop codon in *pro, pol, env* and *orf-X*). Five of the recent enJSRVs maintain an intact genomic organization. Two loci, enJSRV-16 and 18 are identical across their entire genome and are therefore only distinguishable by the location of their integration. The most recently integrated provirus, enJSRV-26, is believed to be a virus that has integrated in a single animal. All of the information derived from the genomic organization of enJSRVs is summarized (Arnaud, Murcia et al. 2007). The genomes of all 27 enJSRVs identified to date are summarised in Figure 8. All of the insertionally polymorphic enJSRV loci with an intact genomic organization are able to produce virus particles *in vitro* and *in vivo* (Black, Arnaud et al. 2010).

1.4.8 enJSRVs play a critical role in the reproductive biology of the host

Endogenous retroviruses are by definition non-pathogenic, otherwise they would be counter-selected during evolution. The exogenous JSRV is an oncogenic virus whose Env is a dominant oncoprotein. However, as expected, none of the enJSRV Env glycoproteins are oncogenic and they all lack the SH2 binding domain within the cytoplasmic tail that has been found to be a major determinant of oncogenicity (Palmarini, Gray et al. 2001). In addition, the enJSRVs LTR lack those enhancer binding regions that are essential to drive lung-specific expression of JSRV. Expression of enJSRVs is widespread throughout the reproductive tract and has been detected within the oviduct, uterus, cervix and vagina (Palmarini, Gray et al. 2001). Thus, it appears that the high degree of divergence between the LTRs of the enJSRVs and JSRV contribute to their different patterns of expression. Early studies of enJS56A1, enJS59A1 and enJS5F16 LTRs show that the HNF-3 and C/EBP binding sites (found important for JSRV lung-specific expression) are interrupted by an insertion of 13 and 15 nucleotides respectively (Palmarini, Datta et al. 2000; McGee-Estrada and Fan 2007). A comparison of the enJSRV LTRs with that of MMTV has also confirmed the presence of hormone responsive elements (HRE) (Lieberman, Bona et al. 1993) within enJSRV LTRs. The enJSRVs HRE can be linked to their identified expression *in vivo* during pregnancy. In addition, another conserved transcription binding domain among the LTR of enJSRVs is Activator Protein 2 (AP2) which has been identified to regulate bovine binucleate trophoblast-specific genes during the placental development in pregnancy (Ushizawa, Takahashi et al. 2007).

In vivo, enJSRV expression has been found to peak in correspondence to high levels of progesterone and progesterone receptor. *In vitro*, the LTR of various enJSRV loci have shown enhanced expression of the corresponding enJSRV following progesterone treatment (Palmarini, Hallwirth et al. 2000; Dunlap, Palmarini et al. 2006; Spencer, Sandra et al. 2008). enJSRVs are particularly abundant within the luminial epithelium and glandular epithelium cells of the ovine genital tract from day 12 -18, coinciding with an upregulation of progesterone. Expression of enJSRVs also coincides with that of Interferon-tau

(IFNtau), the pregnancy recognition signal in ruminants (Bazer, Burghardt et al. 2008). IFN-tau expression is crucial in order to prevent luteolysis from occurring and allows establishment of the developing conceptus (Spencer, Burghardt et al. 2004). The involvement of HERV-W Env protein Syncytin-1 expression in the fusion event that leads to the formation of the syncytiotrophoblast during placental formation has already been shown (Frendo, Vidaud et al. 2000; Frendo, Olivier et al. 2003). Although human and ruminant pregnancy differs significantly in anatomy (Barry and Anthony 2008) it appears that they share the presence of ERVs during placental morphogensis. Inhibition of enJSRV *env* transcription and translation from day 12 of pregnancy results in the incorrect expansion of the conceptus during pre-implantation development from a solid form to that of a filamentous. This results in no attachment to the uterine wall, (the correct process is summarised in Figure 9) demonstrating the essential role of enJSRVs in this process (Dunlap, Palmarini et al. 2006). In preparation for the implantation of the conceptus, mononuclear/binuclear cell fusion occurs leading to trinucleate cells which fuse in order to produce placentomes in support of conceptus development (see Figure 10 for summary). This occurs in the presence of enJSRV and HYAL2 expression and it is speculated that they play an important part in this process (Dunlap, Palmarini et al. 2005). How enJSRVs elicit their action is not yet fully understood, although fusion mediated solely by the enJSRVs Env and HYAL-2 receptor seems unlikely, as to date enJSRV Env has not been found capable of inducing syncytia in vitro.

Together, JSRV-enJSRVs and the domestic sheep provide a rare opportunity to study the on-going co-evolution of retroviruses and their host. This is a rare opportunity and the only other species for which there is evidence of ongoing endogenisation is the koalas (Tarlinton, Meers et al. 2006).

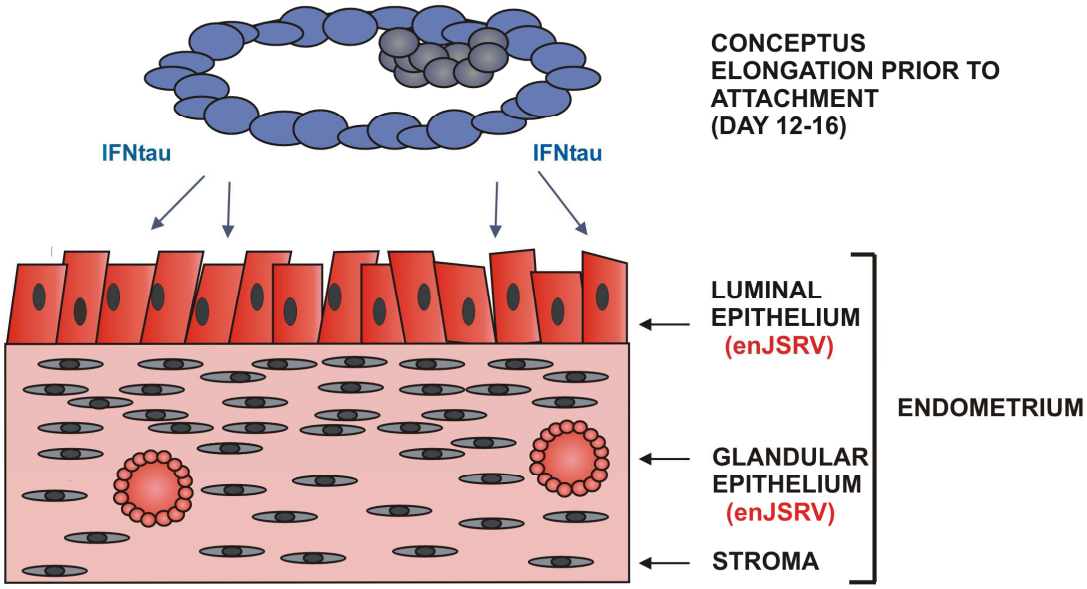


Figure 9. Schematic diagram illustrating the pattern of enJSRVs expression during early pregnancy.

The developing conceptus emits IFNtau as a signal for the endometrium to prepare for the implantation event. enJSRVs are found expressed in the luminal and glandular epithelium of the endometrium as indicated in red as well as in the conceptus. A crucial part of development is the elongation of the conceptus into a filamentous form for successful implantation mediated by enJSRVs.

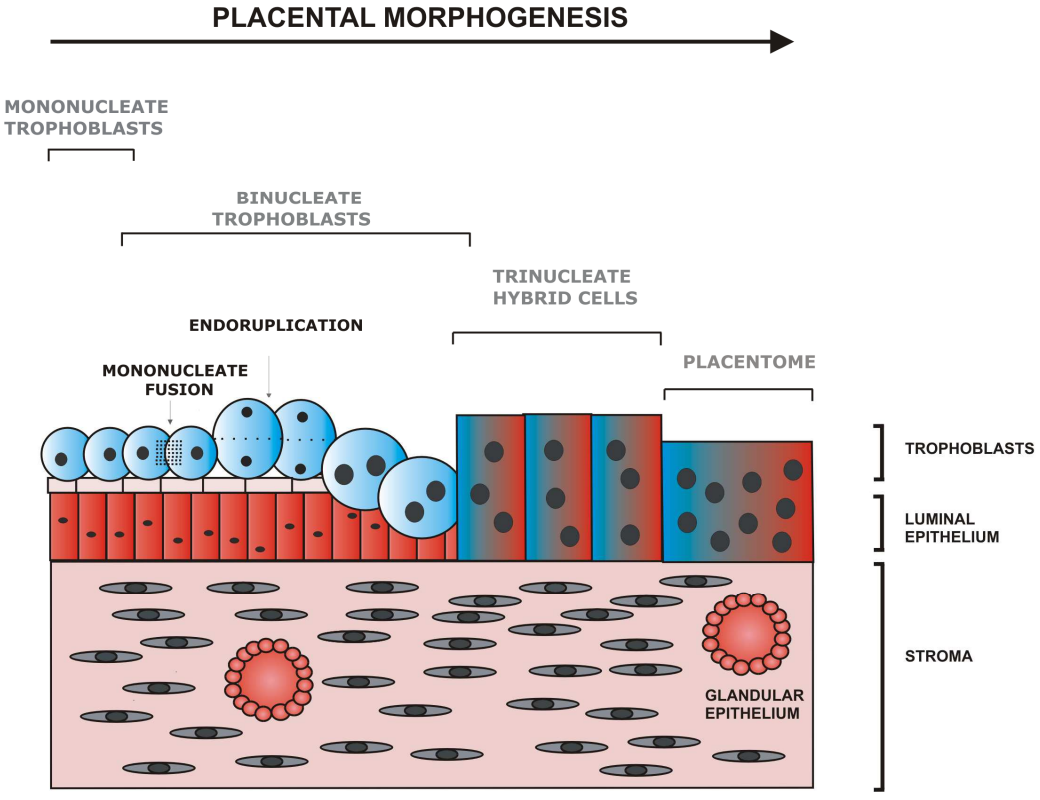


Figure 10. Schematic diagram illustrating the major phases of placental morphogenesis.

Fusion of mononucleate and binucleate trophoblast cells of the conceptus leads to trinucleate hybrid cells which invade the endometrium. This is a prerequisite for placentome formation necessary to support conceptus development and occurs during the expression of enJSRV and its receptor HYAL2.

1.5 Host vs. Virus

Host cells possess a complex array of antiviral mechanisms with which to respond to viral detection to fight virus infections. In addition to the constitutive expression of “intrinsic” antiviral factors, the recognition of a virus infection triggers intracellular signalling pathways that result in the induction of the “innate” immune response and subsequently an “adaptive” immune response. The continuous challenge to the host provided by pathogens results in a strong selective pressure to develop sophisticated barriers to infection which, in turn, has major evolutionary implications for viral populations. Thus, a key requisite for any successful virus is the ability to evade recognition by the host innate immune system (Bieniasz 2004).

1.5.1 Intrinsic immunity

Intrinsic viral restriction factors are constitutively expressed proteins which interfere with the viral lifecycle. ‘Viral interference’ is a form of intrinsic immunity mediated by intact or defective endogenous retroviruses. Viral interference has not been reported in humans but there is evidence of this process in both mice and sheep. In mice, Fv1 and Fv4 are two host genes which control susceptibility to the Friend strain of Murine Leukemia Virus (F-MLV). Fv1 is derived from a resident endogenized retroviral Gag (Best, Le Tissier et al. 1996). Fv1 has two alleles, Fv1n and Fv1b present in mice, which exert their action over specific subtypes of MLV (Hartley, Rowe et al. 1970) and block MLV infection at a stage after reverse transcription but before integration takes place by a mechanism that has not been entirely clarified (Bieniasz 2004). Fv4 instead is closely related to the Env of MuLV and restricts viral infection by blocking access to and downregulating the F-MLV receptor from the cell surface, as reviewed by (Bieniasz 2004).

The enJSRVs of sheep also provide a 'viral interference' restriction mechanism by engaging the HYAL-2 receptor (Spencer, Mura et al. 2003). Furthermore, two of the 27 enJSRVs identified to date, enJSRV-20 and en56A1, provide an additional form of intrinsic viral restriction mediated by their Gag protein. Unique to both enJSRV-20 and en56A1 is a tryptophan in position 21 of Gag which results in a transdominant phenotype defective for exit (Mura, Murcia et al. 2004). The exogenous JSRV has an arginine in position 21 of Gag that is considered essential for directing the protein towards the centrosome for assembly. The presence of tryptophan at position 21 of Gag of the transdominant en56A1 results in a significantly different intracellular distribution to that of exogenous Gag. It is suspected that both of these transdominant enJSRVs entered the germline with a tryptophan at position 21 in two distinct events. They then became fixed within the species thereafter due to the positive host function they are associated with. In the presence of en56A1, exogenous JSRV Gag does not assemble at the centrosome region. Instead the JSRV Gag is found to colocalise with en56A1 Gag and, when the proteosomal machinery is inhibited, en56A1/JSRV Gag aggresomes are observed (Mura, Murcia et al. 2004). This restriction mechanism is independent of the Late domain and membrane binding domains of Gag and is called JSRV late restriction (JLR) in recognition of the effect it has over the late phase of the replication cycle (Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007). The most recent sheep ERV acquisition , enJSRV-26, , is capable of escaping the JLR and this is due to the substitution of a single amino acid in the signal peptide of its envelope protein (Armezzani, Arnaud et al. 2011). The JLR proves to be a mode of restriction unique to sheep which gives specific protection against the continuous challenge JSRV poses. The escape by enJSRV-26 is an excellent example of the continuous struggle between the host and virus for dominance.

1.5.2 Innate Immunity

Innate immunity is a mechanism with ancient origins established prior to the evolutionary division which formed the plant and animal kingdoms and, as such, it is represented in both. In its most concise definition, innate immunity is an immediate, uniform, potentially inducible response inherited from the germline which can efficiently discriminate between host DNA/RNA and virus/pathogen

DNA/RNA (Janeway 1989). Innate immunity is distinguishable from intrinsic viral restriction as it requires induction by Interferon (IFN) to elicit widespread expression and effects, unlike intrinsic factors which are constitutively expressed. Innate immunity is regulated predominately by plasmacytoid dendritic cells (pDC) which are a large source of IFN following viral infection (Barchet, Cella et al. 2002). The innate immune response bridges the gap between host detection of virus and the time taken for the adaptive immune system to respond (Tough 2004). IFN induces an antiviral state in nearby uninfected cells following viral detection, upregulating a range of restriction factors which target various aspects of the retroviral lifecycle. Many studies on innate immunity have been conducted in the fruit fly drosophila due to the absence of an adaptive immune response. Simple invertebrates, such as *Drosophila melanogaster*, rely on RNA interference (RNAi) as the major antiviral mechanism (Wang and Ligoxygakis 2006)

In contrast to the adaptive immunity, the innate immune response is an inherited uniform response "embedded" within the germline. In many ways the IFN response is therefore a uniform response. However, the IFN induced genes are by no means a static population with both positive and negative selection acting upon IFN stimulated genes (ISGs) and with many examples of the recent acquisition of further ISGs. The addition of further downstream genes activated by the innate immune response provides further antiviral related activity (see 1.5.8 IFN stimulated genes with antiretroviral activity). Innate immunity provides a first line of defence while the adaptive response generates the potential to evade new and emerging viruses faster than the selective process to establish an innate response within the germline.

1.5.3 Interferon (IFN)

Interferons (IFNs) are a family of secreted cytokines that induce an antiviral cellular state following viral infection as part of the innate immune response (Shirazi and Pitha 1993). IFNs can be distinguished into three classes: type I, II and III IFNs. The type I IFNs found within placental mammals include IFN-α, IFN-β, IFN-ω, IFN-δ, IFN-ε, IFN-τ, IFN-κ, IFN-ζ subtypes. The exact biological effect and signalling pathways utilised by IFNs varies and is dependent on their

expression pattern, local host environment and subtype cellular targets. IFN-α and IFN-β can be induced in most cell types as a direct response to viral infection. However, IFN–δ is unique to pigs and IFN- ζ is unique to mice whilst IFN-τ is specific to ruminants. Within humans, IFN-α has expanded considerably during evolution leading to a family consisting of thirteen genes whilst IFN-β, IFN-ω, IFN-ε, IFN-κ subtypes maintain only one gene {Pestka2004 #558}. Amplification of the IFN-α gene suggests this gene is of pivotal importance during initiation of innate immunity. Type II IFNs typically display less antiviral activity than that of IFN type 1.

Although the majority of cell types are capable of IFN production following antiviral detection, pDC cells are a specific subset of DCs (Fitzgerald-Bocarsly 1993) which have a prominent role in IFN production. They produce large quantities of type I IFN which in turn regulates the induction of both innate and adaptive immunity. In addition to IFN, pDCs also produce other cytokines with antiviral function. IFN plus IL-12 induces the secretion of IFN-γ from natural killer (NK) cells, CD8+ T cells and CD4+ T helper cells (Avice, Demeure et al. 1998; Berg, Cordes et al. 2002; Kamath, Sheasby et al. 2005), whereas memory B cells are stimulated to produce antibodies in the presence of IFN and IL-6 (Jego, Palucka et al. 2003). Furthermore, IFN leads to the stimulation of T cells as IFN promotes the development of pDCs to DCs (Grouard, Rissoan et al. 1997). In short pDCs have three major functions: the production of IFN, antigen presentation and T cell priming, as summarised by (Barchet, Blasius et al. 2005).

1.5.4 Intracellular recognition of retroviruses via TLR and RIG-I

Recognition of viral entry occurs via viral “pathogen associated molecular patterns” (PAMPs) detected by cellular host ‘pattern recognition receptors’ (PRRs). There are several groups of PRRs which are capable of recognizing different forms of viral DNA/RNA including Toll like receptors (TLRs), retinoic-acid inducible gene (RIG-I) like receptors (RLR), nucleotide binding domain, leucine-rich repeat containing proteins (NLR) and cytoplasmic sensors (Yoneyama and Fujita 2010). Of particular significance to retroviruses is the recognition of RNA by TLRs and RLRs. Certain cells will preferentially utilise one

pathway over the other for activation of an antiviral response following viral detection. RIG-I is utilised by a wide range of host cells while pDCs preferentially use TLR signalling as reviewed by (Kato, Sato et al. 2005).

1.5.5 Toll like receptors

TLRs are found both at the plasma membrane and within intracellular endosomes. Those members of the TLR family which are most significantly involved in the detection of invading retroviruses are those found within endosomes (TLRs 3, 4, 7, 8 and 9) with TLR3 and 4 specific to DCs and TLR7, 8 and 9 for pDCs. Double stranded RNA (dsRNA) is a hallmark of viral infection and is recognised by TLR3, which is linked as a factor for DC maturation (Matsumoto and Seya 2008). In contrast, TLR7/8 are crucial for the recognition of ssRNA and TLR7/9 are found to be specific to pDCs. TLRs stimulate cytokine and IFN production via the adaptor molecules TRIF for TLR3 and Myd88 for TLR7/8/9, which act upon the transcription factors IRF3/7 and NF-κB. It is specifically the expression of IRF-7 in pDCs which is linked with the rapid secretion of IFN-α/ß, as reviewed by (Beutler, Eidenschenk et al. 2007) and summarised in Figure 11.

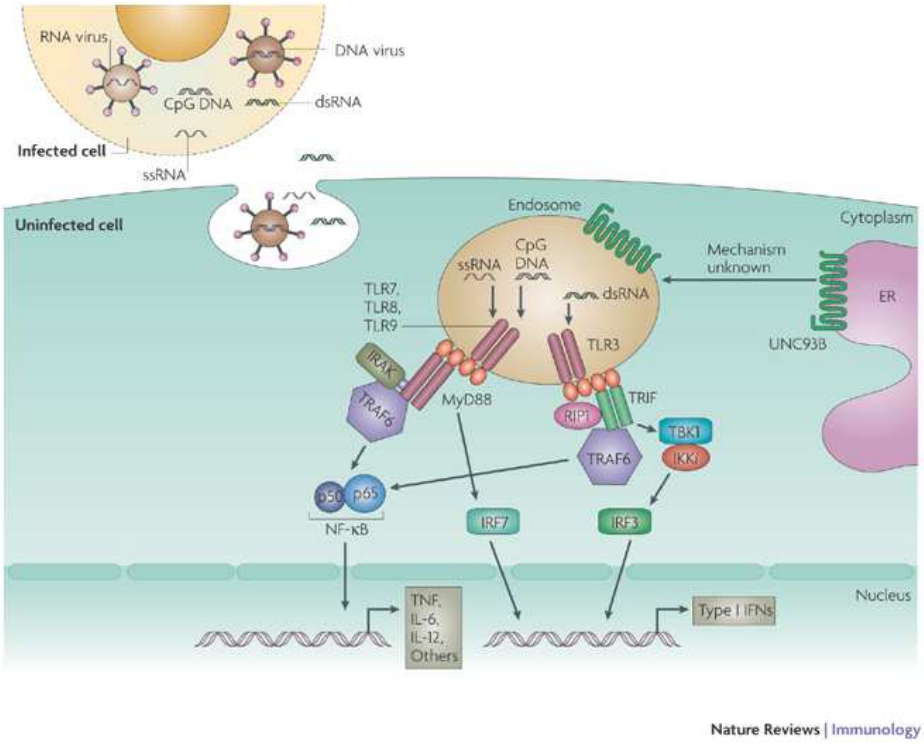


Figure 11. Schematic diagram illustrating intracellular recognition of retroviruses by TLRs. Reproduced with permission from Nature.

1.5.6 Retinoic-acid inducible gene I (RIG-I) like receptors

Unlike the endosome based TLRs, RIG-I like receptors (RLR) which include Retinoic-acid-inducible-protein 1 (RIG1) and Melanoma-differentiation-associated gene 5 (MDA5) are cytosol based proteins. Similarly to TLRs, they are also involved in the recognition of RNA viruses leading to the upregulation of cytokines and induction of IFN. It has been proposed that RIG-I recognizes intracellular viral non-capped ds/ssRNA, that is distinguishable from host capped mRNA. In addition, the identification of uridine and adenosine rich regions within viral sequences, as well as RNAseL products, upregulates the RLR response. RIG-I binds short viral RNAs, whilst MDA5 binds those longer than 1Kb. Both RIG-I and MDA5 remain within the cytoplasm in an inactive state until RNA binding triggers a conformational change and exposure of the Caspase activation and recruitment domain (CARD) for downstream signalling. The initiation of the RLR response is best understood to date for RIG-1 while the activation of MDA5 has yet to be fully characterised. The CARD and repressor domain (RD) of RIG-I are held in an inactive state until the recognition of viral infection. The RD

domain of RIG-I recognises and engages with PAMPs. This leads to K63 linked ubiquitination of RIG-I by TRIM-25 and RING finger protein which switches RIG-I to an active state by an alteration in conformation. The new conformation induces a signalling cascade involving oligomerisation of RIG-I and a CARD dependent interaction with IPS-1, resulting in IFN upregulation as summarized by (Loo and Gale 2011) in Figure 12. However, several studies with knockout mice have revealed that the requirement of RIG-I/MDA5 varies depending on the virus in question. Whether activation occurs by TLRs or by RLRs both pathways act on transcription factors NF-κB, IRF-3 and IRF-7 which in turn induces the expression of IFN-α/β, as reviewed by (Beutler, Eidenschenk et al. 2007).

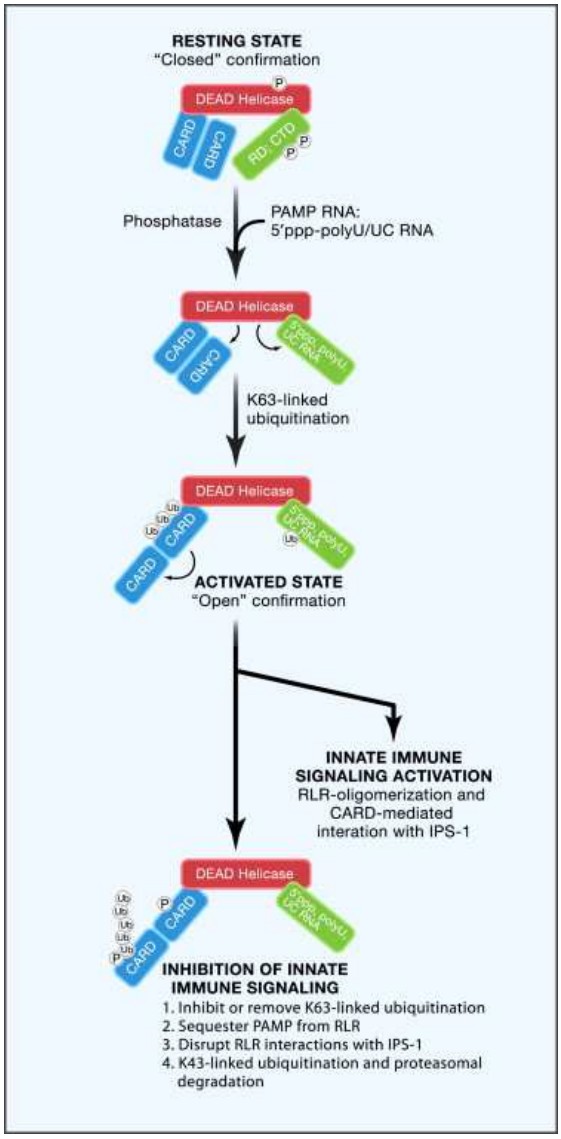


Figure 12. Schematic diagram illustrating the induction of IFN by RLR signalling via RIG-I. Reproduced with permission from Cell Press.

1.5.7 Propagation of the IFN response

Upon upregulation, IFN-α/ß is secreted and induces dimerization of the IFN-α/ß receptor (IFNAR-1 and -2) of neighbouring cells. Ligand-induced dimerization initiates a signalling cascade via the JAK/STAT pathway activating ISGF-3 (Interferon stimulated gene factor, a heterodimer of STAT1/2). ISGF-3 binds to the IFN-stimulated response element (ISRE), present in the promoters of most IFN stimulated genes (ISGs). IFN elicits a very broad regulation of gene response to viral infection. There are hundreds of genes that are upregulated in response to IFN treatment that, in combination, determine the antiviral state. The INTERFEROME database (Samarajiwa, Forster et al. 2009) collects all the IFN regulated genes. There are 1996 human genes thought to be influenced by IFN (1581 upregulated, while 415 appear to be downregulated). These gene products are further divided as roughly 676 being nuclear, 626 cytoplasmic, 379 are membrane based, 151 mitochondrial, 64 are Golgi based, 34 ribosomal and finally 35 are lysosomal (Hertzog, Mansell et al. 2011).

1.5.8 IFN-stimulated genes with antiretroviral activity

Intrinsic and innate immunity have a significant impact on retroviral evolution. There are several examples of retroviruses which have refined their genome in order to overcome the action of restriction factors that threaten their existence. Similarly, there is evidence of the expansion of innate immune restriction factors in response to the pressure exerted on the host by the consistent challenge that retroviruses present. There are several IFN induced restriction factors which have a significant role in the antiviral response elicited by the host following the discovery of infection, including APOBEC, TRIM and BST-2 (Neil and Bieniasz 2009).

1.5.8.1 APOBECs

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like proteins (APOBECs) are a group of RNA/DNA editing enzymes. Two proteins belonging to

this family have a significant role in the antiviral innate immune response, APOBEC3F(1-3) and 3G, are induced or maximised by IFNα signalling in T cells, macrophages and DCs, (Chen, Huang et al. 2006; Peng, Lei et al. 2006; Wang, Huang et al. 2008). APOBEC3F and 3G are cytosine deaminases that target the early stages of viral replication and are incorporated into viral particles exiting the cell (Figure 13). Upon infection of new cells APOBEC exerts its antiviral effect during reverse transcription by editing cytosine residues to uracil. This has a significant impact on reverse transcription, creating a disproportionate amount of uracil in minus strand DNA. This in turn creates G to A 'hypermutations' during second strand synthesis with a negative impact, eventually inducing a 'crash' in the viral population as reviewed by (Harris and Liddament 2004).

There is evidence of the restriction of ancient gammaretroviruses by APOBEC3G as significant G to A hypermutation of the endogenous retroviruses of chimpanzee ERV-1 and 2 has been observed. These data led to the speculation that APOBEC had an impact on the evolution of these viruses and limited their host range (Perez-Caballero, Soll et al. 2008). Multiple copies of APOBEC proteins are found within primate genomes, indicating the presence of antiviral genes under positive selection.

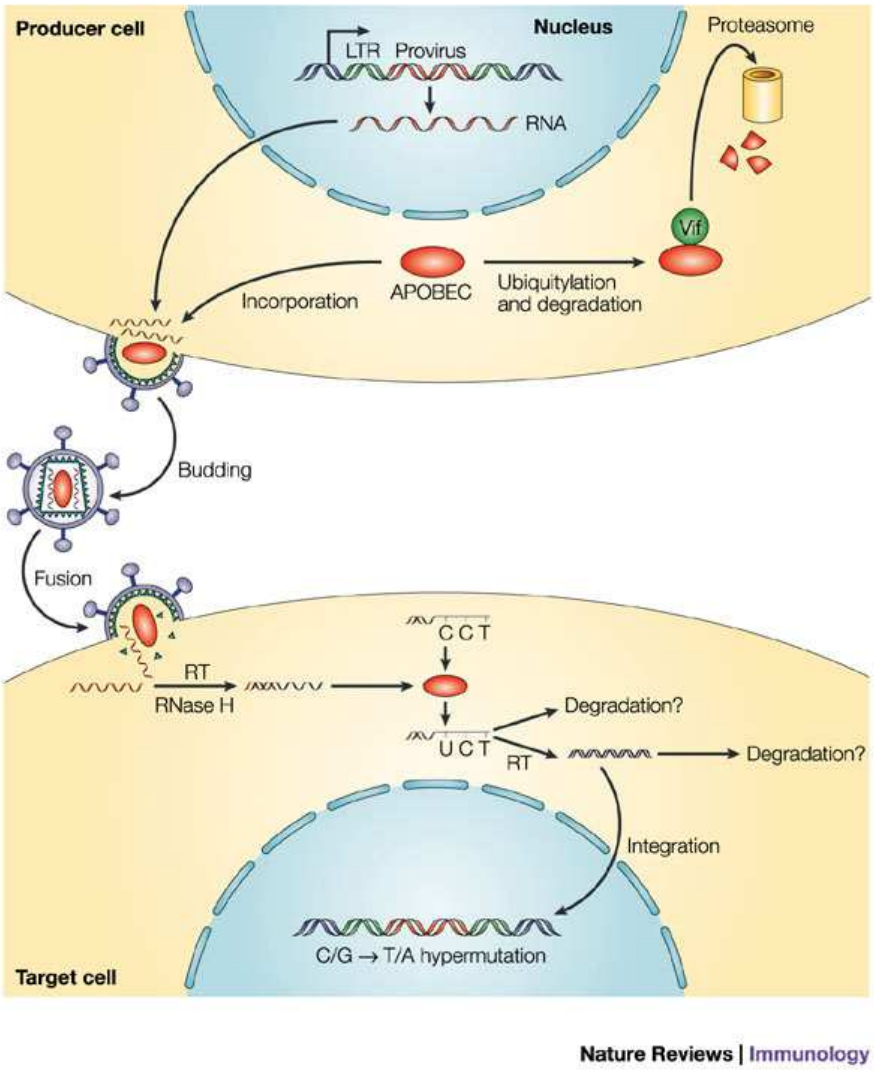


Figure 13. Overview of the antiviral properties of APOBECs.
Reproduced with permission from Nature.

1.5.8.2 TRIMs

Tripartite motif proteins (TRIMs) are a family of IFN-induced proteins with a potent antiretroviral activity (Figure 14). Of all the TRIM proteins the most notable is TRIM5α, which acts soon after viral entry but before reverse transcription (Asaoka, Ikeda et al. 2005). TRIM5α is composed of four distinct domains necessary for its function: an N-terminal RING domain involved with protein-protein interactions, two central B box domains with unspecified function and a C terminal coiled-coil domain associated with protein oligomerization. This restriction factor interacts with the Capsid protein of viral particles following entry (Sebastian and Luban 2005), although the exact

mechanism by which TRIM5α 'disarms' the entering virus is not completely clear. Whilst premature Capsid disassembly of entering viral particles, inhibition of RT and inhibition of proteosome activity have all been associated with TRIM5α activity, none of them alone appear to be essential for the antiviral function, reviewed by (Neil and Bieniasz 2009).

The effect exerted by TRIM19 is an example of a nuclear antiviral mechanism. It has several isoforms which differ in their C-terminal regions and it is based within the nuclear bodies. The presence of different TRIM19 isoforms is linked to the range of functions that has been associated with this protein, including transcription, translation and signalling for cellular functions, reviewed by (Nisole, Stoye et al. 2005).

A rapid expansion of TRIM proteins among vertebrates has been observed to the extent that TRIM is believed to have originated primarily as an antiviral mechanism rather than by subfunctionalization of a normal host physiological function. TRIM5α in primates has been under positive selection likely driven by episodes of retroviral infection within the species (Si, Vandegraaff et al. 2006).

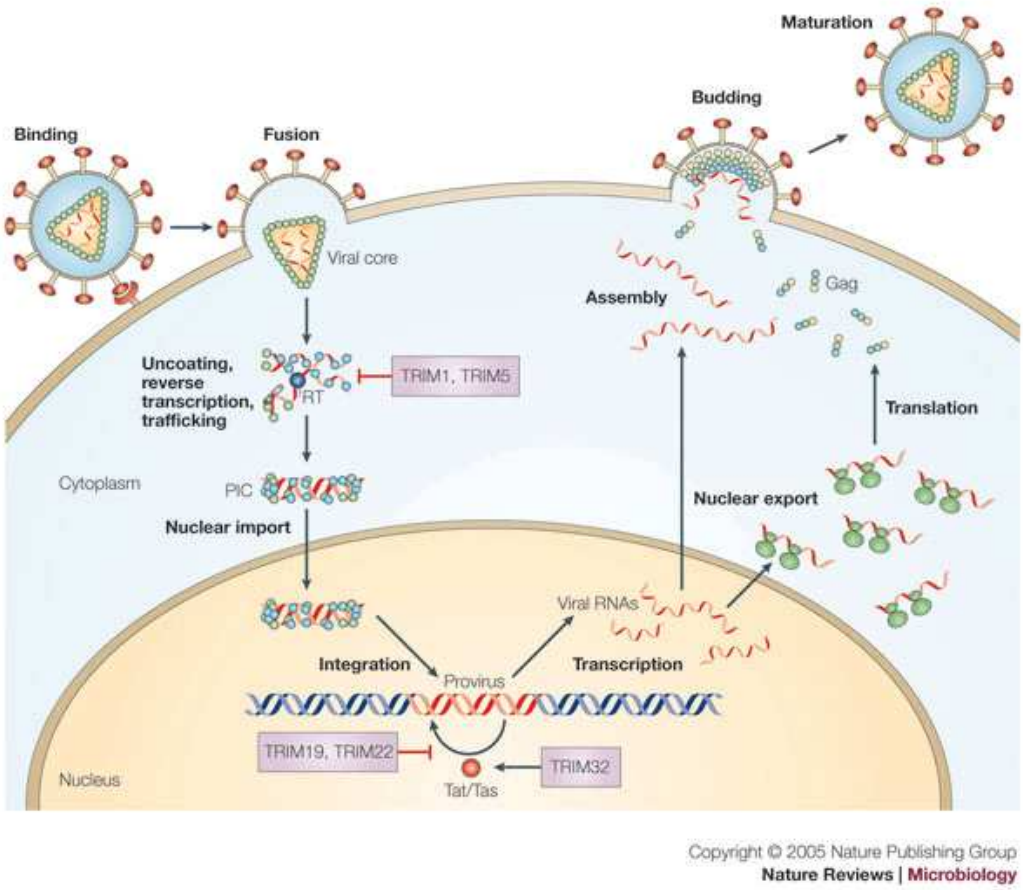


Figure 14. An outline of the antiviral mechanisms induced by TRIM proteins. Reproduced with permission from Nature Reviews.

1.5.9 Viral evasion of the host immune response

Retroviruses have evolved a set of viral proteins and tuned their replication cycle with unique characteristics that best equip them to counteract the host intrinsic, innate and adaptive immune response. Common to most retroviruses (especially lentiviruses) for example, is the intrinsic ability to rapidly evolve Env in order to evade the host humoral immune responses and HIV-1 shows high variability within VR1 and VR2 as a result of host immune selection (Rambaut, Posada et al. 2004; Curlin, Zioni et al. 2010). Retroviral populations have a high turnover that is coupled with the genomic variability introduced by the reverse transcriptase (approximately 1 every 10,000 bases). This, combined with the possibility of recombination events, results in poor immune recognition by the host.

Cell-to-cell transmission can also allow retroviruses to evade the host immune response. For example, cell-to-cell transmission increases viral transfer efficiency from infected to uninfected cells in the presence of host restriction factors (Jolly, Booth et al. 2010{Jolly, 2011 #706; Jolly 2011) and allows the virus to “escape” from neutralising antibodies.

Retroviruses may also target the immune cells that are responsible for driving the host response. For example, a significant clinical feature of HIV-1 infection during disease progression is the loss of both CD8 + and CD4+ T cells, both of which form part of the adaptive immune response of the host. In addition, it is suspected that for simple retroviruses ERVs also offer a degree of immune evasion through tolerance offered by the mandatory expression of enJSRVs during physiological processes which is tolerated by the host.

It is possible that viral proteins may evade host restriction mechanisms by directly antagonising host restriction factors. Retroviruses with a complex genome have evolved a variety of mechanisms in order to evade immune recognition. HIV is probably one of the best understood examples of a retrovirus that has evolved a variety of countermeasures that allows the evasion of recognition and elimination by the host immune system.

The HIV accessory genes (*nef, vif, vpu* and *vpr*) have been acquired in order to evade the host restriction mechanisms. In particular, the HIV accessory protein Vif counteracts the antiviral action of APOBEC-3F and -3G by linking them with the ubiquitin dependent proteosomal degradation pathway (Conticello, Harris et al. 2003; Sheehy, Gaddis et al. 2003). Distinct domains of both APOBEC-3F and -3G interact with separate regions of HIV Vif (Russell, Smith et al. 2009). SIV Vif mutants have demonstrated the impact of this protein in viral pathogenesis and its importance in evading G to A hypermutation induced by APOBEC (Schmidt, Fritz et al. 2011). In theory, the activity of APOBEC is damaging to viral replication, although it can also be considered an important factor in shaping viral adaptation and evolution following transmission (Wood, Bhattacharya et al. 2009).

Retroviruses can also modify global cellular transcription levels, not only for viral particle production but also for inhibiting signalling pathways that are

involved in the induction of the innate immune response (Taylor, Ghorbel et al. 2009). Certainly the response of IFN following infection appears to be delayed by HIV (Abel, Rocke et al. 2005).

1.6 BST-2 /Tetherin

Bone marrow stromal cell antigen 2 (BST-2) is a type II transmembrane protein with unique properties and potent antiviral activity. During viral replication, BST-2 blocks viral particle release from cells by 'tethering' virions to the plasma membrane as they bud from it (Neil, Zang et al. 2008; Van Damme, Goff et al. 2008). To date, one copy of the Bst-2 gene has been found in most vertebrates (splice variants have been identified in human and canines) with the exception of ruminants (see below). BST-2 has been termed over the past decade in several ways including CD317, HM124 and, more recently, tetherin. In light of its discovery in bone marrow stromal cells, the original term Bst-2 will be used throughout this thesis (Goto, Kennel et al. 1994).

1.6.1 Identification of BST-2

BST-2 was originally identified as a surface protein preferentially expressed during B cell development in humans (Goto, Kennel et al. 1994; Ishikawa, Kaisho et al. 1995). Overexpression of BST-2 on the surface of terminally differentiated B cells was a consistent feature of multiple myeloma. For this reason, BST-2 was considered as a potential target for antibody-based therapy and considerable efforts were spent by several groups towards this goal (Ozaki, Kosaka et al. 1997; Ozaki, Kosaka et al. 1998; Ohtomo, Sugamata et al. 1999; Ono, Ohtomo et al. 1999). These early studies were conducted before it was understood that BST-2 is an IFN-inducible gene that possess antiviral activity. However, at that time there were clues which indicated that BST-2 was involved in more than B cell development. The BST-2 promoter contains tandem repeats that can bind STAT3, known also to regulate the expression of IL-6, a differentiation factor of B cells. The binding region for STAT3 is highly conserved in the promoter regions

of human, rhesus and murine BST-2 orthologs. IRF-1/2, ISGF3 and five potential AP2 binding sites are additional transcription factor binding regions identified for BST-2 (Ohtomo, Sugamata et al. 1999).

1.6.2 BST-2 is an IFN induced restriction factor

The identification of a link between IFN and BST-2 established the possibility that BST-2 acted as a viral restriction factor (Blasius, Giurisato et al. 2006). Interestingly, the idea of a cellular restriction factor blocking HIV-1 exit was proposed following the observation that the HIV accessory protein Vpu was necessary for viral particle release in some cell lines, but dispensable in others (Neil, Eastman et al. 2006). Viral exit of HIV-1ΔVpu mutants was blocked in IFN-α induced T cells and macrophages to the plasma membrane (Smith, Thresher et al. 1991). Later, it was shown that BST-2 was an IFN induced restriction factor, capable of 'tethering' particles of HIV-1ΔVpu mutants to the plasma membrane (Neil, Zang et al. 2008; Van Damme, Goff et al. 2008). The restriction model proposed was that BST-2 restricted HIV-1 by forming a link between the plasma membrane and the virion envelope thus preventing viral exit. The release of restricted viral particles was obtained by proteolytic treatment of cell membranes, strongly suggesting that a protein tether was active in the restriction process (Ohtomo, Sugamata et al. 1999). There is some evidence suggesting that BST-2 is incorporated into virions (Fitzpatrick, Skasko et al. 2010), although data with opposing conclusions are also present in the literature (Miyagi, Andrew et al. 2009).

1.6.3 BST-2 has a broad restriction activity

Human BST-2 has been found to block not only HIV but also several other retroviruses belonging to different genera including alpharetrovirus, Betaretrovirus, deltaretrovirus and spumaviruses (Jouvenet, Neil et al. 2009). In addition, BST-2 has been shown to restrict several other enveloped viruses including Arenaviruses (Sakuma, Sakurai et al. 2009; Radoshitzky, Dong et al. 2010) and Herpesviruses (Mansouri, Viswanathan et al. 2009). There are conflicting data available for Filoviruses (Jouvenet, Neil et al. 2009) and

Paramyxoviruses, while BST-2 clearly does not block the exit of Poxviruses and Bunyaviruses (Radoshitzky, Dong et al. 2010).

The proposed model of BST-2 restriction is summarised in Figure 15 and will be discussed in more detail in Chapter 4.

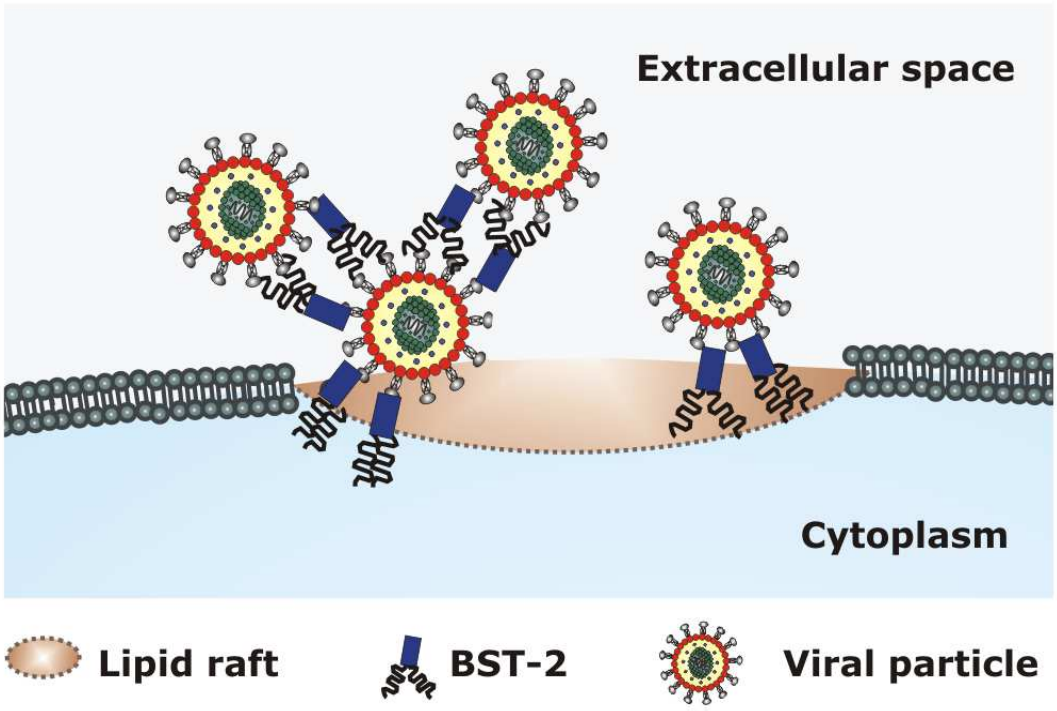


Figure 15. Schematic diagram illustrating the 'tethering' viral restriction model induced by BST-2.

BST-2 is highlighted in blue. Adapted from (Evans, Serra-Moreno et al. 2010). Viral particles can be retained individually or in aggregates, reducing dissemination of virions within the host.

1.6.4 Conserved features of BST-2

A comparison of human, rhesus, rat and murine BST-2 orthologs revealed four major conserved features (Kupzig, Korolchuk et al. 2003). BST-2 is composed of a short N terminal cytoplasmic region preceding a transmembrane region and an ectodomain, followed by C terminal GPI anchor. The ectodomain has several features including glycosylation sites and cysteine residues involved with dimerization and a coiled-coil motif.

The hydrophobic transmembrane region and C terminal GPI anchor create two points of contact with the plasma membrane. The only other protein to share

this unique topology is the PrP (prion) protein. This unusual topology supports the hypothesis that BST-2 tethers fully formed infectious viral particles to the plasma membrane of host cells, ultimately by creating a bridge between the plasma membrane and virion. In other words, BST-2 can 'crosslink' both the plasma membrane and virion creating a stable bridge resulting in the retention of the tethered particles at the plasma membrane.

Interestingly, Paul Bienasz and colleagues (Perez-Caballero, Zang et al. 2009) demonstrated that the configuration of BST-2 has more importance than the primary sequence for efficient restriction. An artificial 'art tetherin' with no sequence homology to BST-2 and yet still maintaining the configuration of BST-2 is still capable of restricting the release of enveloped viral particles. This is significant evidence supporting the notion that it is the configuration of BST-2 which leads to the efficient 'tethering' of viral particles on the cell surface.

Whilst there are clear conserved features across BST-2 orthologs, there are contrasting views on the importance of these different regions on the antiviral restriction mechanism (Andrew and Strebel 2011). It has been suggested that the discrepancies which have arisen in the literature may be due to the different experimental settings used in different laboratories. For example, (Andrew, Miyagi et al. 2009) describe the necessity of glycosylation for the BST-2 mechanism, whereas it was reported as dispensable for its action in the study of (Perez-Caballero, Zang et al. 2009). The key difference between these studies is the location of the tag used for detection within the protein (Andrew and Strebel 2011). The major structural features and the predicted topology of hBST-2 are summarised in Fig 14.

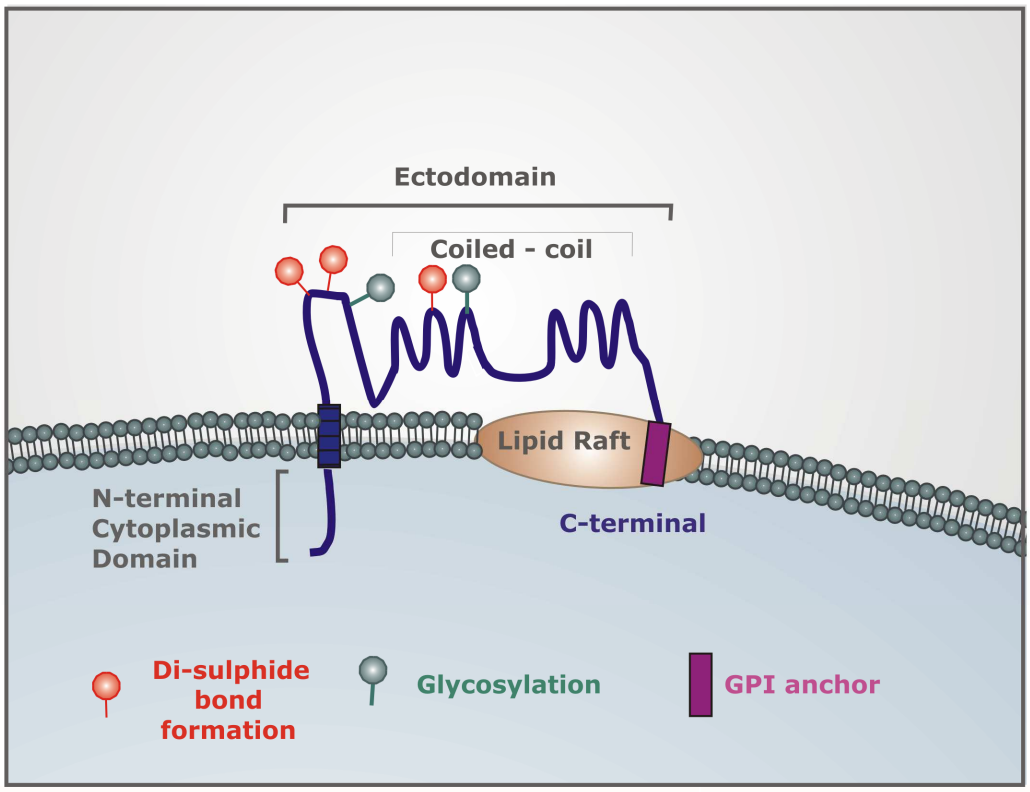


Figure 16. The four major conserved regions and membrane topology of BST-2.

1.6.5 N-terminal Cytoplasmic tail

The N-terminal cytoplasmic tail of BST-2 is important for the internalisation from the plasma membrane surface via the clathrin mediated pathway. Two tyrosine residues are conserved in BST-2 orthologs that may contribute to an internalisation motif influencing the intracellular localisation (see below) (Kupzig, Korolchuk et al. 2003; Rollason, Korolchuk et al. 2007).

1.6.6 Transmembrane region & C terminal GPI anchorage

The model of BST-2 restriction predicts the formation of a bridge between the viral envelope and the plasma membrane (Neil, Zang et al. 2008). Based upon the distinctive topology of BST-2, it is logical to assume that the bridge is provided by the N terminal transmembrane region and the C terminal GPI anchorage. Indeed, mutagenesis experiments have found the transmembrane region to be essential for the structure and restriction (Hinz 2010). In addition, the mere presence of a GPI anchor is a strong indicator of a protein with a

plasma membrane association (Paulick and Bertozzi 2008). Introduction of a GPI anchor into a protein not of plasma membrane origin has been shown to result in its redistribution to the plasma membrane (Brown, Cowen et al. 2000). A BST-2ΔGPI mutant localises to the ER and it appears that the GPI anchor is an essential factor for the correct folding and export of the BST-2 protein to the plasma membrane (Kupzig, Korolchuk et al. 2003). However, upon cleavage and disruption of the GPI anchor by the enzyme PI-PLC, BST-2 still maintains its membrane association, confirming the presence of an additional transmembrane domain (Kupzig, Korolchuk et al. 2003). Cleavage of the GPI anchor of BST-2 during viral 'tethering' does not release viral particles attached to the plasma membrane, presumably because of the terminal transmembrane domain and BST-2 dimers (Fitzpatrick, Skasko et al. 2010). Together, these data are highly supportive of the predicted mechanism of BST-2 restriction suggested by (Neil, Zang et al. 2008).

1.6.7 Ectodomain- Glycosylation

N-linked glycosylation is feature of BST-2 that appears to be conserved. Glycosylation results in three isoforms of BST-2, (ranging from approximately 25-35kDa) commonly observed following transient transfection and western blot analysis with BST-2 expression plasmids. These were confirmed as di- and monoglycosylated forms following incubation with PNGase F, an enzyme which removes N-linked sugars from glycol proteins (Kupzig, Korolchuk et al. 2003). When human BST-2 was compared with isolates from rhesus, rat and mouse BST-2, two similarly positioned N glycosylation sites could be found within the extracellular domain. Glycosylation has not been considered an important factor of function as studies with hBST-2 have demonstrated that altering the glycosylation status does not alter the distribution or impact upon the ability of BST-2 to restrict viral particle exit (Andrew, Miyagi et al. 2009; Sakuma, Sakurai et al. 2009). In contrast, others have reported that unglycosylated BST-2 is void of restrictive properties and suggests that this is likely as a result of trafficking defects (Perez-Caballero, Zang et al. 2009).

1.6.8 Ectodomain - Disulphide bond formation

Human, rhesus, rat and murine orthologs of BST-2 all share three cysteine residues, which can mediate the potential dimer formation induced by disulphide bonds (Ohtomo, Sugamata et al. 1999; Kupzig, Korolchuk et al. 2003). Interestingly, BST-2 dimerization has been shown to be essential in the block of HIV release induced by human BST-2 (Andrew, Miyagi et al. 2009) and yet it is dispensable for the restriction of Lassa or Marburg viruses (Sakuma, Sakurai et al. 2009).

1.6.9 Ectodomain - Coiled-coil motif

A comparison of murine and human BST-2 appears to suggest that the coiled-coil motif of the ectodomain is an evolutionary conserved feature (Swiecki, Scheaffer et al. 2011). This motif is essential for virion retention and it provides the flexibility required for the two point contact model envisaged for BST-2 restriction (Hinz, Miguet et al. 2010; Yang, Lopez et al. 2010). The ectodomain region of tetherin forms a single α-helix which assists in the formation of parallel BST-2 homodimers (Yang, Lopez et al. 2010), as previously described (Ohtomo, Sugamata et al. 1999). The observation of BST-2 tetramers has been excluded from having any functional role in viral restriction and it is most probably the result of non-specific interactions. While there is the suggestion that tetramers may confer stability to BST-2, it appears clear that dimerization alone is sufficient for viral particle retention (Schubert, Zhai et al. 2010).

1.6.10 Bst-2 intracellular localisation and expression

BST-2 is found to be expressed to varying degrees within a range of tissues in the absence of other IFN induced proteins (Erikson, Adam et al. 2011). BST-2 is constitutively expressed on the plasma membrane of cells which are targeted by retroviruses e.g. T cells. BST-2 can be induced on the surface of many other cells in response to IFN (Blasius, Giurisato et al. 2006). BST-2 is found concentrated within lipid rafts of the plasma membrane (Kupzig 2003). Indeed, plasma membrane based localisation of BST-2 is crucial for the ‘tethering’ antiviral effect to take place. BST-2 is then internalised from the plasma

membrane via clathrin mediated endocytosis (Rollason, Korolchuk et al. 2007; Masuyama, Kuronita et al. 2009) before being passed to the Golgi where an independent pool of BST-2 has been identified (Kupzig, Korolchuk et al. 2003). The localisation of BST-2 is summarized in Figure 17 and will be discussed in more detail in Chapter 3.

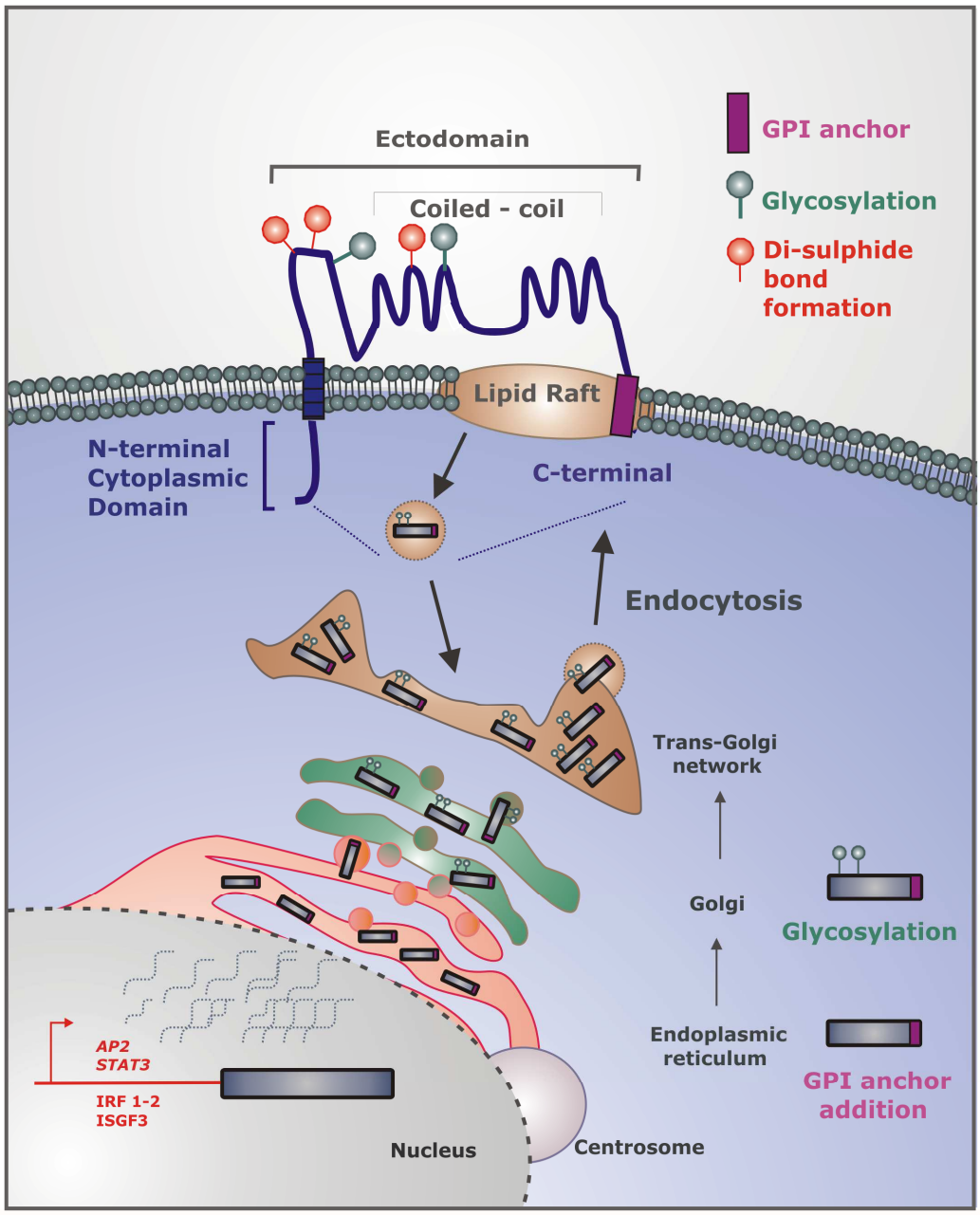


Figure 17. Illustration detailing the processing and localisation of BST-2.

1.6.11 Viral evasion of BST-2 restriction

The search for a restriction factor blocking HIV exit was stimulated by the observation that the accessory protein Vpu was required for successful viral exit in certain cell lines (Van Damme, Goff et al. 2008). In addition to downregulating CD4 from the cell surface, Vpu actively engages BST-2 and downregulates it from the plasma membrane (Iwabu, Fujita et al. 2009; Iwabu, Fujita et al. 2010; Ruiz, Guatelli et al. 2010). Vpu directs CD4 to the E3 ubiquitin ligase complex driven by a signal within the cytoplasmic domain (Andrew and Strebel 2010; Goffinet, Homann et al. 2010). Depletion of BST-2 by Vpu has been suggested to occur via proteosome degradation, (Goffinet, Allespach et al. 2009) also as a result of a combination of late endosomal targeting and degradation by lysosomes (Mitchell, Katsura et al. 2009). Many studies have considered the interaction of BST-2 and Vpu in vitro with contrasting results. Vpu affects BST-2 expression in HeLa cells and macrophages while BST-2 levels in H9 and CEMx174 cells are unaffected (Andrew, Miyagi et al. 2009). The specificity of Vpu for BST-2 is linked to three amino acid residues in the transmembrane domain of BST-2 (Kobayashi, Ode et al. 2011; Lv, Wang et al. 2011). There is evidence that Vpu promotes release of HIV without affecting the turnover of BST-2. However, Vpu appears to slow down its transport to the plasma membrane and redistributes BST-2 to the intracellular pool located within the trans-Golgi network (TGN) compartment by the recycling pathway common to BST-2 (Dube, Roy et al. 2010; Schmidt, Fritz et al. 2011). The antagonism Vpu provides is specific to human BST-2 and it does not antagonise simian BST-2 orthologs. Indeed Vpu is not present in many simian lentiviruses. Gain of function studies, which swapped domains between human and simian BST-2, concluded that the transmembrane region of BST-2 was responsible for the antagonism elicited by Vpu (Yoshida, Kao et al. 2011). Interestingly, SIVtan (but not SIVmac) and HIV-2 utilise Env to overcome human BST-2 restriction by sequestering it within the TGN (Gupta, Mlcochova et al. 2009; Le Tortorec and Neil 2009). The inability of SIVmac Env to overcome human BST-2 is an elegant demonstration of the species specific nature of virus and host factor co-evolution. SIVagm/blu/cpz/mac utilise the accessory protein Nef in a species-specific manner to counteract BST-2 restriction by the recruitment of the AP2 adaptor complex to downregulate BST-

2 (Jia, Serra-Moreno et al. 2009; Zhang, Wilson et al. 2009; Zhang, Landford et al. 2011). In addition, the Ebola virus glycoprotein and the K5 protein of Kaposi's sarcoma associated herpesvirus also facilitates escape from BST-2 restriction by mechanisms that have not been completely clarified (Lopez, Yang et al. 2010) (Mansouri, Viswanathan et al. 2009).

The accessory proteins acquired by viruses to overcome BST-2 restriction have led in turn to the selection of proteins that escape virus blockage. This is particularly prominent for human BST-2. There is evidence to suggest that deletion of 5 amino acid residues in the cytoplasmic tail of hBST-2 (when compared with other simian primates) allows it to avoid the counteracting action of Nef. This is considered an important factor in preventing transmission of SIV to humans (Sauter, Vogl et al. 2011). In response, HIV-1 Vpu has developed a series of modifications within the N terminal region which account for the nature of its specifc interaction with hBST-2 (Lim, Malik et al. 2010). In a manner similar to human BST-2, both murine and rodent orthologs were capable of restricting the release of Vpu deficient HIV-1 when expressed in 293T cells, although this restriction was not overcome in the presence of the accessory protein Vpu. Murine and rodent BST-2 share 71% homology with each other but only 41% and 36% homology respectively with human BST-2 The critical regions of BST-2 for the evasion of Vpu are spread across the structural domains (Goffinet, Allespach et al. 2009). Sheep Betaretroviruses, have no accessory proteins to overcome the antiviral action of the ovine BST-2 (Arnaud, Black et al. 2010). The interaction of both exogenous and endogenous JSRVs with oBST-2 is the subject of this thesis. In addition, there are data suggesting that BST-2 is unable to block cell to cell transmission by retroviruses. This will also be discussed as part of this thesis.

Chapter 2 - Material and Methods

2.1 Cells

HEK-293T cells are immortalized human embryonic kidney cells (referred throughout the text simply as 293T cells) and were maintained in Dulbecco's modified Eagle's medium (DMEM) Glutamax (Gibco), supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% Penicillin-Streptomycin (Gibco). CPT-tert cells (ovine choroid plexus cells immortalised with h-tert, a gift from David Griffiths, Moredun) were maintained in Iscove's modified Dulbecco's medium (IMDM Glutamax, Gibco) supplemented with 10% FBS + 1% Pen-Step. Cells were incubated at 37^{o}C, with 5% CO_2 in 95% humidity. Cells were routinely cultured in 10cm diameter tissue culture dishes and were passaged when monolayers reached ~95% confluency. All adherent cells were rinsed three times with phosphate buffered saline (PBS) pH7.4 (Gibco), once with trypsin (Gibco) and then incubated with 1 millilitre (ml) of trypsin at 37^{o}C until detachment of the monolayer from the dish. Cells were resuspended in 10ml of the appropriate growth medium and pelleted by low speed centrifugation (Eppendorf Centrifuge 5810) at 1000g for 5 minutes (mins). Cells were then washed twice in PBS, resuspended in 10ml of fresh growth media and seeded at the required density into a new 10cm Petri culture dish.

For the long term storage, cells were resuspended in 1ml of 90% FBS/10% Dimethyl sulfoxide (DMSO, Sigma Aldrich) and aliquoted into fully labelled cryovials (Nunc). Vials were frozen at -80 ^{o}C in a cryofreezer (Mr Frosty, Nalgene) before being transferred into liquid nitrogen storage 24h later. The recovery of cells stored in liquid nitrogen was initiated by thawing vials for the minimum time necessary at 37^{o}C. Cells were then added drop wise into 10 ml of suitable growth medium and pelleted by low speed centrifugation 1000g for 5 mins. The resulting pellet was resuspended in 10mls of appropriate growth medium and seeded into either a 6 cm or 10 cm tissue culture dish as appropriate.

2.2 Plasmids

JSRV plasmids

Table 1. Summary of the main JSRV and enJSRV expression plasmids employed in this study.

Name	Description	Source/ reference
pCMV2JSRV$_{21}$	full length molecular clone of JSRV$_{21}$	Palmarini 1999
pCmv435024	full length molecular clone of enJSRV7	Arnaud, Caporale et al. 2007
pCmv457M3	full length molecular clone of enJSRV15	Arnaud, Caporale et al. 2007
pCmv46F9	full length molecular clone of enJSRV18	Arnaud, Caporale et al. 2007
pCmv4102B15	full length molecular clone of enJSRV26	Arnaud, Caporale et al. 2007
pCMV2JSRV21FLAG	pCMV2JSRV21 with HA tag between signal peptide and SU of Env	This thesis
pCMV5a-JS21Flag	JSRV Env with C terminal FLAG epitope	Varela, 2008
pCMV3DGPP2NFlag	JSRV Env with N terminal FLAG epitope	Varela, 2008
pCMVN-FLAG57m4Env	enJSRV18 Env tagged at the N terminal	Dr. F Arnaud
pCmv4js21XSstoM3	full length molecular clone of JSRV provirus with 2 stop codons before Env	Dr. M. Caporale
pCmv467f9ds	full length molecular clone of enJSRV-18 provirus with 2 stop codons before Env	Dr. M. Caporale

pCMV2JSRV21 is a full length molecular clone of JSRV driven by the Cytomegalovirus (CMV) immediate early promoter and has been described previously (Palmarini, Sharp et al. 1999). Similarly, full length proviral clones of enJSRV-7, 15, 18 & 26 (pCmv435024, pCmv457M3, pCmv46F9, pCmv4102B15), driven by the CMV promoter, have also been described (Arnaud, Caporale et al. 2007). pCMV2JSRV21HA is a construct generated from pCMV2JSRV21 which contains the haemagglutinin (HA) epitope inserted within the Env protein of

JSRV, preceding the SU and after the signal peptide in the context of the full length proviral clone. This epitope was inserted by site directed mutagenesis using Fw 5‘-CCAGACTACGCTGCGGCTTTTTGGGCGTACATTCCTGATCC-3’ and Rw 5‘-GACGTCGTATGGGTAAGCTGCCCCATTTTGTACCCGCTGTAAC-3’ primers.

pCMV5a-JS21Flag and pCMV3DGPP2NFlag express the JSRV Env protein tagged with the FLAG epitope at either the C or N terminus respectively, and have been described previously (Varela 2007). pCMVN-FLAG57m4Env (enJSRV-18 Env tagged at the N terminal) was provided by Dr. F. Arnaud. pCmv4js21XSstoM3 and pCmv467f9ds express the full length exogenous JSRV and enJSRV-18 proviruses respectively but contain two stop codons preceding the envelope open reading frame (provided by Dr. M Caporale).

BST-2 plasmids

Two ovine Bst-2 genes (designated oBST-2A and -2B) were isolated and cloned into the pCI mammalian expression vector (Promega) to obtain pCI-oBST2A and pCI-oBST2B respectively as already described (Arnaud, Black et al. 2010). pCIoBST2AHa and pCIoBST2BHa express oBST-2A and -2B containing an HA epitope tag prior to the predicted ω site for GPI cleavage.

The Vpu gene of HIV-1 within a mammalian expression vector was provided by Dr. S.J. Neil (Kings College). The empty pCI-DNA mammalian expression plasmid was used as a control for transfection to equilibrate DNA volumes. In addition, peGFP-C1, which expresses the enhanced green fluorescent protein (eGFP), was used as a control for transfection efficiency. Cav-1GFP is an expression plasmid for Caveolin-1, a plasma membrane based protein, fused with GFP at the C terminus (Addgene plasmid 14433). E-Cad-GFP–c1 is an expression plasmid for Cadherin-1, a plasma membrane based protein fused with GFP and was kindly provided by Dr. K. Anderson.

oBST-2B mutants were generated by site directed mutagenesis (Figure 18 and Table 2).

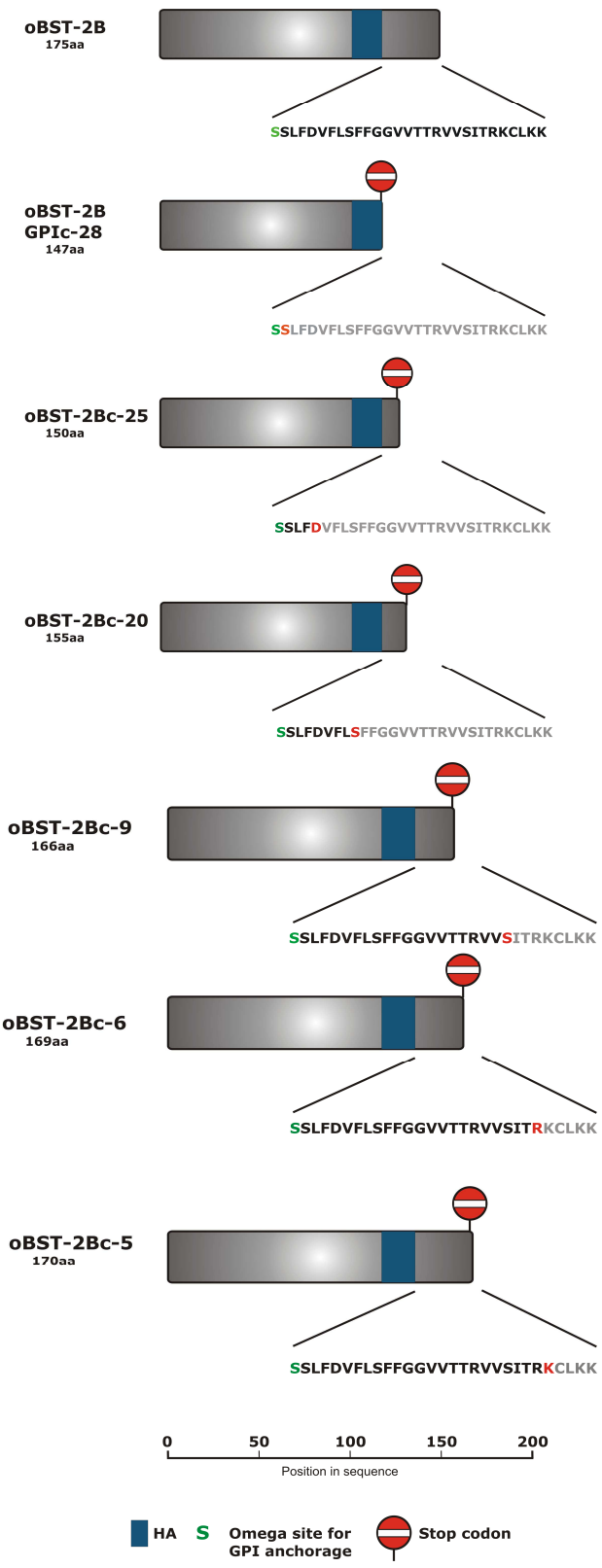

oBST-2B
175aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2B
GPIc-28
147aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2Bc-25
150aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2Bc-20
155aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2Bc-9
166aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2Bc-6
169aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
oBST-2Bc-5
170aa
SSLFDVFLSFFGGVVTTRVVSITRKCLKK
0
50
100
150
200
Position in sequence
HA
S
Omega site for
GPI anchorage
Stop codon

Figure 18. Illustration of the oBST-2B mutants employed in this thesis

Table 2. Summary of the primers employed for the construction of the oBST-2B mutants employed in this thesis

Plasmid	Primers 5'-3'
oBST-2BGPlc-28	Fw gactacgctgctaggtccTGAttgttcgacgtgttcctc Rw gaggaacacgtcgaacaaTCAggacctagcagcgtagtc
oBST-2Bc-25	Fw gctaggtccagcttgttcTAGgtgttcctctccttttc Rw gaaaaaggagaggaacacCTAgaacaagctggacctagc
oBST-2Bc-20	Fw tgttcgacgtgttcctctGATttttcgggggtgtggtga Rw tcaccacacccccgaaaaaTCAgaggaacacgtcgaaca
oBST-2Bc-9 -	Fw cgggttgtgtccatcaccTGAaagtgtctgaagaaatga Rw tcatttcttcagacacttTCAggtgatggacacaacccg
oBST-2Bc-6	Fw cgggttgtgtccatcaccTGAaagtgtctgaagaaatga Rw tcatttcttcagacacttTCAggtgatggacacaacccg
oBST-2Bc-5	Fw gttgtgtccatcacccgcTAGtgtctgaagaaatgag Rw ctcatttcttcagacaCTAgcgggtgatggacacaac

All PCR primers were purchased from MWG Biotech. For cloning purposes, all PCR reactions were performed using Hotstar Hifidelity (Qiagen) according to the manufacturer's protocol.

PCR purification and Gel extraction was performed with the QIAquick PCR purification kit (Qiagen) or QIAquick Gel extraction kit (Qiagen) respectively. Ligations were performed using the Rapid DNA Ligation Kit (Roche). Plasmids were transformed into either DH5α or TOP10 strains of *E.Coli* (Invitrogen). Minipreps of plasmid were prepared using the QIAprep Miniprep kit (Qiagen), and

Maxipreps with Purelink HiPure Plasmid Maxiprep kit (Invitrogen). DNA concentrations and quality were measured and verified using a Nanodrop 2000 spectrometer.

The authenticity of each plasmid was verified first by restriction digestion with appropriate restriction enzymes (Roche) and subsequently by DNA sequencing (Dundee Sequencing Service).

All sequence predictions for oBST-2A/ -2B were made by submitting sequences to programs available on the Expasy Proteomics server http://expasy.org/tools. TMHMM Server v2 predicted potential transmembrane regions, NetNGly 1.0 Server sites for possible N-linked glycosylation, and GPI-SOM for residues targeted for cleavage and GPI anchor addition.

For site directed mutagenesis, PCR reactions were performed using the Quikchange II site directed mutagenesis kit (Agilent Technologies) according to the manufacturer's protocol. When necessary, PCR products were precipitated at -20^{o}C with isopropanol in the presence of 3M Sodium Acetate pH5.5 (Ambion). The DNA was precipitated by centrifugation at 20,000g for 30mins at 4 ^{o}C (Eppendorf Centrifuge 5810-R) and resuspended in double deionized water (ddH2O) (Millipore) prior to transformation into competent cells.

2.3 Transient transfections

Transient transfections for western blot analysis were performed using the CalPhos™ mammalian transfection kit (Clontech) adhering to manufacturer's protocol. 293T cells were plated 24 hours prior to transfection in 10cm plates so that 80% confluency was achieved prior to transfection. An hour prior to transfection the cells were washed with 1X PBS and maintained in media containing no antibiotics. Transfection complexes were formed by first mixing appropriate amounts of plasmid DNA (0.25-4μg) with 2M Calcium phosphate. This mixture was then added dropwise to a 2X Hepes-buffered saline solution and incubated for 20 mins at room temperature. Transfection complexes were then added to cells and incubated overnight at 37^{o}C. Following overnight incubation, the cells were washed with 1X PBS and fresh medium containing antibiotics was

added. Cells were incubated for a further 24 hours before cell supernatant/lysates were harvested.

2.4 Cell lysates

Cells lysates were harvested in general 24 hours post transfection for protein analysis by standard molecular techniques. Briefly, cells were rinsed with cold PBS and lysed on ice for 10 minutes with modified RIPA buffer ((15mM Tris-HCl, pH 7.4; 1% NP-40; 1mM EDTA; 150mM NaCl, 1mM PMSF; 1mM NaF; protease inhibitors cocktail (COMPLETE-Roche)). Cells were collected by scraping and subsequently sonicated on ice. Protein concentration was measured using the BioRad protein assay kit according to manufacturer's protocol.

2.5 PNGaseF treatment of lysates

Peptide N-Glycosidase F (PNGaseF, Sigma-Aldrich) is commonly used to study the glycosylation status of proteins as it removes N-linked carbohydrate chains added during the process of post translation modification. 293T cells were transfected as previously described using CalPhos with 1μg of pCI-oBST2A or -2B. 24 h post-transfection, cell lysates were harvested in 1% NP-40 (Fisher). 200μg of the appropriate cell lysate (mock transfected, or transfected with expression plasmids for oBST-2A and oBST-2B) was aliquoted in duplicate. The samples were diluted to a final volume of 35μl before adding 10μl of 250mM PBS pH7.5, 2.5μl of 2% Sodium Dodecyl Sulphate, 1M 2-mercaptoethanol. The samples were then boiled at 100 ^{o}C for 5 min and cooled on ice. 2.5μl of 15% Triton X100/PBS (v/v) was added before the addition of 2μl of PNGaseF solution, or PBS as a mock control. All samples were then incubated at 37^{o}C for 6h. Reactions were stopped by adding 4μl of 4x SDS-PAGE reducing buffer (250mM Tris-HCl pH6.8, 8% SDS, 40% Glycerol (v/v), 0.008% Bromophenol Blue, 20% 2-Mercaptoethanol) and boiling for 5 min prior to SDS-PAGE/western blot analysis.

2.6 Cell lysate fraction preparation

293T cells were transfected as described above with expression plasmids for oBST-2A or -2B. The cells were then washed with PBS and treated for 30 min at room temperature with 3,3'-dithiobis sulfosuccinumidylpropionate (DTSSP) (Thermo Scientific). DTSSP crosslinks cellular proteins to membranes and this treatment was used in an attempt to preserve oBST-2A/-2B in their native environment during the fractionation process. DTSSP treatment was stopped by the addition of 1M Tris (pH7.5) prior to harvesting the lysates. The cells were then scraped from the Petri dish and washed with PBS. The cells were sheared using a 21G needle (BD Microlance) with 30 strokes per sample. Lysates were then processed using the Plasma Membrane Protein Extraction Kit (Biovision) as suggested by the manufacturer. Cytosolic fractions and plasma membrane fractions were retained for western blot analysis.

2.7 Viral particle preparations

Cell culture supernatants were collected 24 hours after transfection with the appropriate plasmid. The viral particles were concentrated by ultracentrifugation at 35,000rpm (SW41 rotor Beckmann) for 60 minutes at 4^{o}C on a 29% sucrose cushion. Viral pellets were resuspended in 1x TNE buffer (diluted from 10X stock, 1M NaCl; 0.1M Tris; EDTA final solution pH 7.5) at a final volume 200x concentrated compared to the initial volume of the centrifuged supernatants. The viral pellets were resuspended overnight at 4^{o}C in 4μl of 4x SDS-PAGE reducing buffer (250mM Tris-HCl, pH 6.8; 8% Sodium Dodecyl Sulphate; 40% glycerol (v/v); 0.008% Bromophenol Blue; 5% β-Mecarptoethanol) and boiled for 5 minutes prior to SDS-PAGE western blot analysis, or storage at -80^{o}C for subsequent use.

In some cases, viral pellets were purified by isopycnic centrifugation on 20 to 55% (w/w) sucrose gradients (25,000 rpm (SW41 rotor Beckmann) for 16h at 4^{o}C). Fractions of approximately 500 μl were then collected and the virus concentrated by ultracentrifugation prior to further analysis.

2.8 SDS-PAGE and western blotting

200μg of cell lysate or an appropriate volume of viral pellet material was separated by SDS-PAGE before transferring to a Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare). Membranes were then incubated in blocking buffer (5% milk in TBS-Tween (ddH2O, 10% Tris Buffered Saline (TBS) 10x, 0.1% Tween 20)) for 1h at room temperature prior to incubation overnight at 4°C with primary antibodies. The primary antibodies used were anti JSRV/enJSRVs Gag (used at either 1:3000 dilution for lysates or 1:300,000 for viral pellet detection) and anti-HA (1:500) in blocking buffer, or anti-JSRV TM (1:500) in BSA blocking buffer (5% BSA, 5% milk, ddH2O, 10% TBS 10x, 0.1% Tween 20). Membranes were then washed three times with TBS-TWEEN solution and incubated with a species-specific horseradish peroxidise-conjugated secondary antibody diluted in blocking buffer for 1h at room temperature. The secondary antibodies used were donkey anti rabbit F(ab')2 fragment (1:30,000; GE Healthcare) or an anti mouse F(ab')2 (1:30,000; AbD Serotec). Detection was performed by chemiluminescence (ECL, GE Healthcare). When redetection was necessary, the membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) at 37°C for one hour and washed with TBS-TWEEN solution. The stripped membranes were then used as before to blot using the required primary antibody. Whenever necessary, the band strength in western blots was quantified using a Storm 640 (Molecular Dynamics) using ImageQUANT software (GE healthcare).

2.9 Reverse Transcriptase Activity assay

Reverse transcriptase (RT) activity from concentrated viral pellet/virus isolated on sucrose gradient was detected and quantified using the HS-Mg RT Activity Kit (Cavidi) according to the manufacturer's protocol. Positive RT activity was determined with values representing more than twice the negative controls. A rRT of HIV-1 was used to derive a standard curve.

2.10 Dynamic Light Scattering

Dynamic Light Scattering (DLS) was used to estimate the size of viral particles released by enJSRV-18 and JSRV in the presence and absence of oBST-2A/-2B. All samples were checked by western blot prior to DLS analysis and all measurements were made using the Zetasizer Nano zs90 (Malvern). Viral pellets produced following Calphos™ transfection of the appropriate plasmid(s) were diluted to a final volume of 500µl in 1x TNE buffer. Samples were loaded into the cuvette provided with the apparatus, inserted into the Zetasizer Nano zs90 and a run was initiated. All of the measurements were made at 37^{o}C. The Zetasizer Nano zs90 software compiled the data and generated a Z average and count rate. The Z average is representative of the predicted hydrophobic diameter and the count rate is indicative of sample quality, respectively. An average value and standard error was calculated from three individual measurements per sample.

2.11 Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed with a Nanosight (LM-20). NTA was used to estimate the size of viral particles released by enJSRV-18 and JSRV in the presence and absence of oBST-2B. All samples were checked by western blotting prior to NTA analysis. Three measurements of each sample were made and the results captured and analyzed with the NTA 2.0 Build software (Nanosight). Mean values and standard errors for the average size of all particles within the sample were calculated.

2.12 Confocal microscopy

CPT-tert cells were plated 24 hours prior to transfection on 2-chamber slides (Cornell) in IMDM-Glutamax media (Invitrogen) supplemented with 10% FBS + 1% Penicillin-Streptomycin. Cells were transfected with the appropriate plasmids using Lipofectamine 2000 as recommended by the manufacturer. Transfection mixtures were incubated at room temperature for 20 minutes to allow

Lipofectamine 2000 complexes to form before each reaction was added dropwise to the chamber. The cells were incubated for 5 h at 37°C before replacing the media containing transfection mixture with fresh complete media. 18h post-transfection the cell monolayer was washed twice with cold PBS, fixed with 5% formaldehyde in PBS at 4°C for 20 min, and quenched in 10mM ammonium chloride.

For the analysis of intracellular protein distribution, the cells were permeabilised with PBT (PBS + 0.5% Trition X-100; Sigma-Aldrich) for 10 min at room temperature and then blocked with solutions 1 and 2 (Table 3) consecutively for 5 minutes each to minimise non-specific antibody binding. The appropriate primary antibodies (Table 4) were then diluted in solution 2 and incubated overnight at 4°C in a lightproof container.

Table 3. Composition of blocking solutions used for confocal microscopy.

Solution 1	Solution 2
200µl Fish Gelatin (Sigma-Aldrich) + 50mls 1%PBS + 2% Tween20	1250 µl Goat Serum (Biosera) + 50mls 1%PBS + 2% Tween20

Table 4. Primary and secondary antibodies used in this thesis.

Primary Antibody	Antibody species and type	Dilution
p23 GAG	polyclonal anti-rabbit	1:200
FLAG	polyclonal anti-rabbit (Sigma)	1:750
HA	monoclonal anti-mouse (Covance)	1:100
TGN46	polyclonal anti-rabbit (Abcam)	1:1000
Golgi -Giantin	polyclonal anti-rabbit (Abcam)	1:100
Golgi - p115	anti-rabbit (Abcam)	1:100
Rab7	Polyclonal anti-rabbit (Abcam)	1:100
GFP	polyclonal anti rabbit (Abcam)	1:500
Secondary Antibody		
Alexa Fluor 488	goat anti-rabbit (Invitrogen)	1:3000
Alexa Fluro 594	donkey anti-mouse (Invitrogen)	1:3000

The next day, the cells were washed three times in PBS-T (PBS + 2% Tween20) before repeating the blocking step with solutions 1 and 2. The cells were then incubated with the appropriate secondary antibodies in Solution 2 (as indicated below) at 37^{o}C for 30 min and then washed again three times with PBS-T solution. Finally, the chamber walls and adhesive were removed from slides before adding one drop of Vectashield with 4', 6-diamidino-2-phenylindole dihydrochloride DAPI (Vector Laboratories). Coverslips (22x22mm, BDH Laboratory Supplies) were then applied and sealed with nail varnish. Cells were examined and imaged with a Leica TCS SP2 confocal microscope. Image analysis was performed with Image Pro Analyzer 7.0 (Media Cybernetics).

Colocalisation was measured using Pearson's Correlation (a value reflecting the degree of overlap between two channels) calculated by Image Pro Plus software (Media Cybernetics). The value ranges from -1 to 1. Anything below 0.5 was considered non-significant and anything above as a significant degree of colocalisation. At least fifty cells were measured for each experiment. All values presented represent an average percent and include standard error values.

2.13 Brefeldin A treatment

Whenever necessary cells were treated with Brefeldin-A, a fungal metabolite which fragments the Golgi stacks leading to the concentration of proteins within the endoplasmic reticulum and redistribution of Golgi based proteins. Brefeldin-A was used at a concentration of 200ng/ml. Transient transfections were performed as described in section 2.12. At the point where the medium was changed, the replacement media was either serum free medium (SFM) alone (control), or SFM containing 200ng/ml Brefeldin A. The cells were incubated for 90mins before fixation and immunolabelling for confocal microscopy (see 2.12 Confocal microscopy).

Chapter 3 - Investigating the intracellular distribution of the oBST-2 paralogs

3.1 Summary

In this chapter, we defined their intracellular distribution of the oBST-2 paralogs. We also highlight key differences between the oBST-2A/ -2B proteins which may contribute to the difference in intracellular localisation observed.

3.2 Introduction

3.2.1 BST-2 localisation and intracellular trafficking.

As discussed in Chapter 1, BST-2 is a cellular restriction factor with a broad antiviral activity. To date, the majority of knowledge of BST-2 expression and mode of action has been derived from studies involving the human ortholog. *In vivo*, human BST-2 is found constitutively expressed to varying degrees on the surface of a range of cells including hepatocytes, pneumocytes, Leydig cells, plasma cells and bone marrow stromal cells (Erikson, Adam et al. 2011). This is in direct contrast to the original reports following its discovery when it was considered a surface marker of terminally differentiated human B cells (Goto, Kennel et al. 1994). It is not found to be constitutively expressed on the surface of T cells, B cells, macrophages, monocytes or plasmacytoid dendritic cells (pDC) in humans. There is strong evidence to suggest that constitutive expression is species specific as it is found expressed on the surface of murine pDCs (Swiecki and Colonna 2010), but is absent from human pDCs (Erikson, Adam et al. 2011). BST-2 is found expressed in a wide variety of cells including pDCs following treatment with IFN (Kupzig, Korolchuk et al. 2003; Blasius, Giurisato et al. 2006), although in vivo it may only partially regulate expression (Erikson, Adam et al. 2011). In vitro studies have shown that BST-2 is constitutively expressed in HeLa and Jurkat cells but its expression can also be induced in 293T and HT1080 cells following IFN treatment.

BST-2 appears to reside specifically within the lipid rafts of the plasma membrane (Kupzig, Korolchuk et al. 2003). Lipid rafts or 'membrane rafts' are small heterogenous regions of the plasma membrane (10-200 nm in diameter) with a high content of sterol and sphingolipids. They are essential for efficient membrane segregation, protein trafficking and signalling (Viola and Gupta 2007). Interestingly, lipid rafts are considered hotspots for viral particle release during the retroviral replication cycle (Nguyen and Hildreth 2000). The localisation of BST-2 at the plasma membrane is fundamental for the 'tethering' antiviral mechanism.

As previously mentioned, BST-2 is composed of a short N terminal cytoplasmic region preceding a transmembrane domain and an ectodomain followed by a C-terminal GPI anchor (Kupzig, Korolchuk et al. 2003). GPI anchorage is a major determinant of BST-2 localisation. BST-2 mutants with the GPI signal deleted localise to the ER. Thus, the GPI signal is essential for the correct export of BST-2 to the plasma membrane and for its restriction capability. The property of BST-2 to make two connections with the plasma membrane, provided by the N-terminal transmembrane region and GPI anchorage, forms the basis of the 'tethering' restriction mechanism. BST-2 inserts one end of the protein within the host plasma membrane and the other within the virion envelope, in this way creating a bridge of retention (Neil, Zang et al. 2008).

BST-2 has been shown to be internalised via clathrin-mediated endocytosis into early recycling endosomes (not late) before passing to the trans-Golgi network (TGN). The N-terminal region of BST-2 contains two conserved tyrosine residues that likely form part of an internalisation motif (Rollason, Korolchuk et al. 2007). Pull down experiments demonstrated the specific interaction of the N-terminal cytosolic region of BST-2 with the μ2 subunit of the adaptor protein AP2 (Masuyama, Kuronita et al. 2009). Adaptor protein complexes are cytosolic heterodimers that regulate protein sorting in the post Golgi network. AP2 mediates endocytosis from the plasma membrane whilst AP1/2 and 4 are involved in endosomal/lysosomal pathways (Nakatsu and Ohno 2003). Disruption of the GPI anchor alters the internalisation pathway of BST-2, demonstrating that the GPI/lipid raft association is an important and established factor for

successful internalisation (Rollason, Korolchuk et al. 2007; Masuyama, Kuronita et al. 2009).

As mentioned above, BST-2 has been found to localise also at the TGN, an unusual property for a GPI anchored protein. The localisation of a second pool of BST-2 to the TGN raises the question of whether this pool is independent from the one recycling from the plasma membrane and TGN.

3.2.2 The Golgi and Trans Golgi Network

The Golgi is formed by several layers of membranous stacks arranged in ribbon like structures juxtaposed to the centrosome (cis and medial cisternae) in eukaryotic cells (Sengupta and Linstedt 2011). Newly synthesized membrane proteins bud from the ER and concentrate within COP II vesicles. The COP II vesicles bud directly from the ER and target the Golgi for cargo delivery (Haucke 2003). Upon arrival at the Golgi, proteins undergo post-translational modification, progressing through the cis and medial Golgi via COP I vesicles before finally entering the trans-Golgi network (TGN). Arrangement of the Golgi as stacked cisternae is an important feature of its function, promoting an optimum environment for sequential post-transcriptional processing events such as glycosylation and sorting of cargo (Sengupta and Linstedt 2011). The Golgi contains numerous resident proteins (Golgins) which provide structural support and function. For example, p115 is localised to the cis Golgi stack and is a vesicle docking protein assisting COP I cargo entering the Golgi. In addition, p115 also plays a role as a Golgin protein for mitosis and apoptosis (Radulescu, Mukherjee et al. 2011). Giantin is also a Golgin but is found within COP I vesicles moving cargo between Golgi stacks and is involved in vesicles docking to Golgi membranes (Sonnichsen, Lowe et al. 1998), whilst TGN46 is an example of a resident TGN protein.

The TGN is the final sorting compartment which determines the final intracellular location of membrane proteins. The Golgi and TGN should be considered as separate structural entities which perform unique functions,

although they are closely associated (Sengupta and Linstedt 2011). This is emphasised by the differences observed following treatment with Brefeldin-A a fungal metabolite which fragments the Golgi. Upon treatment with Brefeldin-A, the Golgi stacks form tubules that fuse with the ER resulting in the redistribution of all Golgi based proteins to the ER. Similarly, the TGN is also fragmented by Brefeldin A but the resulting tubules fuse with the endosomal network and all TGN based proteins are redistributed to the MTOC (Gu, Crump et al. 2001).

The TGN forms the sorting station for newly synthesized membrane proteins. It differs from the Golgi stacks in that it is a structure with an extensive series of membranes rather than organized stacks and varies in size and structure depending on the cell type. Like the Golgi, it is a highly diverse, mobile element with a high level of protein renewal and not a static structure of the cell. It is absent altogether from cells which have a high turnover of very large secretory granules but it does form extensive multilayer interconnecting stacks in cell types which have a more moderate level of granules for secretion (Sengupta and Linstedt 2011). Once located within the TGN, proteins will be sorted for delivery to their final destination based on protein binding domains and motifs. This results in them either being retained as TGN proteins, trafficked back to the Golgi stacks/E.R./nucleus or onwards to endosomes, ubiquitin proteosome or the plasma membrane (Tekirian 2002). The TGN is also the receiving centre for a select set of proteins (approximately 5%) via retrograde transport from the plasma membrane (Chege and Pfeffer 1990)

3.2.3 Interplay between ovine BST-2 and sheep Betaretroviruses

The enJSRVs are abundantly expressed within the reproductive tract of sheep and have a prominent role in placental morphogenesis (see Chapter 1.4.8). enJSRV expression during pregnancy coincides with that of IFNtau, a type I IFN which, as well as having antiviral activity, is the pregnancy recognition signal in ruminants. Previously, a study conducted by Frederick Arnaud in our laboratory, showed that IFNtau upregulates the expression of BST-2 both in vitro and in vivo (Arnaud, Black et al. 2010). Interestingly, he also found that the BST-2 gene is duplicated in ruminants and designated these paralogs oBST-2A and -2B. Duplication of an IFN-induced BST-2 has not been reported for any other

ortholog to date (although splice variants have been identified for human and canine orthologs). The date of this duplication event is unknown but as it is present in both cows and sheep it has occurred prior to their speciation. Both paralogs block JSRV/enJSRVs viral exit, although oBST-2A appears to be more efficient than oBST-2B based on Gag exit (Arnaud, Black et al. 2010). In vivo, the expression of oBST-2 and enJSRVs was found to be mutually exclusive leading to the speculation that the interplay between the host restriction factors such as oBST-2 and enJSRVs may have influenced the tropism of these viruses.

3.3 Results

3.3.1 Sheep Betaretroviruses are restricted by human BST-2 (hBST-2)

Betaretroviruses, including sheep Betaretroviruses, assemble in the cytoplasm in the vicinity of the MTOC, and not at the cell membrane like the great majority of retroviruses. In order to investigate the relationship between ovine BST-2 and sheep Betaretroviruses we first assessed whether the human ortholog (hBST-2) is able to block these viruses. Studies were conducted with the exogenous JSRV and one of its endogenous counterparts, enJSRV-18. As mentioned in Chapter 1, enJSRV-18 has intact open reading frames and is abundantly expressed in transfected cells in vitro (Chessa, Pereira et al. 2009).

We transiently transfected 293T cells with expression plasmids for either enJSRV-18 or JSRV in the presence and absence of hBST-2 (Figure 19). We observed that hBST-2 induced an efficient block on virion release based on the presence of pelletable Gag in the supernatants of transfected cells.

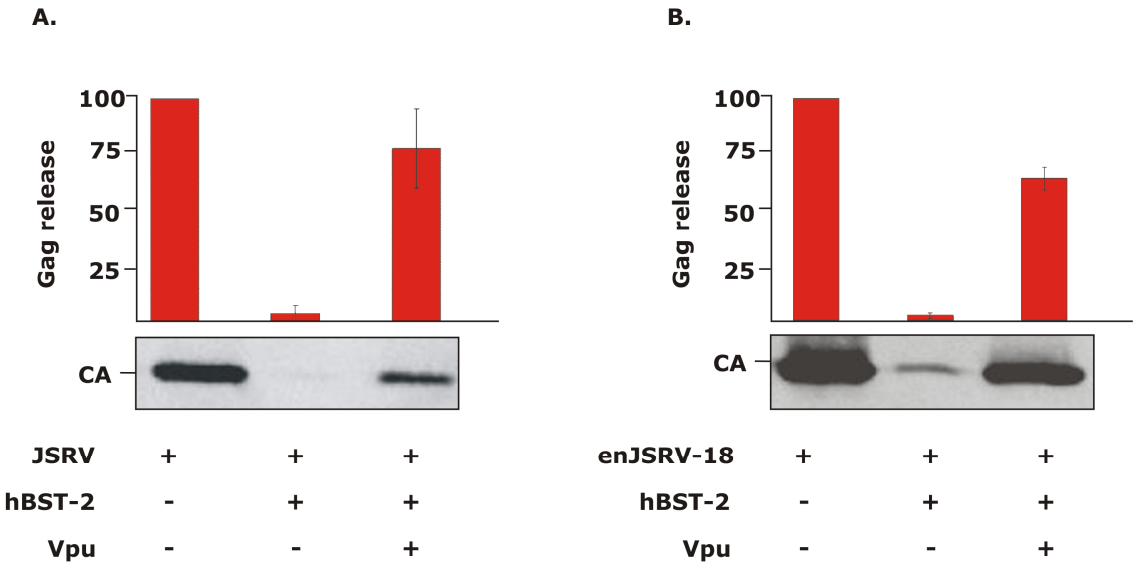


Figure 19. JSRV and enJSRV-18 viral exit is blocked by hBST-2 but rescued by the addition of Vpu *in trans.*

Western blot analysis on concentrated supernatants of 293T co-transfected with expression plasmids for viruses and BST-2/Vpu proteins as indicated in each panel. Cells were transfected in a 6 well plate with 0.5μg of JSRV (panel A) / enJSRV-18 (panel B) and 0.1μg of HA tagged expression plasmids for hBST-2 and Vpu. Blots were incubated with the Gag antisera against the CA domain, indicated in each panel. Viral particle release based on CA release was quantified, and values represent arbitrary units relative to the values of each virus transfected in the absence of BST-2 (which was assigned a value of 100). Representative experiments are shown and were repeated at least three times independently.

In order to determine the specificity of the BST-2 restriction, we also confirmed a similar level of efficiency of restriction for several other insertionally polymorphic, replication competent enJSRVs, including enJSRV-7, 15 and 26, by hBST-2 (Figure 20). As expected based upon previously published data (Neil, Zang et al. 2008), in all cases the provision of the HIV accessory protein Vpu in trans efficiently counteracted the restriction by hBST-2.

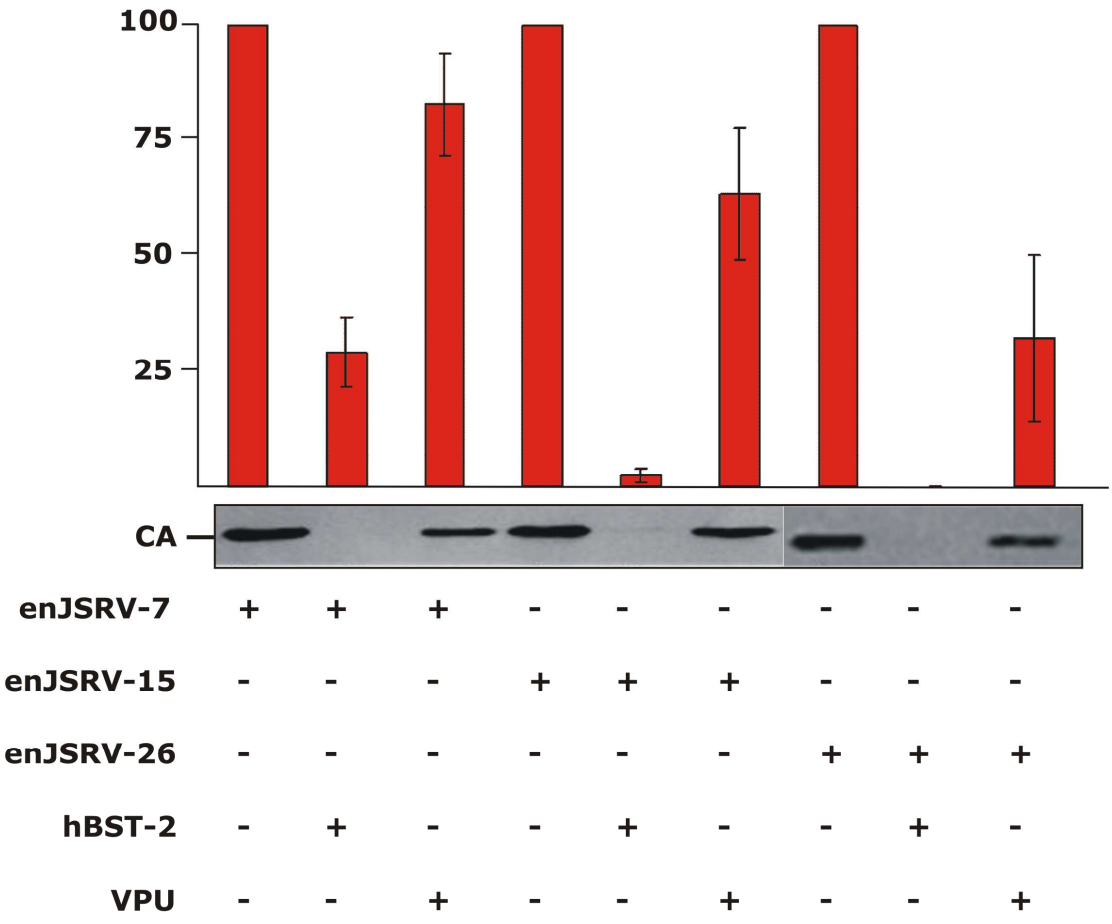


Figure 20. Viral exit of enJSRV-7, 15 and 26 is blocked by hBST-2 but rescued by the addition of Vpu *in trans*.

Western blot analysis on concentrated supernatants of 293T cells cotransfected with expression plasmids for viruses and BST-2/Vpu proteins as indicated in each panel. Cells were transfected in a 6 well plate with 1.5, 0.5 and 4 μg of enJSRV-7, 15 and 26 respectively, and 0.1μg of HA tagged expression plasmids for hBST-2 and Vpu. Blots were incubated with the Gag antisera against the CA domain, indicated in each panel. Viral particle release was quantified based on CA release, and values represent arbitrary units relative to the values of each virus transfected in the absence of BST-2 (which was assigned a value of 100). Representative experiments are shown and were repeated at least three times independently.

As mentioned previously, studies conducted by Frederick Arnaud in our laboratory showed that the BST-2 gene is duplicated in ruminants. oBST-2A blocks JSRV/enJSRVs viral particle exit more efficiently than oBST-2B (Arnaud, Black et al. 2010). In concordance with these data, enJSRV-18 exit was found to be blocked very efficiently in the presence of both oBST-2A and -2B (Figure 21).

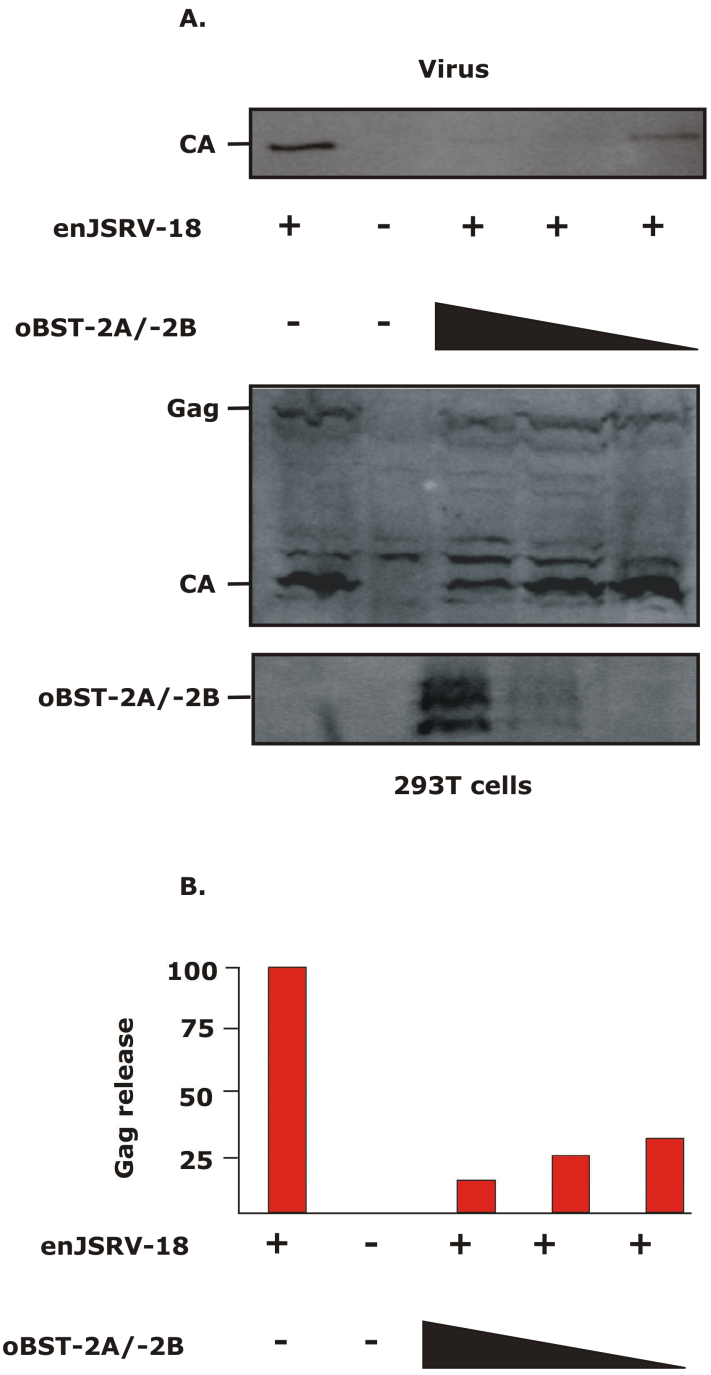


Figure 21. enJSRV-18 viral exit is blocked by oBST-2A/ -2B.

(A) Cells were transfected in 10cm diameter Petri dishes with 1μg of a plasmid expressing enJSRV-18 and a gradient of HA tagged expression plasmids for oBST-2A and -2B ranging from 0.1- 1.4 μg. A ratio of 3:1 for oBST-2A/ -2B respectively was transfected to reflect the expression pattern previously identified in vivo (Arnaud, Black et al. 2010). Blots were incubated with the appropriate antisera, indicated in each panel. (B) Quantification of western blotting of concentrated viral particles from supernatants of 293T co-transfected with expression plasmids for enJSRV-18 and oBST-2A/ -2B. Viral particle release was quantified, and values represent arbitrary units relative to the values of each virus transfected in the absence of BST-2 (which was assigned a value of 100).

3.3.2 oBST-2A and -2B proteins have different glycosylation patterns

Next, we compared the structural features between the ovine BST-2 paralogs and human BST-2. oBST-2A retains many of the structural features typical of the BST-2 proteins including a predicted N-terminal transmembrane region, a C-terminal coiled coil domain, a GPI-Signal Peptide (SP, for cleavage and GPI anchor addition within the ER), as well as N-linked glycosylation sites similarly to hBST-2. In contrast, oBST-2B possesses only an N-terminal transmembrane region and a C-terminal coiled coil region like oBST-2A and hBST-2, and does not appear to contain a GPI-SP. In addition, oBST-2B appears to contain a putative ER retention motif at the C-terminal. Interestingly, no glycosylation sites are predicted in oBST-2B (Figure 22).

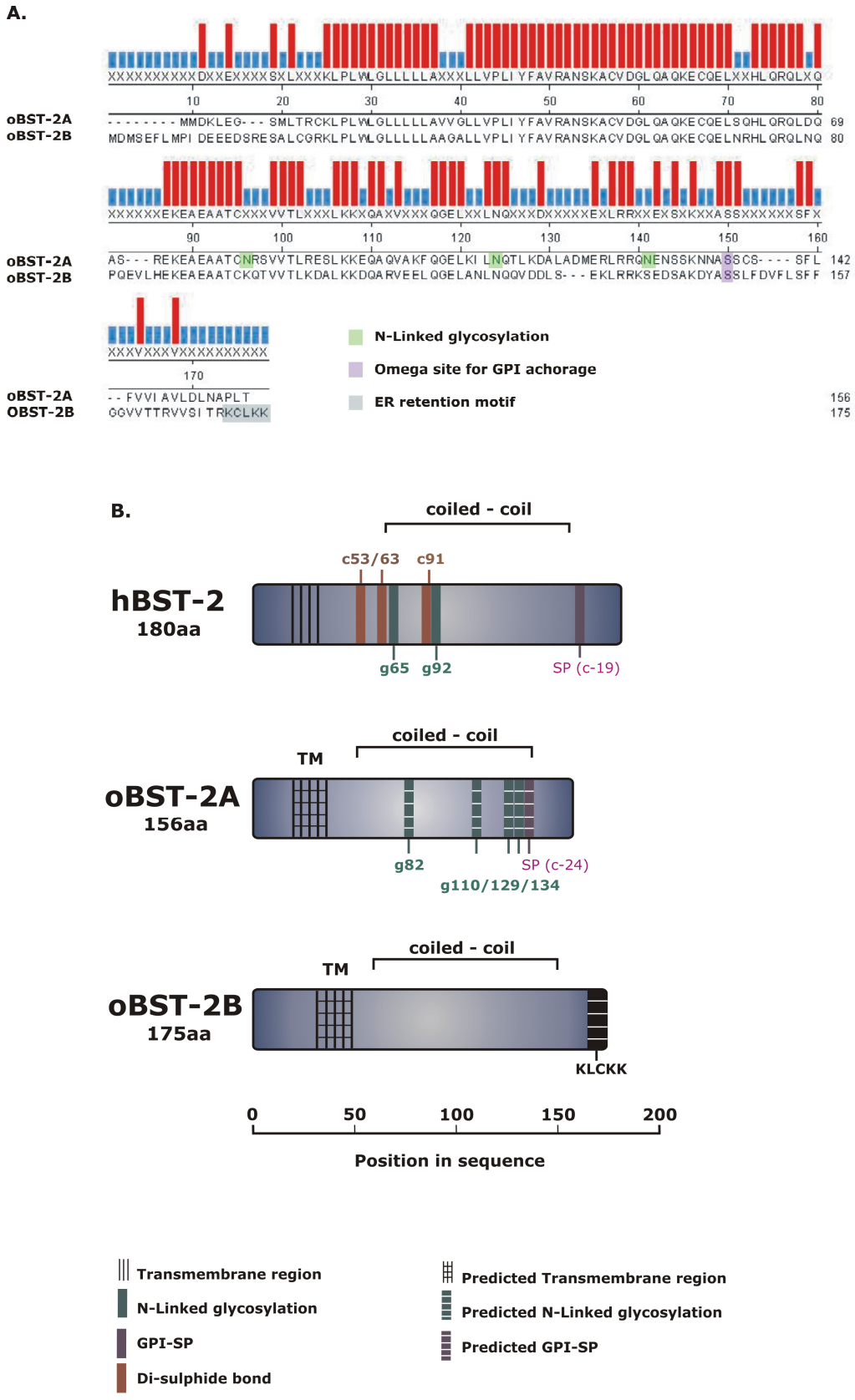


Figure 22. The main features of the BST-2 proteins.

(A) A sequence alignment of oBST-2A/-2B where X depicts non conserved residues. oBST-2B does not contain N-linked glycosylation sites and lacks a GPI-SP yet maintains the omega site for GPI anchorage. (B) A schematic diagram illustrating the main structural features and post-translational modification of hBST-2 and those predicted for oBST-2A/ -2B.

We next experimentally addressed whether the ovine BST-2 paralogs possess a different glycosylation pattern as suggested by the lack of predicted N-glycosylation sites in the latter. Previously, it was observed that oBST-2A and -2B display a different migration pattern by western blotting (Arnaud, Black et al. 2010). oBST-2A appears as a triple band whilst oBST-2B shows a single band (Figure 23).

To establish whether oBST-2A and -2B are glycosylated we transiently transfected 293T cells with expression plasmids for oBST-2A/ -2B (tagged with an HA epitope) and cell lysates were harvested in 1% NP-40 prior to incubation in the presence or absence of PNGaseF (peptide N-glycosidase F), an amidase that removes carbohydrate side chains from N-linked glycosylated proteins. Following overnight incubation, proteins were separated by SDS-PAGE and analysed by western blotting, using an anti-HA antibody for oBST-2A/ -2B detection. The migration patterns of oBST-2A/ -2B in the presence and absence of PNGaseF confirmed a different glycosylation pattern. We found that oBST-2A migrated as a triple band and was reduced to a single band in the presence of PNGaseF, confirming that the observed isoforms were a result of glycosylation as reported for other orthologs (Kupzig, Korolchuk et al. 2003) (Figure 23). In contrast, we observed no difference in the migration pattern of oBST-2B following PNGaseF treatment. Therefore, these data confirm the hypothesis that oBST-2A is a glycosylated protein whilst oBST-2B remains unglycosylated.

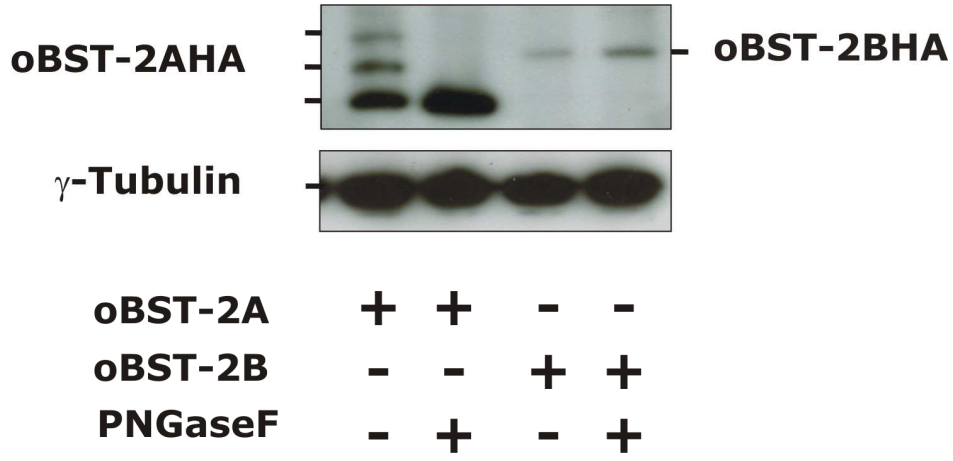


Figure 23. Ovine Bst-2 paralogs show a different glycosylation pattern.

293T cells were transiently transfected with expression plasmids for oBST-2A or -2B. Cell lysates were harvested and incubated in the presence and absence of PNGaseF overnight. Proteins were then separated by SDS-PAGE and visualized by western blotting. Migration patterns indicate that oBST-2A is a glycosylated protein as it is reduced to a single band by PNGaseF. oBST-2B appears to be unaffected by PNGaseF treatment. γ-tubulin was used as a sample quality and loading control.

3.3.3 The oBST-2A and -2B paralogs display a different intracellular localisation

The presence of BST-2 at the plasma membrane is a key factor for the antiviral restriction mechanism displayed by this protein (Neil, Zang et al. 2008; Van Damme, Goff et al. 2008). Although GPI anchorage is a determinant of protein localisation to the lipid rafts of the plasma membrane, we predicted that only oBST-2A has a functional GPI-SP (Figure 22).

We next investigated the intracellular localisation of both oBST-2A and -2B to assess the importance of GPI anchorage for oBST-2. Experiments were performed in sheep CPT-tert cells transiently transfected with oBST-2A or -2B expression plasmids (Arnaud, Black et al. 2010) and analysed by confocal microscopy.

The detection of the BST-2 paralogs with an HA antibody revealed two different staining patterns: (i) a "dispersed", broad based internal and plasma membrane localisation and (ii) a "concentrated" perinuclear staining pattern (Figure 24 (A)).

We noticed that oBST-2A existed primarily as a dispersed localisation whilst in contrast oBST-2B appeared mainly as a significantly concentrated, perinuclear form. We quantified the data by randomly counting the oBST-2A/ -2B staining pattern in 100 cells in two independent experiments (Figure 24 (B)). The results were very consistent in both experiments with more than 90% of the cells transfected with oBST-2A displaying a dispersed phenotype while 90% of the cells transfected with oBST-2B displayed a concentrated phenotype (Figure 24, (B)).

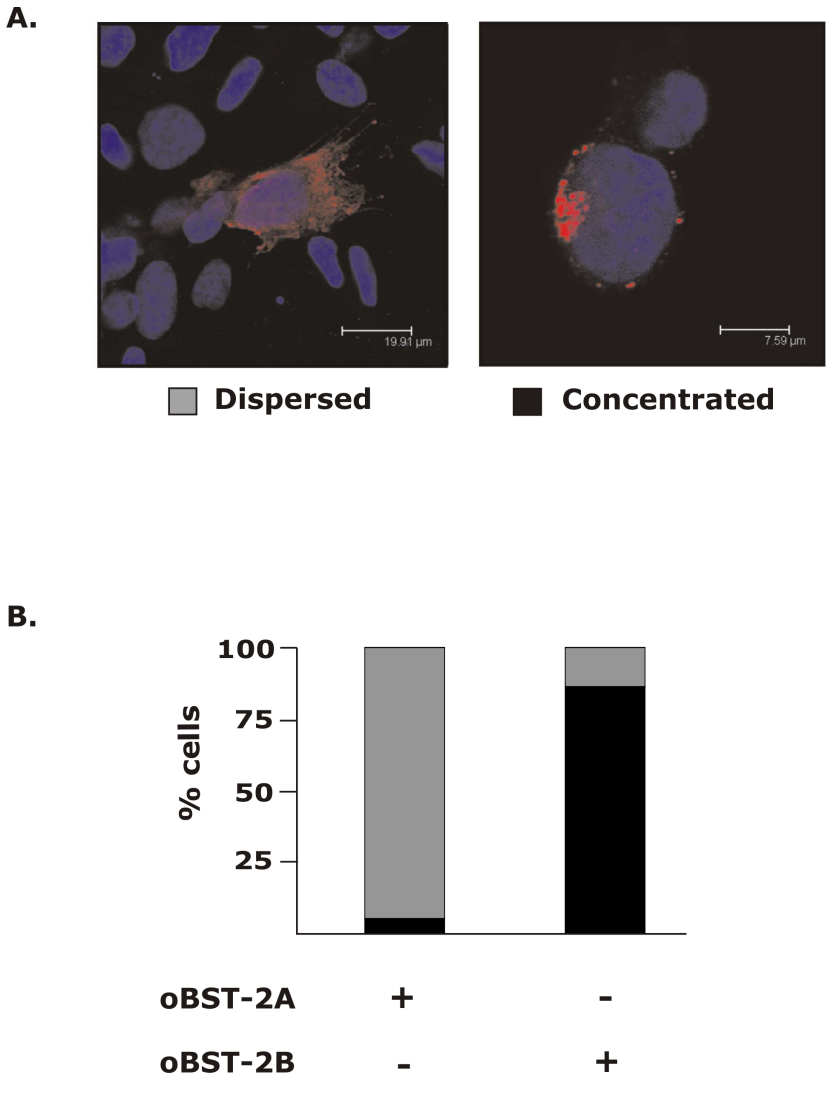


Figure 24. Ovine BST-2 paralogs display a different intracellular localisation.

(A) Confocal microscopy of oBST-2A / -2B in CPT-tert cells transiently transfected with either oBST-2A or -2B expressing plasmids display two different staining patterns: (i) dispersed within the cytoplasm and cell membrane and (ii) concentrated in a perinuclear region. (B) Data were quantified by counting the staining phenotype of 100 cells in random fields. More than 90% of oBST-2A positive cells have a significant dispersed localisation whilst oBST-2B transfected cells display mostly a concentrated, perinuclear association.

3.3.4 oBST-2B has remnants of a once functional GPI anchorage

GPI anchorage ensures protein incorporation within the lipid rafts of the plasma membrane by post translational modification within the ER involving protein cleavage and addition of a GPI anchor at the omega site. Sequence analyses show that oBST-2A has a predicted GPI-SP. oBST-2B lacks a GPI-SP but it has a conserved serine residue. This residue, in the presence of a functional signal peptide, would constitute the 'omega' cleavage site (ω) similar to oBST-2A where cleavage and addition of the GPI anchor would typically occur. To confirm if the presence of a serine omega cleavage site is indicative of a functional site for GPI anchorage addition, we designed an oBST-2B mutant (termed oBST-2BGPIc-28) possessing a premature stop codon in place of the second serine following the omega site (Figure 25). We predicted that insertion of a stop codon at this site would mimic cleavage that would ordinarily occur in the presence of a functional GPI-SP within the ER facilitating GPI anchor addition and consequently modify the oBST-2B protein trafficking patterns.

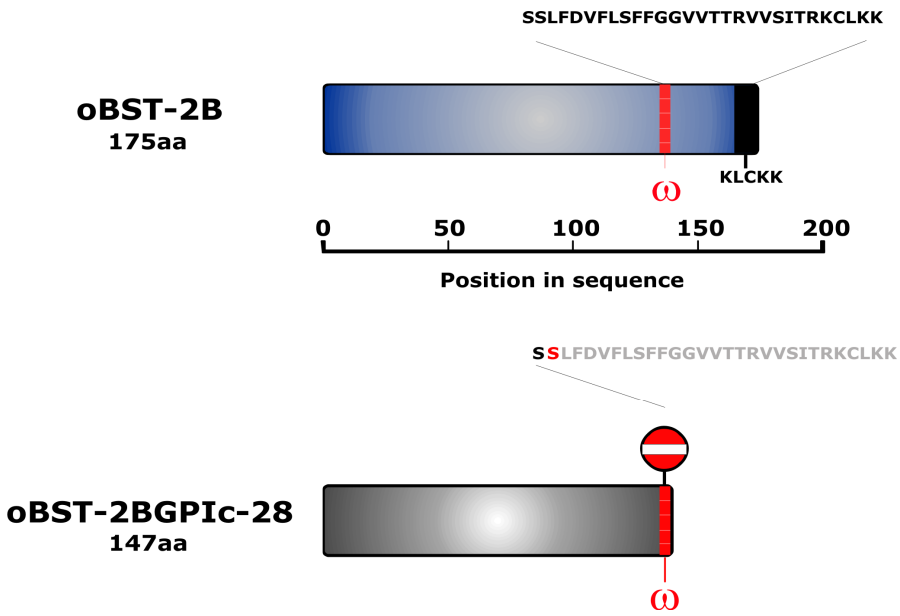


Figure 25. Schematic diagram of the oBST-2B truncated mutant where a stop codon was inserted at ω site .

In order to assess the impact of introducing the premature stop codon, we first transfected CPT-tert cells with oBST-2B (control) or oBST-2BGPIc-28 expression plasmids. Subsequently, we quantified the phenotype of oBST-2B localisation (or the truncation mutant) by confocal microscopy as either concentrated or dispersed (Figure 26A). We found that oBST-2BGPIc-28 had a significant dispersed localisation in comparison to the concentrated perinuclear localisation characteristic of oBST-2B (Figure 26 (B)).

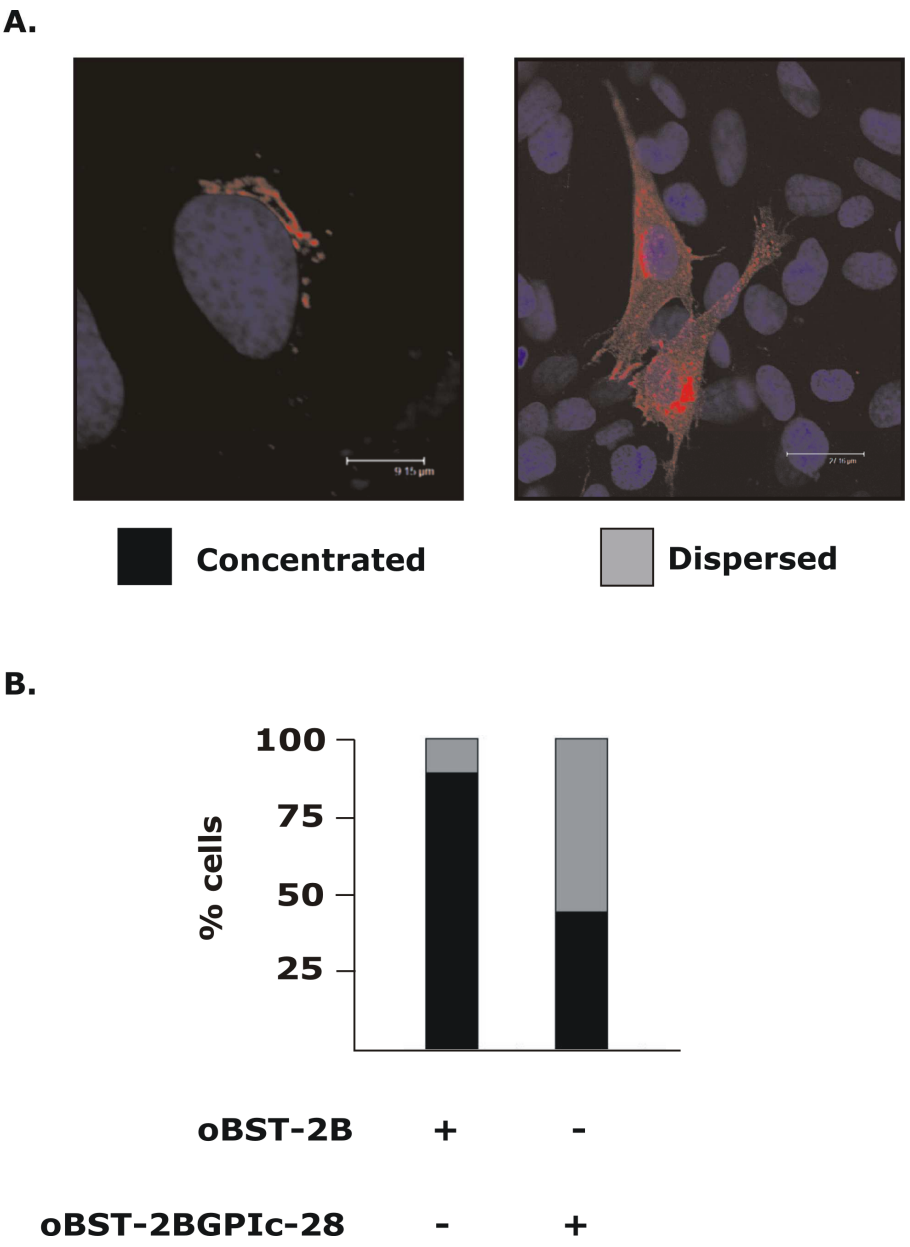


Figure 26. Ovine BST-2GPIc-28 displays an intracellular localisation different to that of oBST-2B.

(A) Confocal microscopy of CPT-tert cells transiently transfected with oBST-2B/oBST-2BGPIc-28 revealed two different staining patterns: (i) dispersed within the cytoplasm and cell membrane and (ii) concentrated in a perinuclear region. (B) Data were quantified by counting the staining phenotype of 100 cells in random fields. oBST-2B transfected cells display mostly a concentrated, perinuclear association whilst oBST-2BGPIc-28 is found as both dispersed and concentrated in roughly equal amounts.

We found that oBST-2GPIc-28 displays an intracellular localisation different to that of oBST-2B, switching from a concentrated to a dispersed phenotype (Figure 26). During the course of these experiments, we also found evidence suggesting that oBST-2BGPIc-28 was able to accumulate at the plasma membrane (Figure 27 (a) and (d)). Thus, oBST-2BGPIc-28 shows a similar phenotype to oBST-2A.

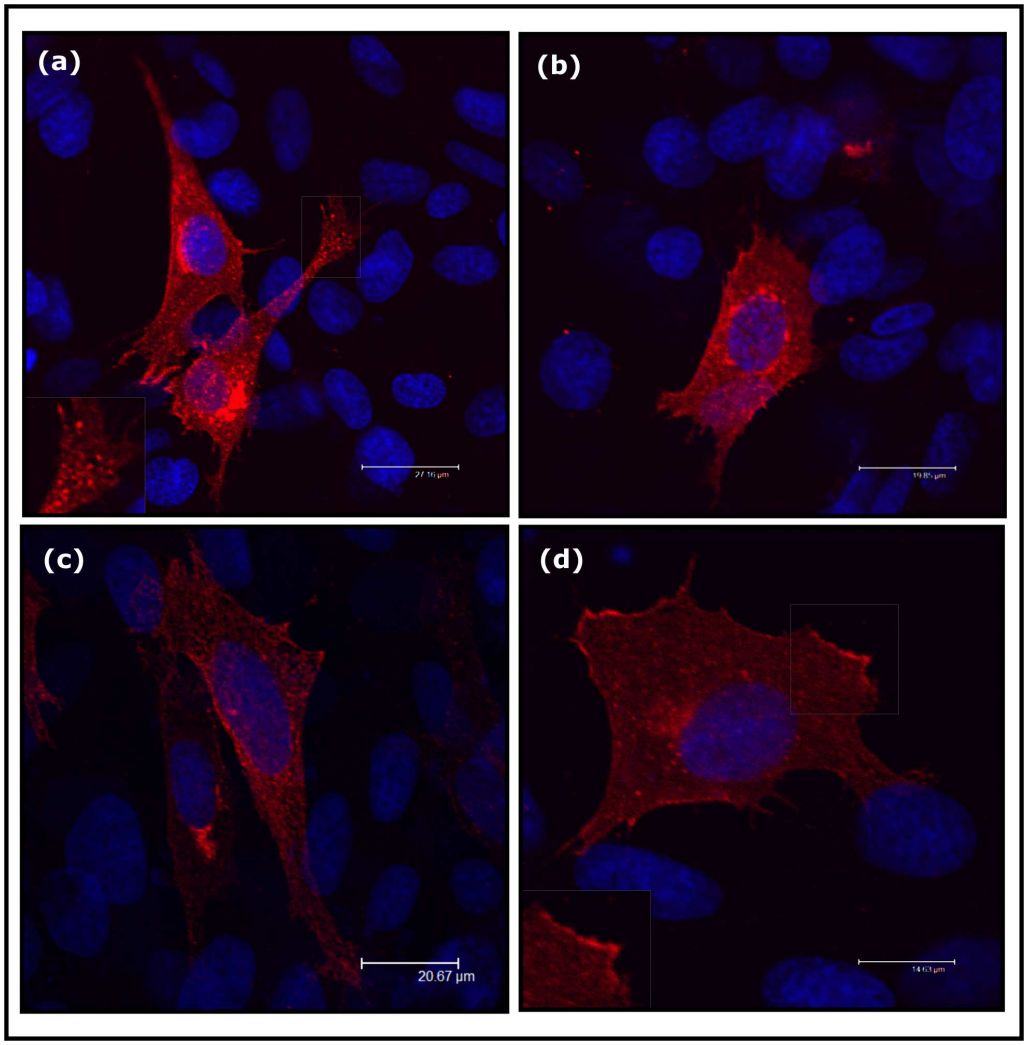


Figure 27. oBST-2B truncation mutants display a distinct localization pattern from oBST-2B

CPT-tert cells transfected with oBST-2BGPIc-28 were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against the HA epitope and appropriate secondary conjugated antibodies. In addition to the 'wild-type' oBST-2B concentrated perinuclear distribution, oBST-2BGPIc-28 also shows a dispersed intracellular localisation (B and C) with plasma membrane association noted (A and D).

3.3.5 oBST-2B has an ER retention motif

Above, we showed that truncation of the last 28 amino acid residues of oBST-2B result in its re-distribution within the cell and the switch from a concentrated to a dispersed phenotype. Perinuclear localization of oBST-2B might be due to a dysfunctional GPI-SP and/or to a predicted ER retention motif (KLCKK) that is present in the C terminal region. In order to test this point we made multiple truncated mutants in oBST-2B after the predicted omega site, as illustrated schematically in Figure 28.

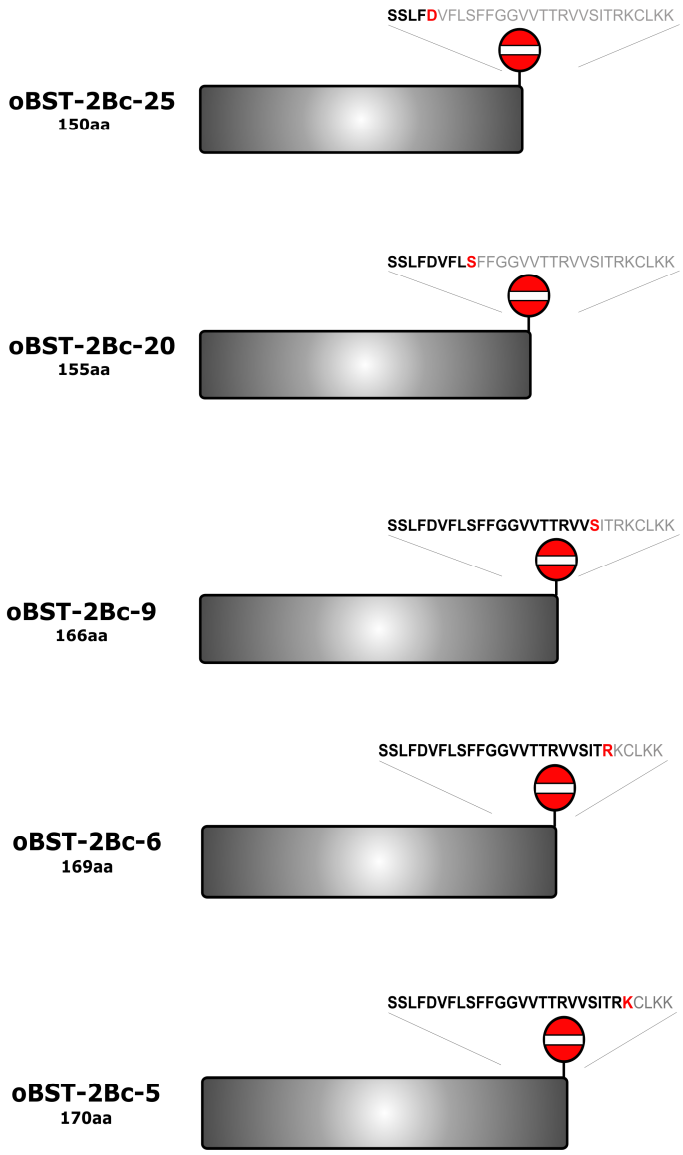
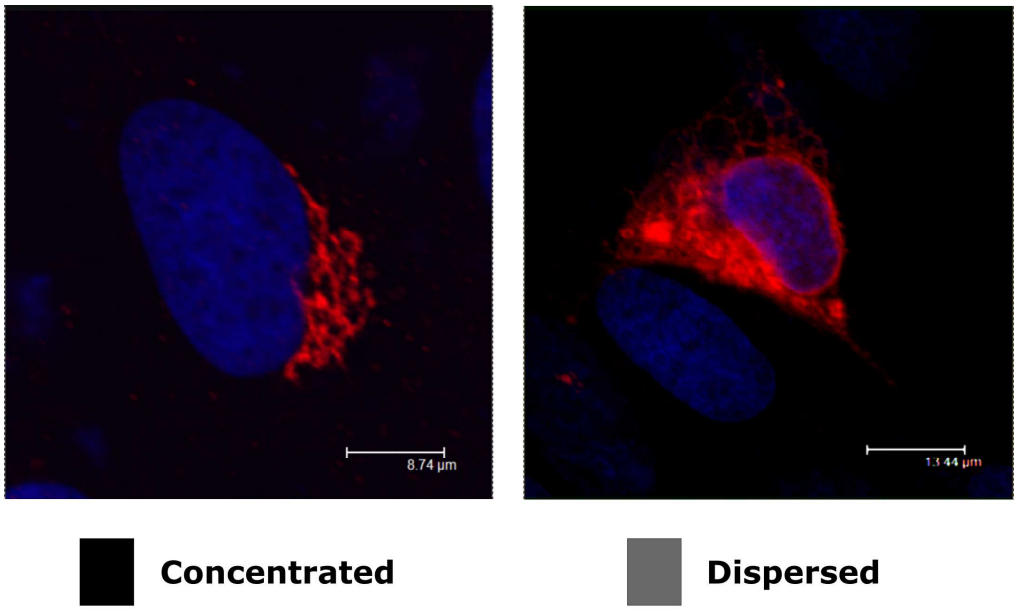


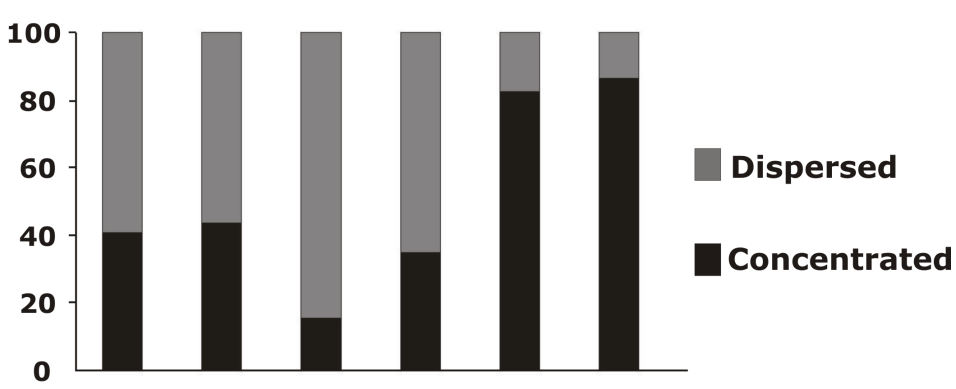
Figure 28. Schematic diagram of the oBST-2B C truncation mutants generated in this study.

To assess the influence of the differing truncations upon oBST-2B distribution, we transfected each mutant into CPT-tert cells and assessed their intracellular localisation as described above. We found that all of the C-terminal truncated mutants, with the exception of oBST-2Bc-5, had a dispersed intracellular localisation whereas oBST-2Bc-5 maintained the localisation typical of the native oBST-2B, predominately concentrated and perinuclear (Figure 29).

A.



B.



oBST-2Bc-25	+	-	-	-	-	-
oBST-2Bc-20	-	+	-	-	-	-
oBST-2Bc-9	-	-	+	-	-	-
oBST-2Bc-6	-	-	-	+	-	-
oBST-2Bc-5	-	-	-	-	+	-
oBST-2B	-	-	-	-	-	+

Figure 29. Ovine BST-2 C truncation mutants display a different intracellular localisation from oBST-2B.

Confocal microscopy of CPT-tert transiently transfected with oBST -2B and C terminal mutants display two different staining patterns: A(i) dispersed within the cytoplasm and cell membrane and A(ii) concentrated in a perinuclear region. (B) Data were quantified by counting the staining phenotype of 100 cells in random fields. oBST-2Bc-25, 20, 9 and 6 positive cells have a significant dispersed localisation whilst oBST-2Bc-5 transfected cells display mostly a concentrated, perinuclear association typical of the native oBST-2B protein.

Interestingly, we also observed that, although oBST-2Bc-5 (deleted of the KLCKK motif) maintains a predominantly perinuclear localisation, it appears to have a more punctuated distribution surrounding the nucleus (Figure 30 panels E and F) rather than the tightly concentrated mass characteristic of oBST-2B (Figure 30, panels A and B). Nevertheless, oBST-2Bc-5, like oBST-2B, could be found in a dispersed form (Figure 30 C and D). It was frequently observed that the punctuated distribution of oBST-2Bc-5 was observed as small individual spherical aggregates, O-rings, around the nucleus (Figure 30, panels G and H). We currently have no explanation as to why this occurs. We conclude that the KLCKK motif is functional and likely represents one of the K(X)KXX retention motifs previously described (Teasdale and Jackson 1996).

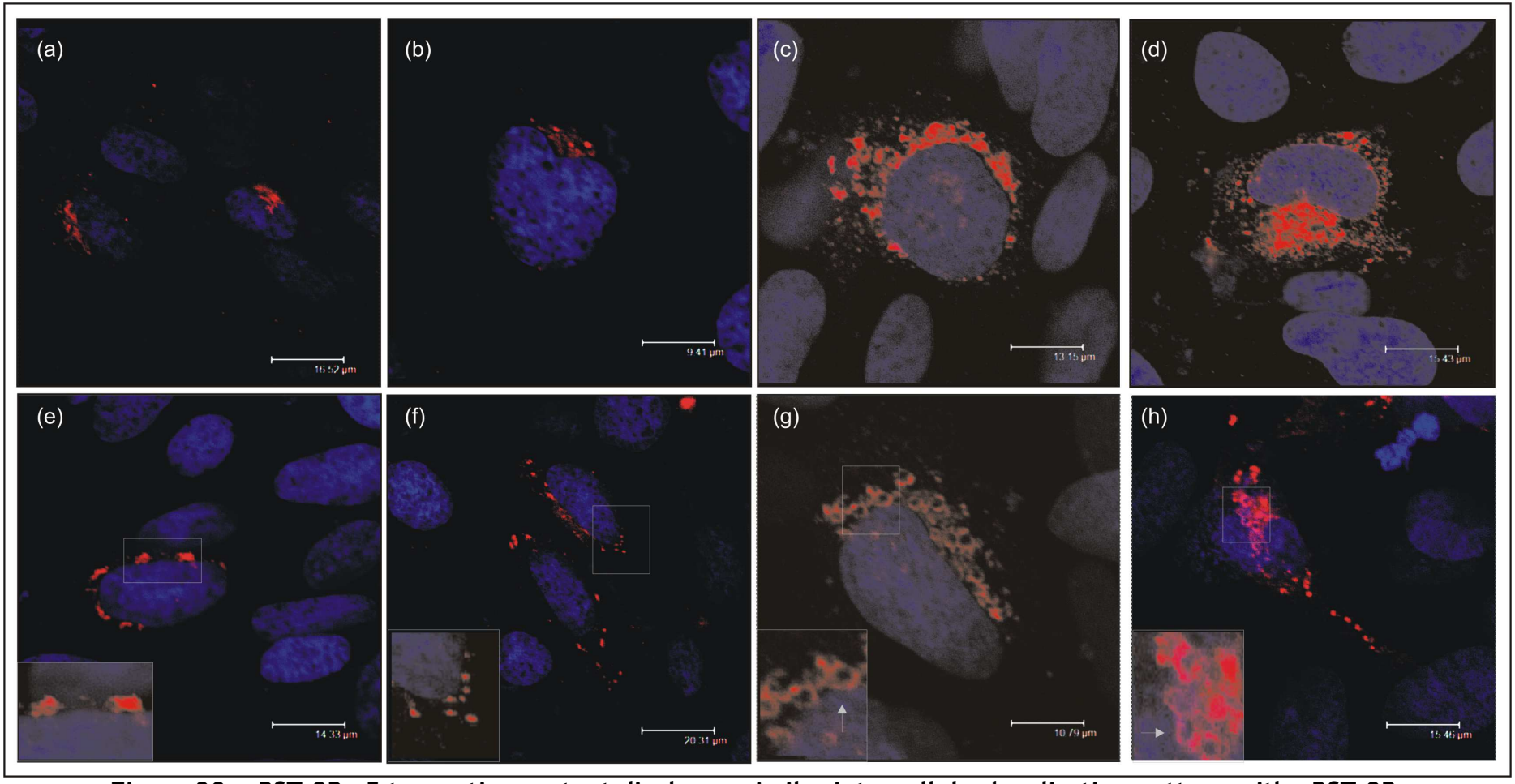


Figure 30. oBST-2Bc-5 truncation mutant displays a similar intracellular localisation pattern with oBST-2B.

Figure 30. oBST-2Bc-5 truncation mutant displays a similar intracellular localisation pattern with oBST-2B.

CP tert cells transfected oBST-2Bc-5 and oBST-2B were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against the HA epitopes and appropriate secondary conjugated antibodies. oBST-2Bc-5 shows a concentrated intracellular localisation (e and f) similar to oBST-2B (a and b) but with a more punctuate appearance around the nucleus. oBST-2Bc-5 is frequently observed with small circular aggregates, O rings (g and h). A small proportion of cells transfected with oBST-2Bc-5 show a dispersed localisation (c and d).

3.3.6 oBST-2B co-localises with Golgi based markers

We have established above that oBST-2B has no functional GPI-SP, it is not glycosylated and displays mostly a perinuclear concentrated intracellular distribution, primarily due to a KLCKK ER retention motif at the C-terminal of the protein. Next, using confocal microscopy we assessed whether oBST-2B localises in the Golgi using different cellular markers: p115, Giantin and TGN46; that localise in the cis, medial and trans-Golgi network respectively (Figure. 31, 32, 33, 34, 35, 36, 37).

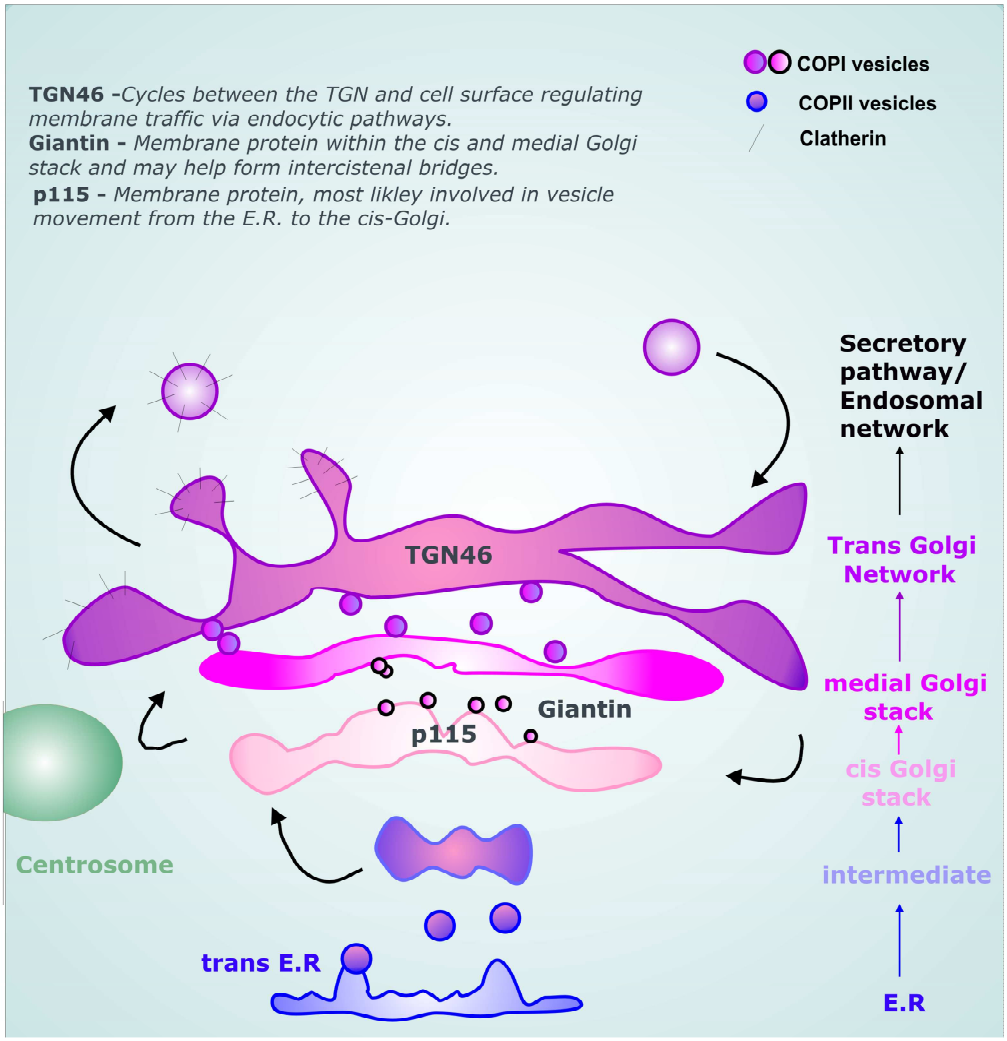


Figure 31. Schematic representation of the Golgi and the cellular markers used in this study.

We transfected CPT-tert cells with expression plasmids for either oBST-2A or -2B and 24h post-transfection cells were fixed and analysed by confocal microscopy using antibodies against the HA epitope (detecting oBST-2A and -2B) and p115 (Figures 32 and 33), Giantin (Figures 34 and 35) or TGN46 (Figures 36 and 37). The extent of co-localisation between oBST-2 and each of the Golgi markers indicated above was performed by calculating the Pearson's correlation coefficient using Image-Pro software (Media Cybernetics).

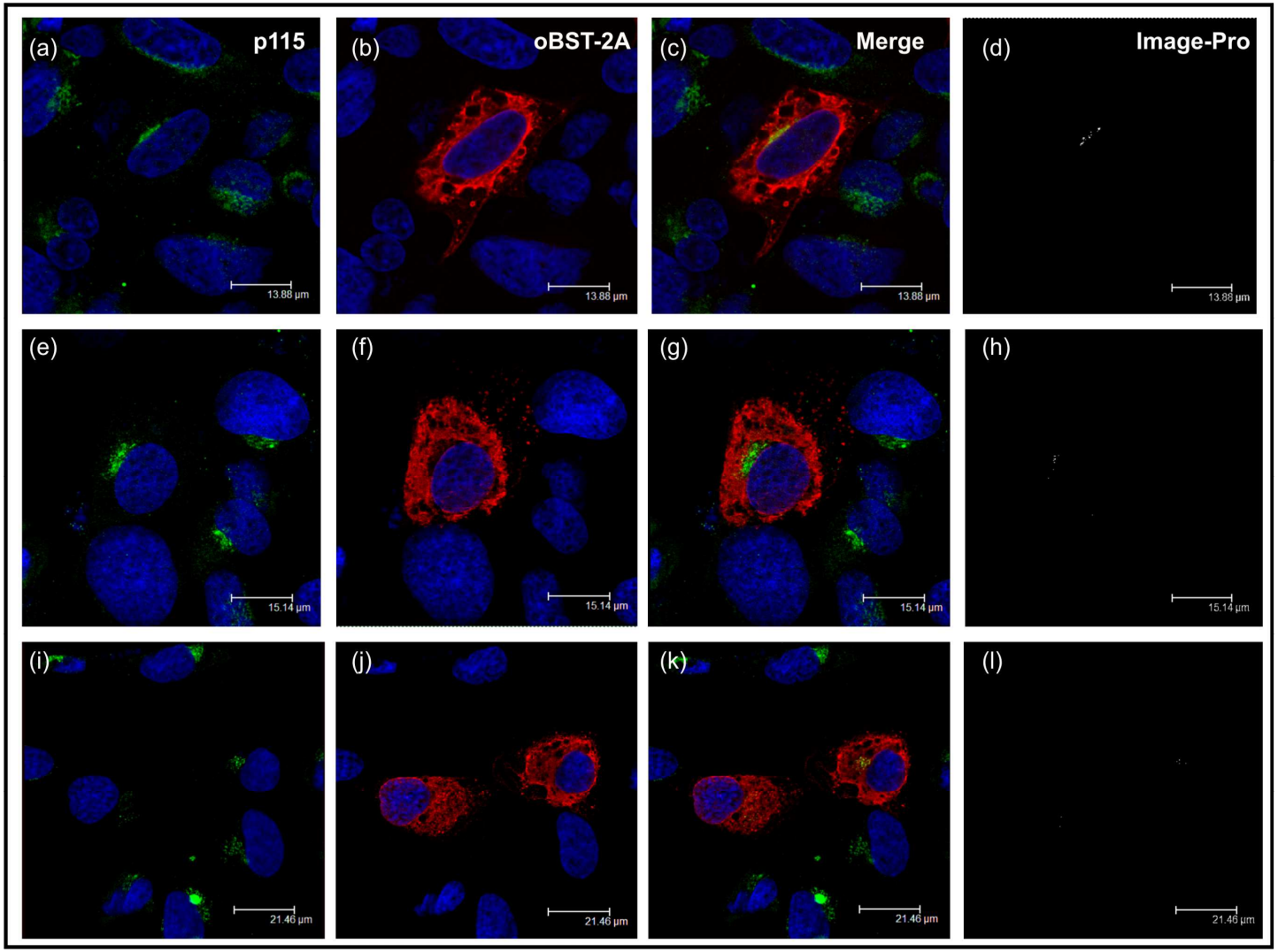


Figure 32. oBST-2A does not co-localise significantly with p115

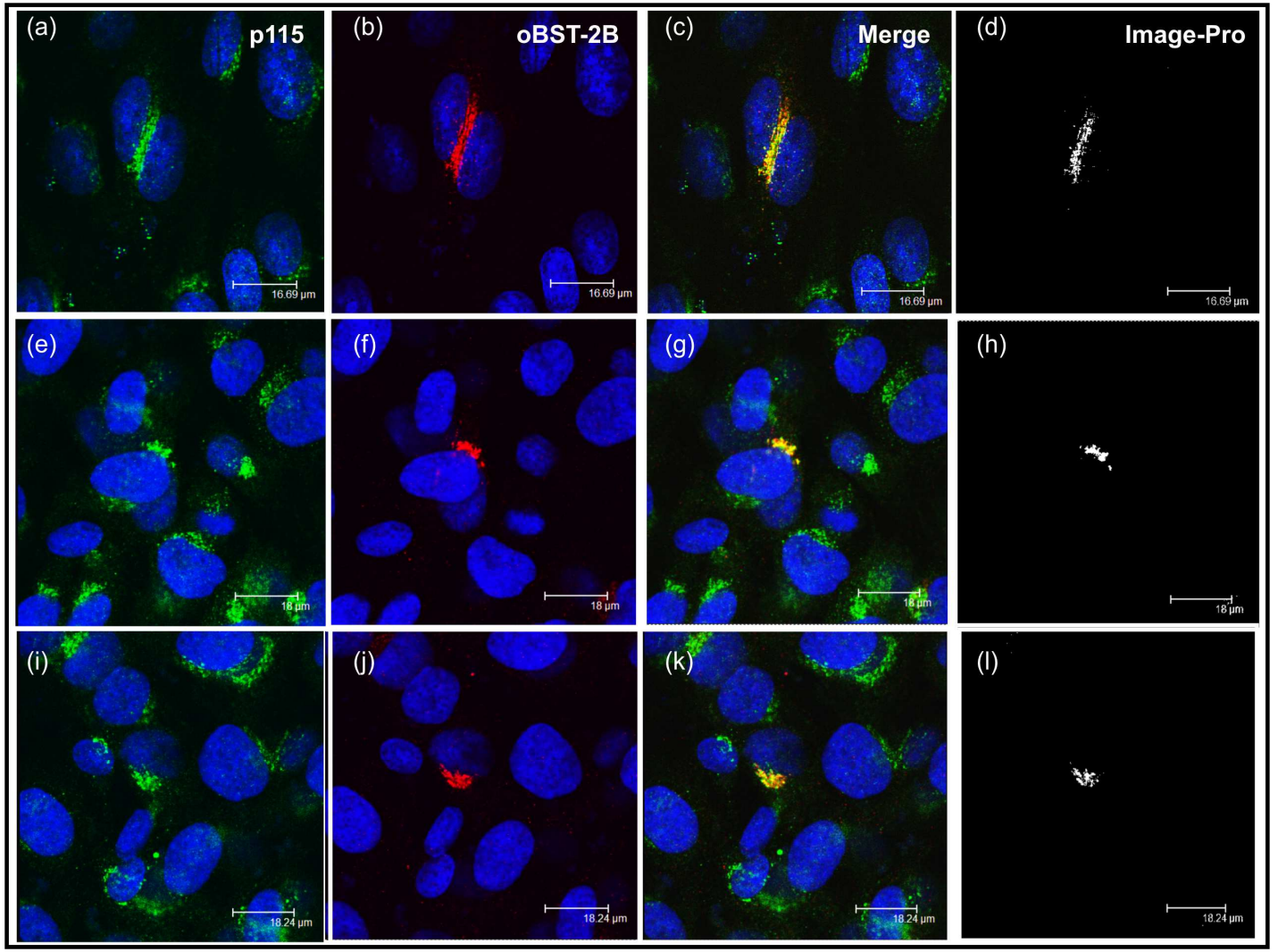


Figure 33. oBST-2B co-localises with p115

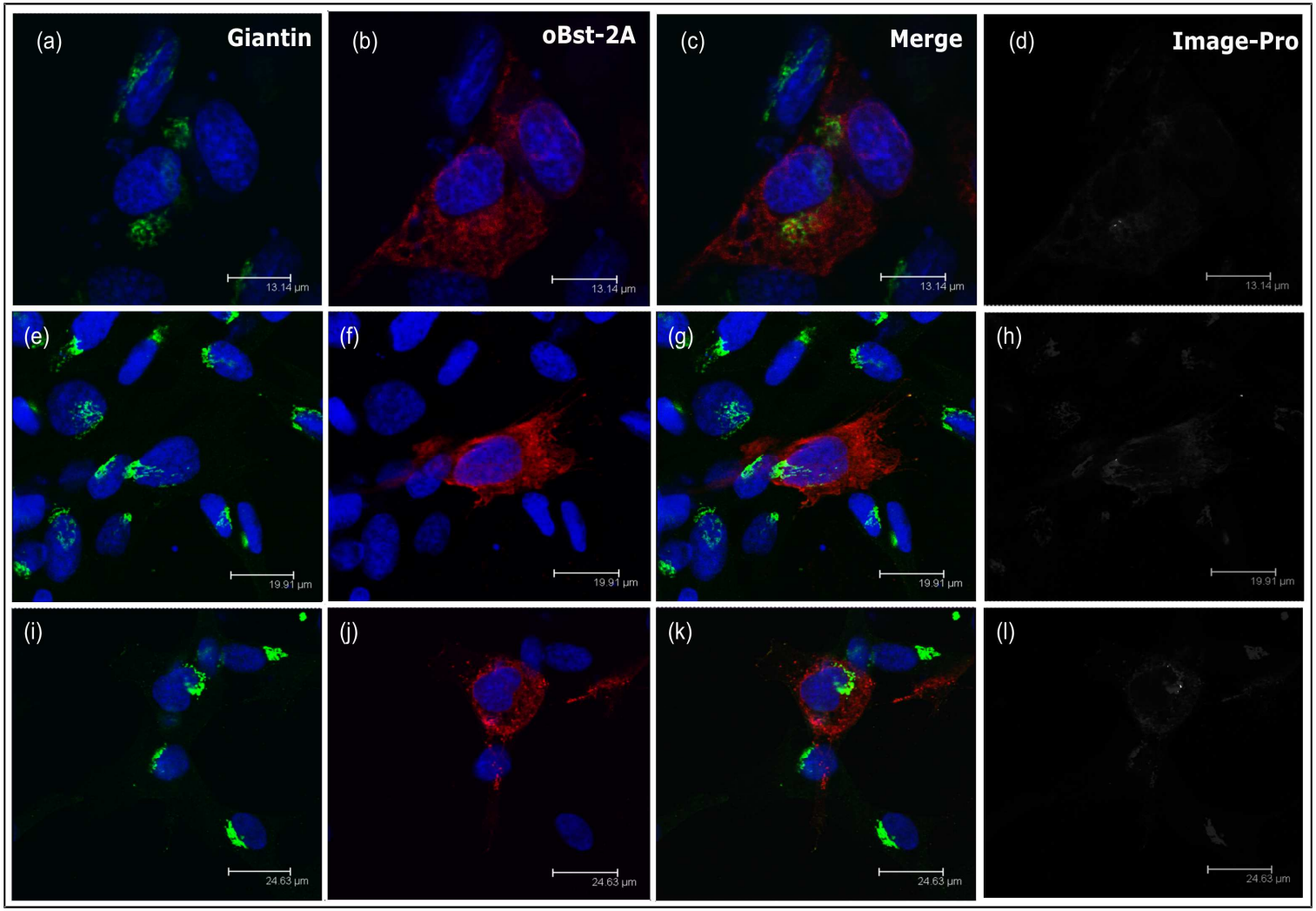


Figure 34. oBST-2A does not co-localise with Giantin

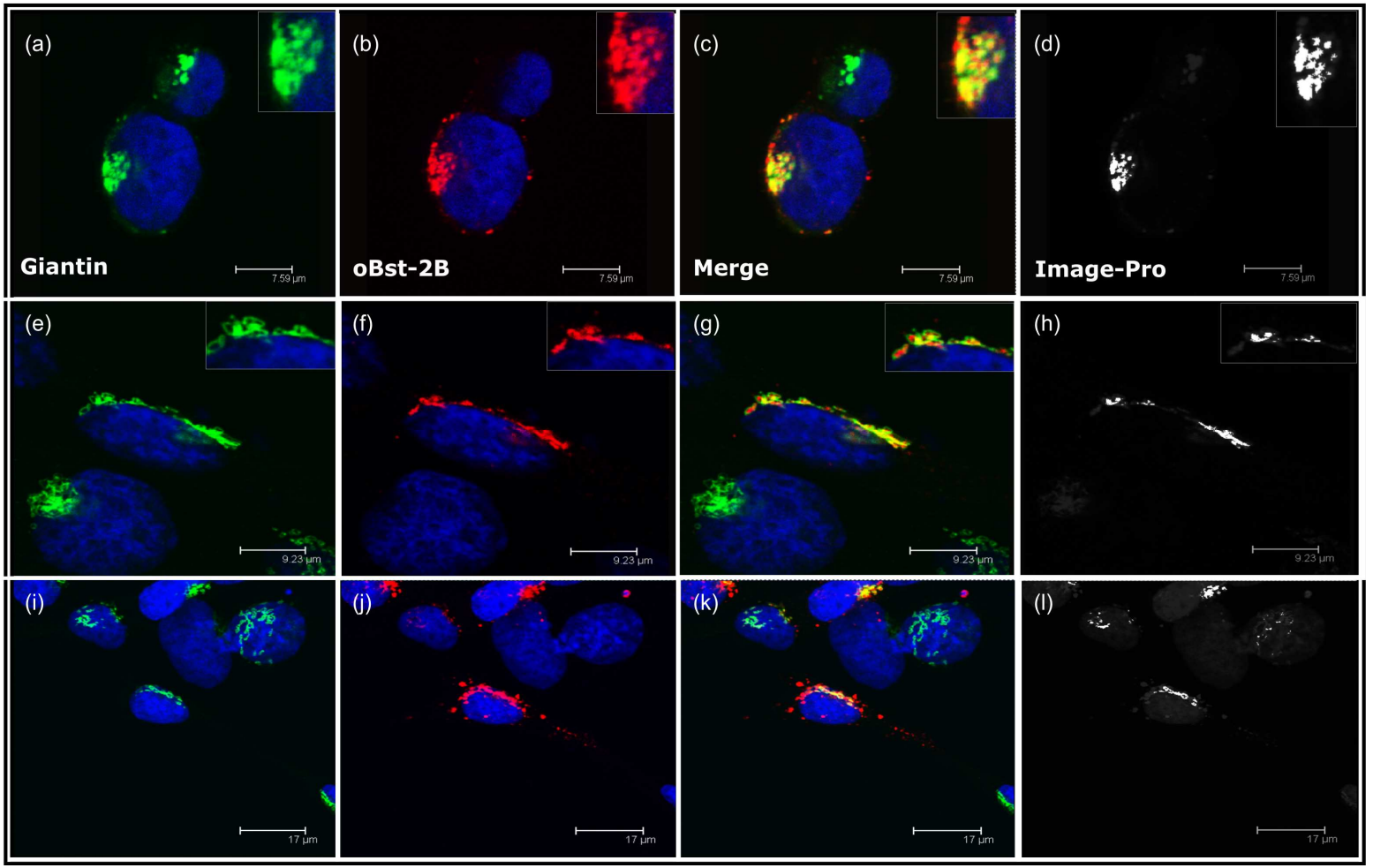


Figure 35. oBST-2B co-localises with Giantin

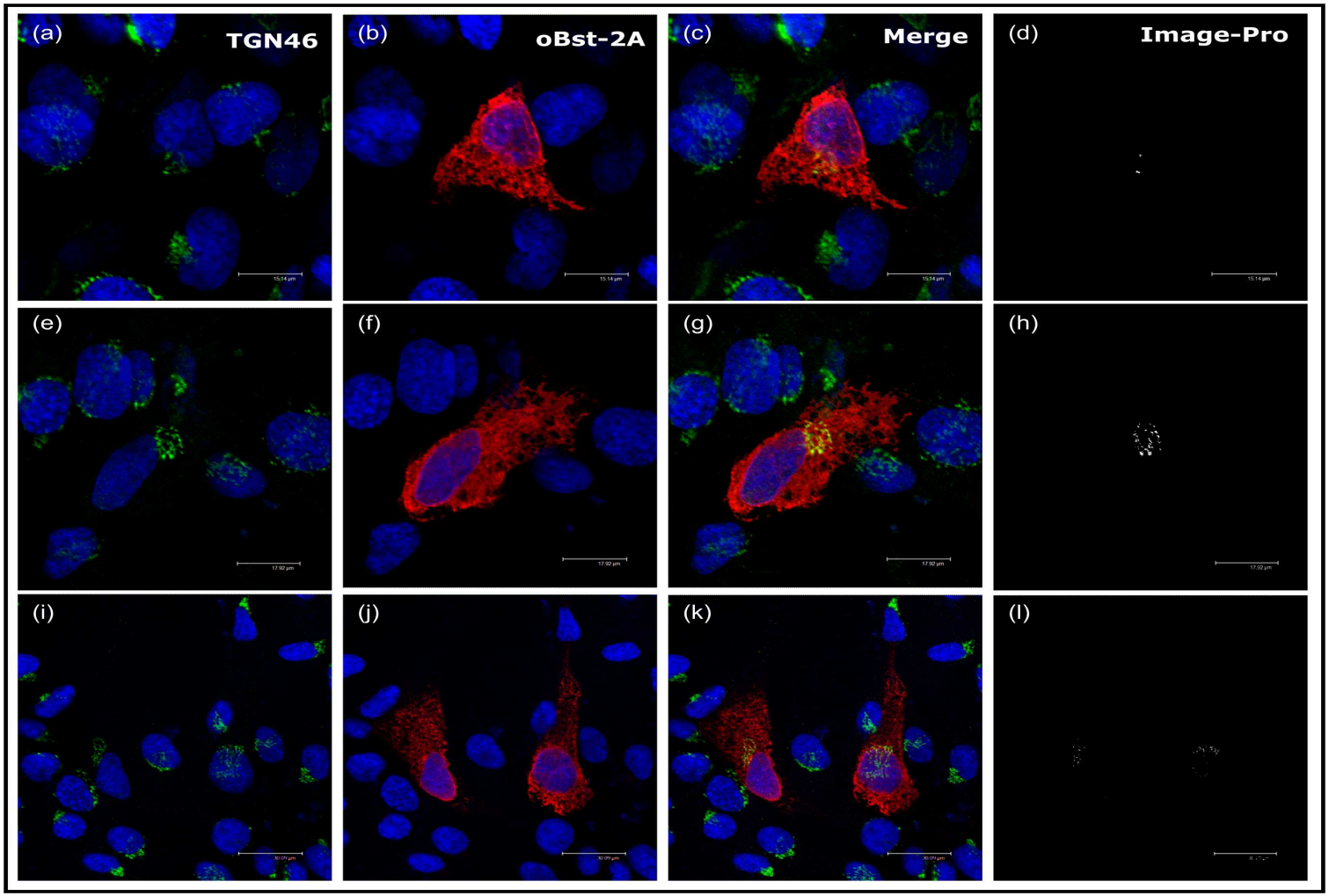


Figure 36. oBST-2A does not co-localise with TGN46

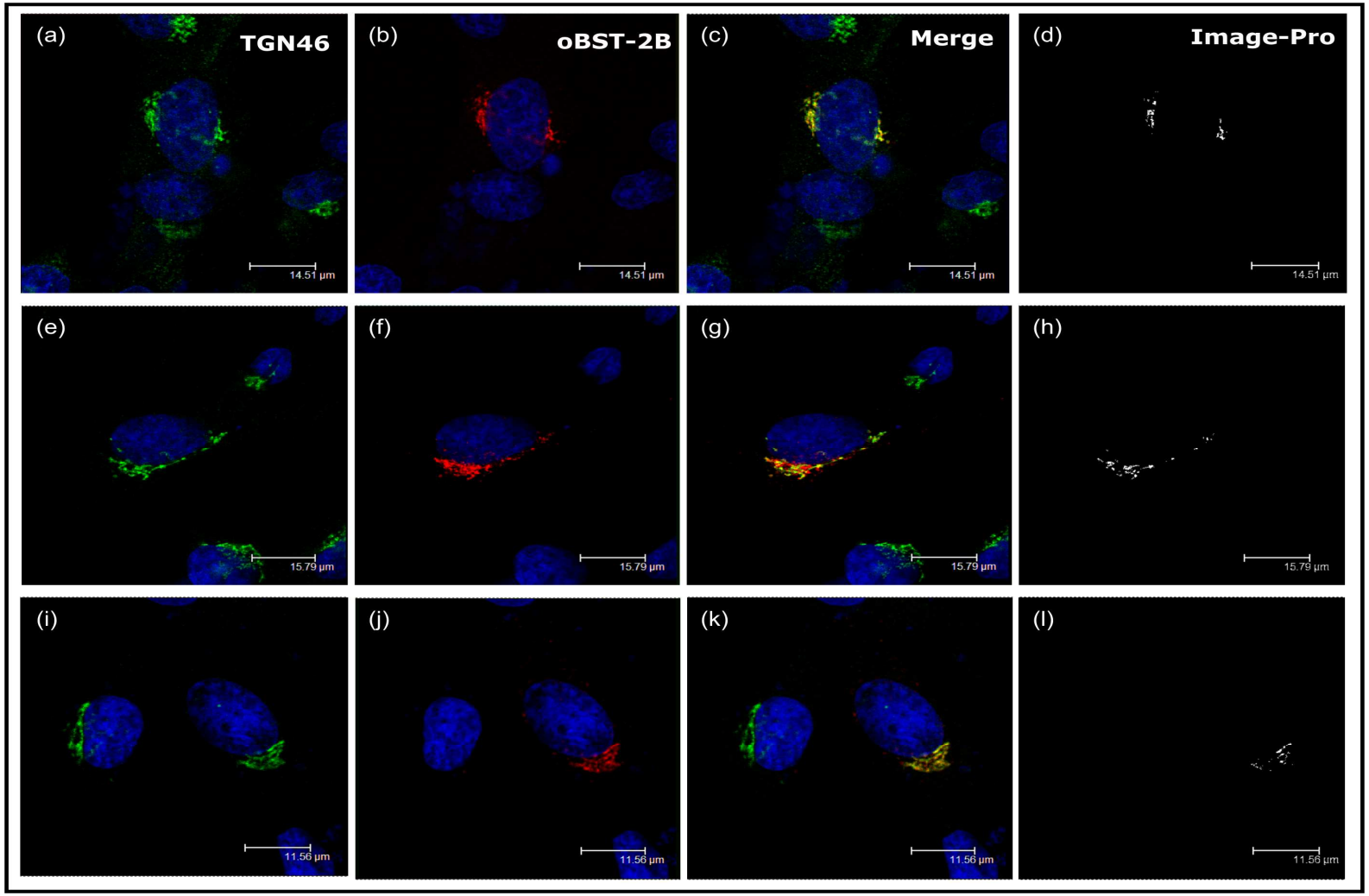


Figure 37. oBST-2B co-localises with TGN46

Figure 32. oBST-2A does not c-localise significantly with p115.

CPT-tert cells transfected with oBST-2A were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against Golgi based p115 (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and no significant colocalisation between p115 and oBST-2A was observed. Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) the absence of white areas indicates there is no significant colocalisation between oBST-2A and p115.

Figure 33. oBST-2B co-localises with p115.

CPT-tert cells transfected with oBST-2B were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against Golgi based p115 (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and the yellow areas indicate significant colocalisation between p115 and oBST-2B. Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) with white areas indicating significant association.

Figure 34 oBST-2A does not co-localise with Giantin.

CPT-tert cells transfected with oBST-2A were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against Golgi based Giantin (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and no significant colocalisation was observed between Giantin and oBST-2A.,Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) with the absence of white areas indicating there is no significant colocalisation between Giantin and oBST-2A.

Figure 35 oBST-2B co-localises with Giantin.

CP tert cells transfected with oBST-2B were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against Golgi based Giantin (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and yellow indicates areas of significant colocalisation between Giantin and oBST-2B. Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) with white indicating areas of significant association Giantin and oBST-2B.

Figure 36 oBST-2A does not co-localise with TGN46.

CP tert cells transfected with oBST-2A were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against TGN based TGN46 (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and no significant colocalisation was observed between TGN46 and oBST-2A. Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) the absence of white areas indicates no significant association

Figure 37 oBST-2B colocalises with TGN46

CP tert cells transfected with oBST-2B were fixed 18h after transfection and analyzed by confocal microscopy using antibodies against TGN based TGN46 (green- a, e and i) and the HA epitopes (red- b, f and j) and appropriate secondary conjugated antibodies. These images were merged (c, g and k) and yellow indicates areas of significant colocalisation between TGN46 and oBST-2B. Co-localisation can also be assessed visually for images with a greyscale merge of individual images (d, h and l) with white indicating areas of significant association.

Data are shown in Figure 38 as the average Pearson's correlation values obtained in 50 random images representative of cells expressing oBST-2A or -2B and incubated with antibodies towards each of the Golgi markers. The Pearson's correlation is a value ranging from -1 to 1, which in this case indicates the degree of co-localisation between the Golgi marker in question and oBST-2A/ -2B. We considered 0.5 as our threshold for significant association. oBST-2B, and not oBST-2A, displayed a significant degree of co-localisation with all three Golgi-based markers (Fig. 38).

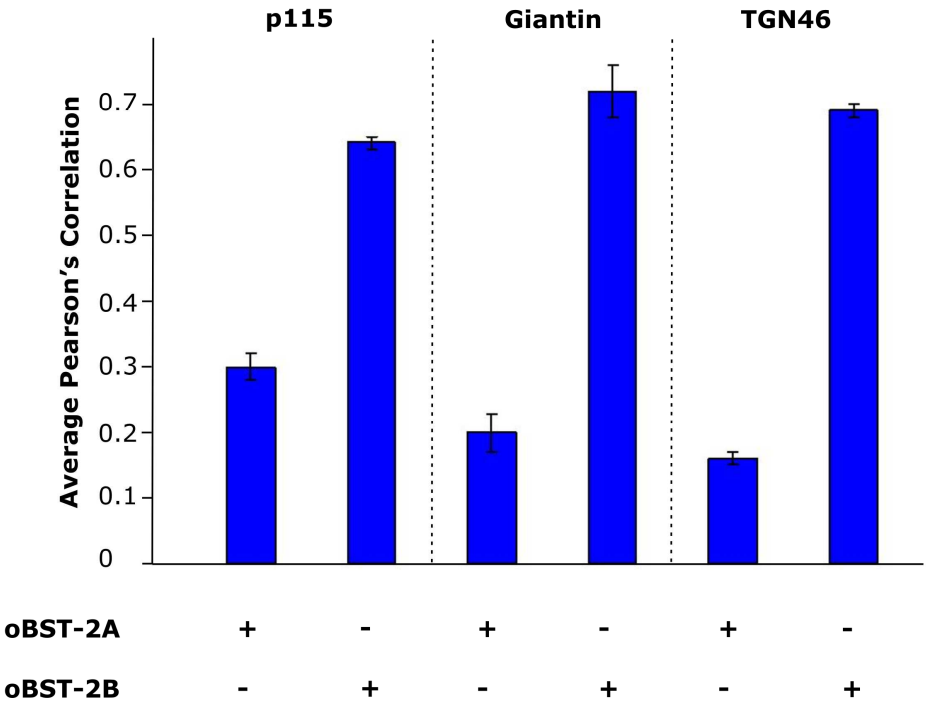


Figure 38. oBST-2B co-localises with Golgi based markers

This figure displays the quantification of the data shown in Figures 32-37. The values represent the average of the Pearson's correlation coefficient. Any values obtained above 0.5 were regarded as representing significant co-localisation.

Next, we used confocal microscopy to assess the localisation of oBST-2B in the presence or absence of Brefeldin-A, a drug which fragments the Golgi apparatus. We observed that oBST-2B behaved in a similar manner to Giantin in the presence of Brefeldin-A as its distribution pattern was redistributed from a predominantly concentrated form (Figure 39) to that of a more dispersed one (Figure 40) results are summarised in Figure 41. Collectively, the data obtained suggest that oBST-2B is a Golgi based protein.

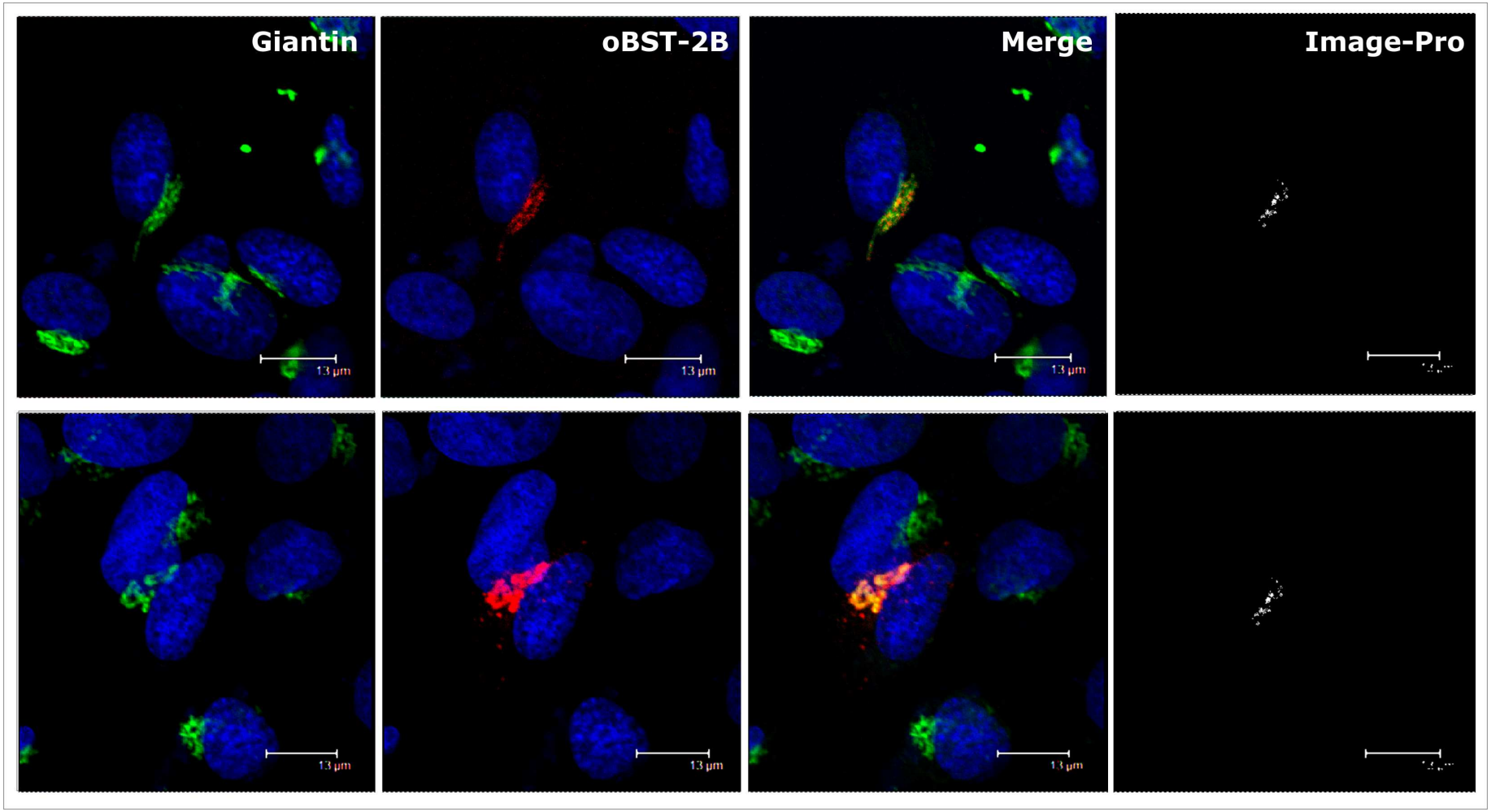


Figure 39. Giantin and oBST-2B have a concentrated, perinuclear distribution

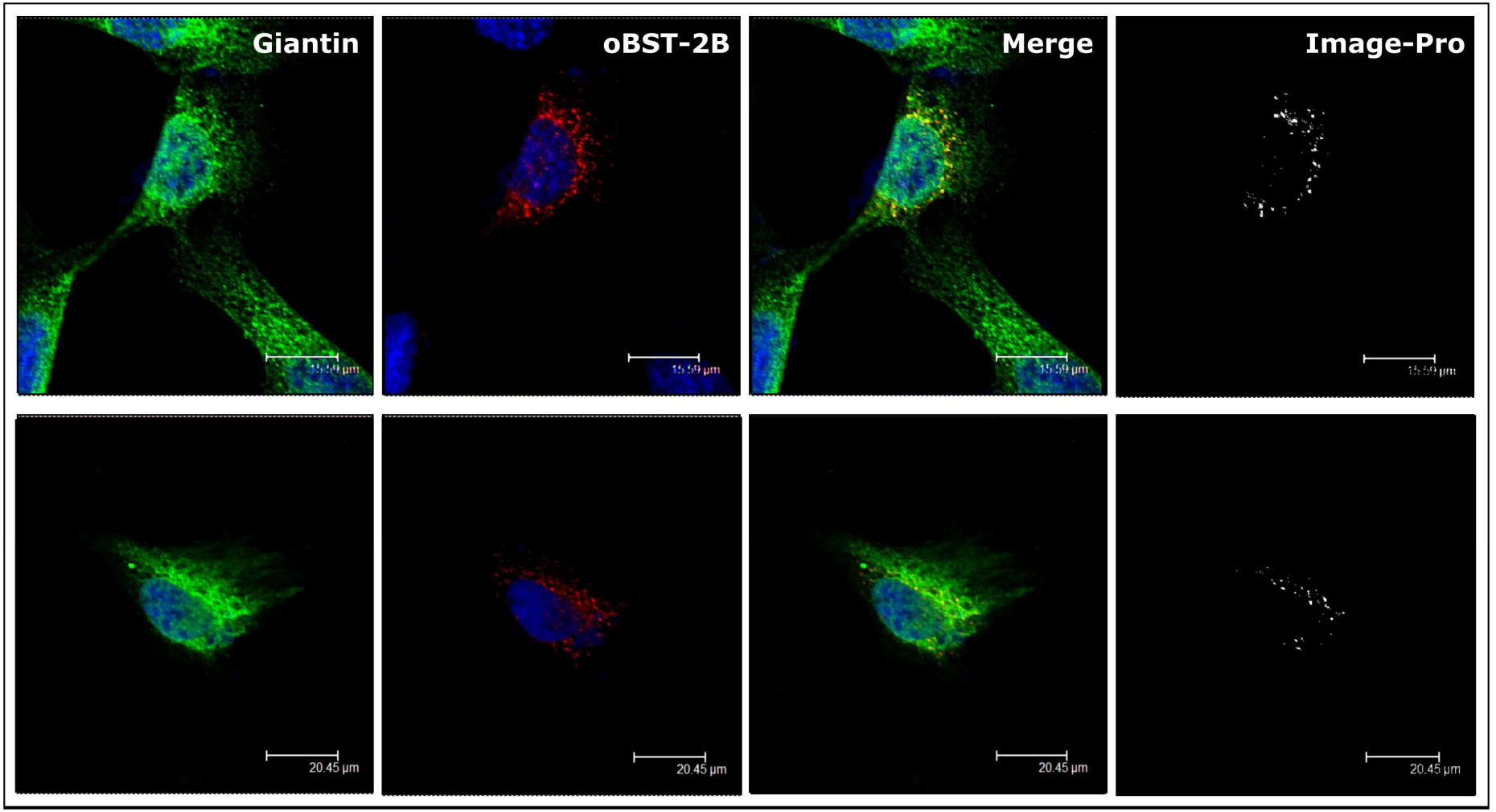


Figure 40. Giantin and oBST-2B localisation is altered by treatment with Brefeldin A

Figure 39. Giantin and oBST-2B have a concentrated, perinuclear distribution.

CPT-tert cells were transfected with the oBST-2B expression plasmid. The cells were fixed and analysed by confocal microscopy using antibodies towards the Giantin Golgi marker and HA epitope as indicated. These images were also merged. oBST-2B and Giantin display a concentrated localisation with a high degree of colocalisation as shown in the merge indicated by yellow and within the Image-Pro greyscale images indicated in white.

Figure 40. Giantin and oBST-2B localisation is altered by treatment with Brefeldin-A.

CPT-tert cells were transfected with either oBST-2A or -2B expression plasmids. Eighteen hours after transfection, the cells were treated in the presence and absence of 200ng/ml of Brefeldin-A for 90mins, fixed and analysed by confocal microscopy using antibodies towards the Giantin Golgi marker and HA epitope as indicated. These images were also merged. Both Giantin and oBST-2B localisation is altered by treatment with Brefeldin-A.

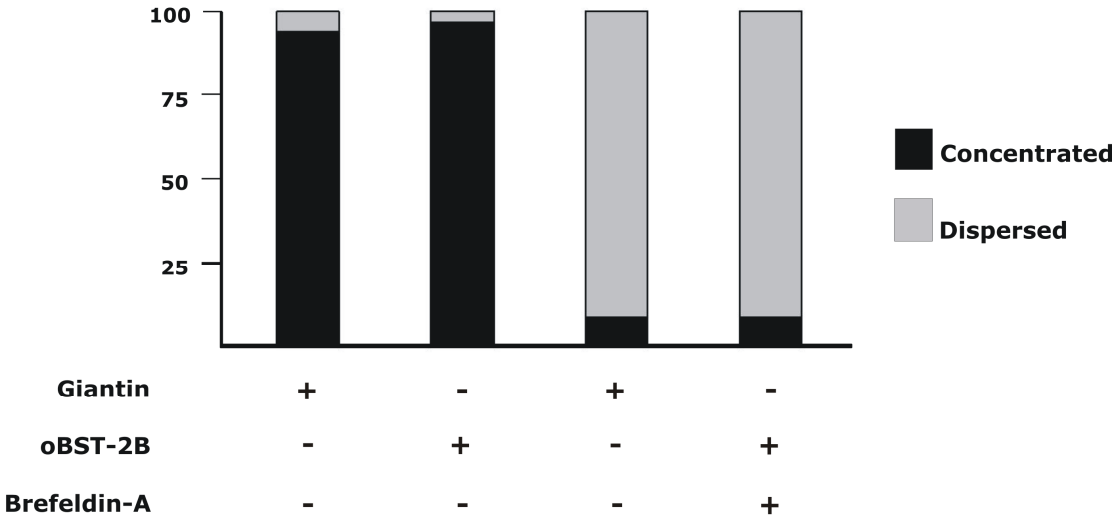


Figure 41. Brefeldin-A treatment leads to an intracellular redistribution of oBST-2B.

This figure displays the quantification data for the images shown in Figures 39 and 40. Data were quantified by counting the staining phenotype of 100 cells in random fields. Cells were grouped as (i) dispersed within the cytoplasm and cell membrane and (ii) concentrated in a perinuclear region. In the absence of Brefeldin-A both Giantin and oBST-2B have a predominantly concentrated localisation. Following treatment with 200ng/ml of Brefeldin-A for 90 mins post transfection the localisation of both Giantin and oBST-2B is altered to be predominantly dispersed pattern.

3.4 Discussion

The Bst-2 gene is an IFN inducible gene with a broad antiviral activity. Interestingly, the Bst-2 gene is duplicated in sheep and in cattle, suggesting that duplication occurred more than 25 million years ago before speciation within the Bovinae subfamily. The fixation and maintenance of oBST-2B in the ruminant genome suggests a biological function for this gene. However, in a previous study, we found oBST-2B to have only a moderate antiviral activity compared to oBST-2A (Arnaud, Black et al. 2010). In this chapter, we aimed to characterise key differences between the ovine BST-2 paralogs, and in particular we investigated their intracellular distribution.

Although oBST-2B appears to have a limited antiviral activity, in co-transfection assays we ruled out that it acts as a negative regulator of oBST-2A. Indeed, sheep Betaretroviruses were efficiently inhibited in the presence of oBST-2A and oBST-2B.

We revealed several properties that are unique to oBST-2B and that might be at the basis of the different biological properties observed in the ovine paralogs. We found oBST-2B not to be glycosylated, to lack a predicted GPI anchor attachment signal and to possess an ER retention motif (KLCKK) at its C-terminal portion.

A lack of glycosylation alone would not seem to be the main reason underlining the different biological properties of oBST-2A and -2B. Mutation of the glycosylation sites in hBST-2 did not affect its distribution nor its common ‘tethering’ antiviral function (Perez-Caballero, Zang et al. 2009). N-linked glycosylation is a common form of protein modification, predicted for more than half of all eukaryotic proteins. It is reported to be involved in a number of processes including protein orientation relative to membranes, protein turnover and regulation of stabilisation against denaturation/proteolysis, enhanced solubility and modulation of immune responses (Helenius and Aebi 2004). Thus, a lack of glycosylation may have a profound impact on BST-2B structure and in turn function.

We found that oBST-2B is present mainly in a concentrated form in a perinucleolar region while oBST-2A is dispersed throughout the cytoplasm and the cell membrane. oBST-2A, similarly to other BST-2 orthologs, appears to maintain a functional GPI-SP and adopt the current "tethering" model where BST-2 is directed via its GPI anchorage to the plasma membrane. However, oBST-2B does not seem to possess a canonical GPI-SP (or at least it is not predicted by current bioinformatic tools), and does not localise to the cell membrane. Consequently, it is difficult to envisage how this protein could restrict viral exit by the classic 'tethering' mechanism.

Despite the importance of GPI anchorage for the BST-2 membrane localisation, this can only account for its absence from the plasma membrane and does not fully explain the differing patterns of intracellular localization that we observed. oBST-2A and -2B share a transmembrane domain but the latter contains a non-canonical ER retention motif (KLCKK). Deletion of this region (e.g. mutant oBST-2Bc-6) resulted in the redistribution of oBST-2B from the characteristic concentrated form to that of a more dispersed form. The KLCKK motif of oBST-2B is similar to the classic K(X)KXX motif, one of several C-terminal ER retention motifs identified. It differs slightly from the standard consensus sequence described which has three lysine residues found at positions -1/-2 and -5 relative to the C-terminus. Therefore, the lack of the KLCKK retention motif may explain the potential redistribution to the plasma membrane rather than the possibility of GPI anchorage.

Proteins with ER retention motifs often require post translational modifications carried out at the Golgi, where they traffic before feeding back to the ER where they are based. ER retention motifs allow re-circulation to the ER from the Golgi and TGN (Jackson, Nilsson et al. 1993). It was puzzling that mutant oBST-2Bc-5 (with a premature termination codon replacing the K residue in position -5) had an apparently concentrated localisation. However, the distribution pattern of oBST-2c-5 was notably different from wild type oBST-2B. Wild type oBST-2B was usually localised mainly in a single perinuclear region while oBST-2c-5 had a series of punctuate dots surrounding the nucleus which may indicate that it

remains trapped within the ER, although further studies will be needed to confirm this point.

Using confocal microscopy, we established that oBST-2B localises in the Golgi stacks. This is not a novel concept for BST-2 as an independent intracellular pool localising at the TGN for hBST-2 has already been described (Kupzig, Korolchuk et al. 2003) and, interestingly, a Golgi based localisation has also recently been described for a BST-2 homolog (GREG) derived from CHO cells (Li, Kaloyanova et al. 2007). oBST-2B localisation was also confirmed by a functional assay. In the presence of Brefeldin-A, oBST-2B is redistributed in the same manner as the Golgi based protein Giantin. Therefore, we conclude from these data that oBST-2B does not progress beyond the Golgi and behaves as a resident Golgin closely associated with the cis/medial cisternae of this organelle. oBST-2A showed a distinct localisation from oBST-2B.

As mentioned above, we noticed a similar intracellular localisation between oBST-2B and GREG (Li, Kaloyanova et al. 2007) However, we find that intracellular localisation is the only feature that these two proteins share. GREG is a constitutively expressed protein which appears to be important for maintaining Golgi structure, while oBST-2B is induced by IFN and displays some antiviral activity (see also Chapter 4). Structurally, GREG and oBST-2B do not share many features. GREG has GPI anchorage like all other BST-2 orthologs and several sites for N-linked glycosylation, but neither of these features are found in oBST-2B. GREG and oBST-2B share a predicted coiled coil region linked to functionality and retention motifs which determine intracellular localisation patterns. GREG possesses a central EQ tandem repeat that is essential for Golgi localisation. oBST-2B has a C-terminal retention motif which we have shown is the determinant of perinuclear localisation.

Therefore we conclude that oBST-2B has some but not all the features conserved for most BST-2 orthologs. oBST-2A is clearly a plasma membrane based protein bearing many of the features of other classical ‘tethering’ BST-2 proteins. If oBST-2B is absent from the plasma membrane, then we expect that it would be difficult to understand how it can contribute to the traditional mode of tethering viral particles to the plasma membrane. However, (Arnaud, Black et

al. 2010) have shown oBST-2B to possess only limited antiviral activity, prompting us to question how oBST-2B can block viral particle release.

A limitation of our studies is that they are conducted by transient transfections. Unfortunately, there is no cell culture system for the propagation of sheep Betaretroviruses.

Chapter 4 - Investigating the antiviral restriction mechanism of the oBST-2 paralogs

4.1 Summary

In this chapter we aimed to identify the antiviral mechanism of the ovine BST-2 paralogs to determine if oBST-2B shared the classical ‘tethering’ antiviral restriction common to other BST-2 orthologs.

4.2 Introduction

4.2.1 Overview of BST-2 induced antiviral restriction.

The discovery of BST-2 as a restriction factor was inspired by the observations that Vpu was mandatory for HIV viral particle release in certain cell lines while it was dispensable in others. These data suggested the presence of a host cellular factor which prevented viral particle release (Smith, Thresher et al. 1991). BST-2 restricts viral replication by ‘tethering’ HIV (and other enveloped viruses) viral particles at the cell membrane, preventing their final release (Van Damme, Goff et al. 2008), (Neil, Zang et al. 2008). Fully formed viral particles are tethered to the cell membrane by BST-2, which creates a bridge of retention between the plasma membrane and virions exiting the cell (Neil, Zang et al. 2008). BST-2 restriction is essentially independent from the cell line in which the assays are carried out, thus ruling out the involvement of essential cofactors for its function (Sato, Yamamoto et al. 2009). There is also evidence to indicate that BST-2 reduces infectivity of the small number of virions released in its presence potentially by interfering with the maturation process (Zhang and Liang 2010). There are conflicting observations regarding the importance of conserved BST-2 structural elements in the tethering mechanism (reviewed by (Andrew and Strebel 2011)). However, it has been demonstrated by mutational analysis of BST-2 with ‘art-tetherin’ (a protein which bears no sequence homology to tetherin but maintains the structural arrangement conserved within BST-2 homologs) that protein configuration is more important than the primary sequence for efficient restriction (Perez-Caballero, Zang et al. 2009).

Human BST-2 possesses a broad antiviral activity extending beyond HIV-1. Human BST-2 has been shown to block the exit of a range of diverse retroviral genera including alpharetroviruses, Betaretroviruses, deltaretroviruses and spumaviruses (Jouvenet, Neil et al. 2009). Further evidence of the restriction of enveloped arenaviruses (Sakuma, Sakurai et al. 2009; Radoshitzky, Dong et al. 2010), herpesviruses (Mansouri, Viswanathan et al. 2009), filoviruses (Jouvenet, Neil et al. 2009) and paramyxoviruses (Radoshitzky, Dong et al. 2010) by human BST-2 has also been demonstrated, although conflicting results have been published in the case of filoviruses (Jouvenet, Neil et al. 2009; Kaletsky, Francica et al. 2009; Radoshitzky, Dong et al. 2010).

Although BST-2 is very efficient in blocking viral exit, some doubts have been cast as to whether this protein can efficiently block viral dissemination within a host. In addition to the ability of BST-2 to 'tether' viral particles to the plasma membrane and thus inhibit cell-free virus transmission, there is also evidence which supports the idea that BST-2 efficiently restricts cell-to-cell virus transmission (Casartelli, Sourisseau et al. 2010). However, it is intriguing that, in contrast to idea that BST-2 may reduce the formation of virological synapses and therefore reduce cell-to-cell transfer, BST-2 does not appear to inhibit their formation and may even promote them (Jolly, Booth et al. 2010). Furthermore, the transfer of virions from an infected to an uninfected cell via a synapse has been observed (Jolly, Booth et al. 2010), although virions that successfully pass through the virological synapses in the presence of BST-2 appear to be heavily aggregated (Casartelli, Sourisseau et al. 2010). The efficient restriction of cell-to-cell transmission by BST-2 has been further disputed by the evidence that ΔVpu HIV-1 mutant viruses switch from cell free to a cell-to-cell transmission mode in the presence of BST-2 (Jolly, Booth et al. 2010). Thus, BST-2 may even promote cell-to-cell virus transmission. An increase in the formation of the virological synapse and cell-to-cell transmission is possibly linked to an increase of virus at the plasma membrane in the presence of BST-2 compared to wild type virus (Jolly, Booth et al. 2010). However, BST-2 restriction may also result in positive selection for viral variants which can spread more efficiently by cell-to-cell transmission, as shown for FIV (Dietrich, McMonagle et al. 2011). Overall, it is important to stress that the antiviral action promoted by BST-2 occurs *in vivo*

in parallel with a range of antiviral factors which target various stages of the replication cycle (Neil and Bieniasz 2009).

Different studies have provided evidence for the positive selection of BST-2 and other restriction factors such as APOBEC-3G and TRIM5α (Sawyer, Emerman et al. 2004; McNatt, Zang et al. 2009). However, these studies have also highlighted the fact that BST-2 does not appear to be as strongly selected as other host restriction factors (Bozek and Lengauer 2010).

4.3 Results

4.3.1 oBST-2A and –2B both redistribute intracellular viral Gag localisation.

In Chapter 3 we established the intracellular localisation of oBST-2B while here we wanted to investigate specifically the effect that they exerted over viral structural proteins. Firstly, using confocal microscopy we looked at the effects of oBST-2A/ -2B on the localisation of enJSRV Gag. Expression plasmids for enJSRV-18 were co-transfected with expression plasmids for either oBST-2A or -2B. Cells were then fixed, permeabilised and subsequently analysed for Gag localisation with a rabbit polyclonal antisera against the capsid (CA) protein. The intracellular distribution pattern of exogenous JSRV and enJSRV-18 Gag proteins, in the presence and absence of oBST-2A and -2B, was based on three previously established distributions: concentrated, dispersed and dispersed with plasma membrane association (Arnaud, Black et al. 2010). We found that both oBST-2A and -2B influence the distribution of intracellular viral Gag proteins, although in apparently different ways. We found a clear redistribution of enJSRV Gag to the plasma membrane in the presence of oBST-2A (Figure 42). These data are in accordance with the ‘tethering’ mechanism of action displayed by BST-2 orthologs. However, we found that a significant proportion of enJSRV Gag displayed a concentrated distribution in the perinuclear region in the presence

of oBST-2B compared to cells expressing enJSRV-18 alone. This concentrated perinuclear distribution observed for Gag is reminiscent of the Golgi-based localisation defined for oBST-2B in Chapter 3.

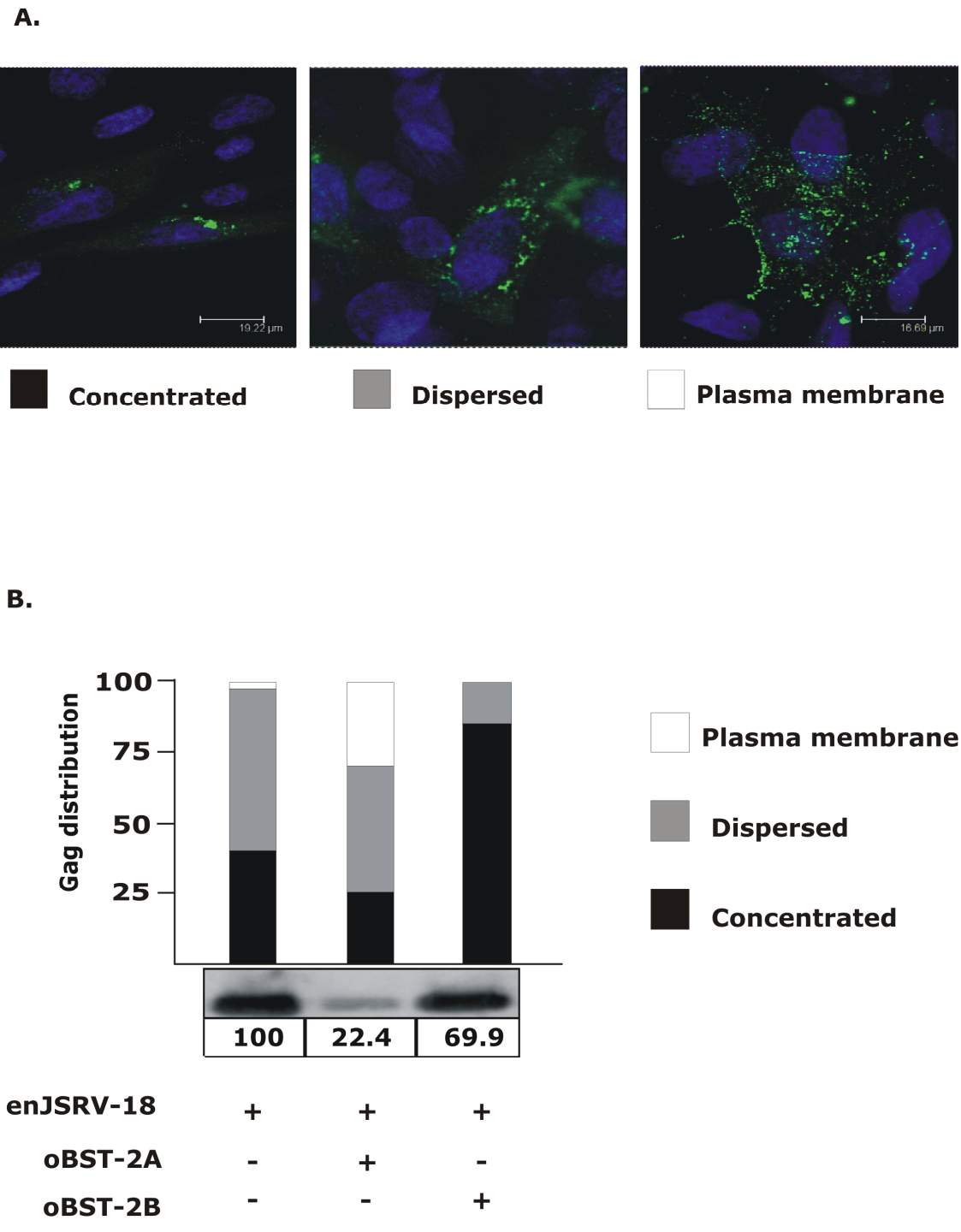


Figure 42. The ovine BST-2 paralogs redistribute enJSRV-18 Gag in different ways.

CPT-tert cells were transfected with expression plasmids for enJSRV-18 alone or with either oBST-2A or -2B (0.25µg of each plasmid). A. The intracellular distribution of Gag (green) was categorised as either concentrated, dispersed or reaching the plasma membrane by confocal microscopy. (Please note the picture representing plasma membrane based distribution was provided by Dr. Frederick Arnaud). B. The phenotype of intracellular gag distribution was counted in 100 cells randomly including blind counts. In the presence of oBST-2A, enJSRV-18 Gag appears to accumulate at the plasma membrane. In the presence of oBST-2B, more than 80% of transfected

cells display enJSRV-18 with a concentrated phenotype. Western blotting of ultracentrifuged supernatants (virus) confirms that oBST-2A/ -2B is actively restricting the release of enJSRV-18 viral particles in those cells counted.

4.3.2 oBST-2B redistributes intracellular Env

Next we sought to determine if oBST-2A/ -2B were also capable of affecting the intracellular distribution of the viral Env protein. This was done by transiently transfecting CPT-tert cells with an expression plasmid for the exogenous JSRV Env (tagged with a Flag epitope at the C-terminus) in the presence or absence of oBST-2A or -2B expression plasmids. The cells were fixed and analysed by confocal microscopy using an anti-Flag antibody to detect Env. The localisation of Env was quantified by confocal microscopy by counting its distribution as ‘concentrated’, ‘dispersed’ or ‘reaching the plasma membrane’ (Figure 43 (A)). This time we found that oBST-2A had no influence over the distribution of viral Env compared to Env alone. However, in the presence of oBST-2B we again observed a notable redistribution of viral Env to the perinuclear region as summarized in Figure 43 (B). We also noted that Env that is localised in the perinuclear region co-localises with oBST-2B, as determined by Pearson’s correlation 0.62 (+/- 0.02), illustrated in Figure 44.

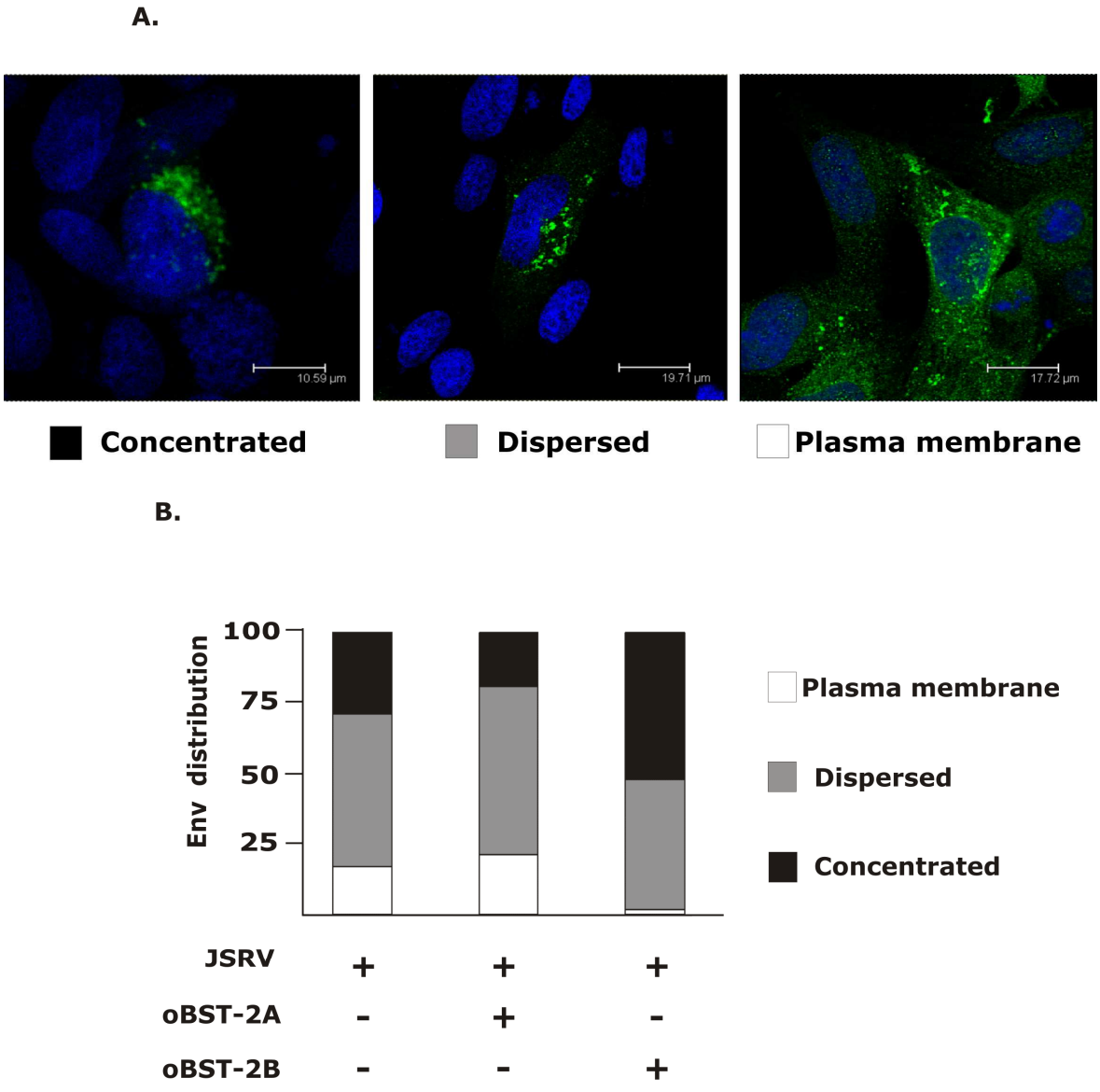


Figure 43. oBST-2B redistributes the intracellular distribution of Env.

CPT-tert cells were transfected with 1µg of JSRV Env in the presence or absence of oBST-2A/ -2B (0.25µg). A. The intracellular pattern of Env (green) was classed and counted as concentrated, dispersed or plasma membrane associated by confocal microscopy. B. oBST-2A had no influence over Env distribution. However, oBST-2B holds a significant proportion of Env in a concentrated form upon comparison to the Env counted alone

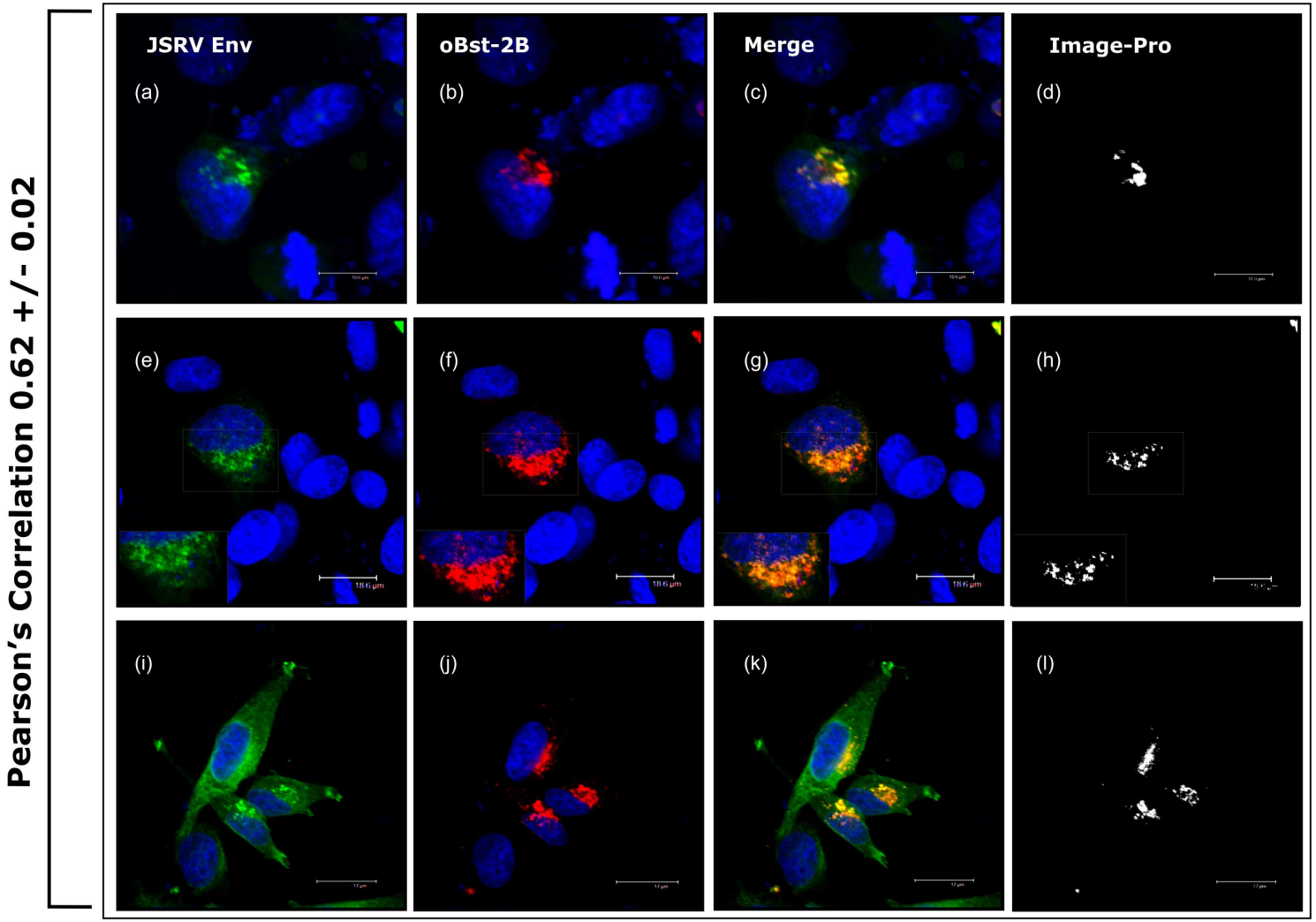


Figure 44. Env held in a perinuclear localisation shows a significant degree of co-localisation with oBST-2B.

Figure 44. Env held in a perinuclear localisation shows a significant degree of co-localisation with oBST-2B.

CPT-tert cells were transfected with 1μg of JSRV Env in the presence or absence of oBST-2B (0.25μg). Eighteen hours after transfection the cells were fixed and analysed by confocal microscopy using antibodies against the Flag (a, e and i) and HA (b, f and j) epitopes. These images were also merged. Env and oBST-2B display a concentrated localisation with a high degree of colocalisation as shown in the merge indicated by yellow (c, g and k) and within the Image-Pro greyscale images indicated in white (d, h and l). The Pearson's correlation value of 0.62 is above the 0.5 threshold and is therefore considered to confirm a significant degree of co-localisation between Env and oBST-2B.

4.3.3 oBST-2B potentially exerts not just an effect over viral proteins but creates a general inhibition of intracellular trafficking

Thus far we have shown that oBST-2B had a significant effect on the trafficking/intracellular localisation of the Gag and Env proteins of both an exogenous and an endogenous sheep Betaretrovirus. Next, we sought to confirm if the influence on protein distribution due to oBST-2B was confined specifically to viral proteins or if it extended to cellular membrane proteins. This would determine if the mode of restriction was virus specific or reflected a more general control over intracellular trafficking of membrane proteins exerted by oBST-2B. We used a GFP tagged form of Caveolin-1 expressed from a plasmid transfected in the presence or absence of either oBST-2A /-2B expression plasmids. Once again, quantification of the intracellular protein localisation was made by confocal microscopy by grouping the expressed proteins as either concentrated, dispersed or based at the plasma membrane (Figure 45 (A)). Caveolin-1 proteins in isolation were found to traffic to the plasma membrane. We found no significant difference in the intracellular distribution of Caveolin-1 in the presence or absence of oBST-2A. Interestingly, the presence of oBST-2B resulted in a greater proportion

of Caveolin-1 being retained in a more concentrated form, as illustrated in Figure 45 (B).

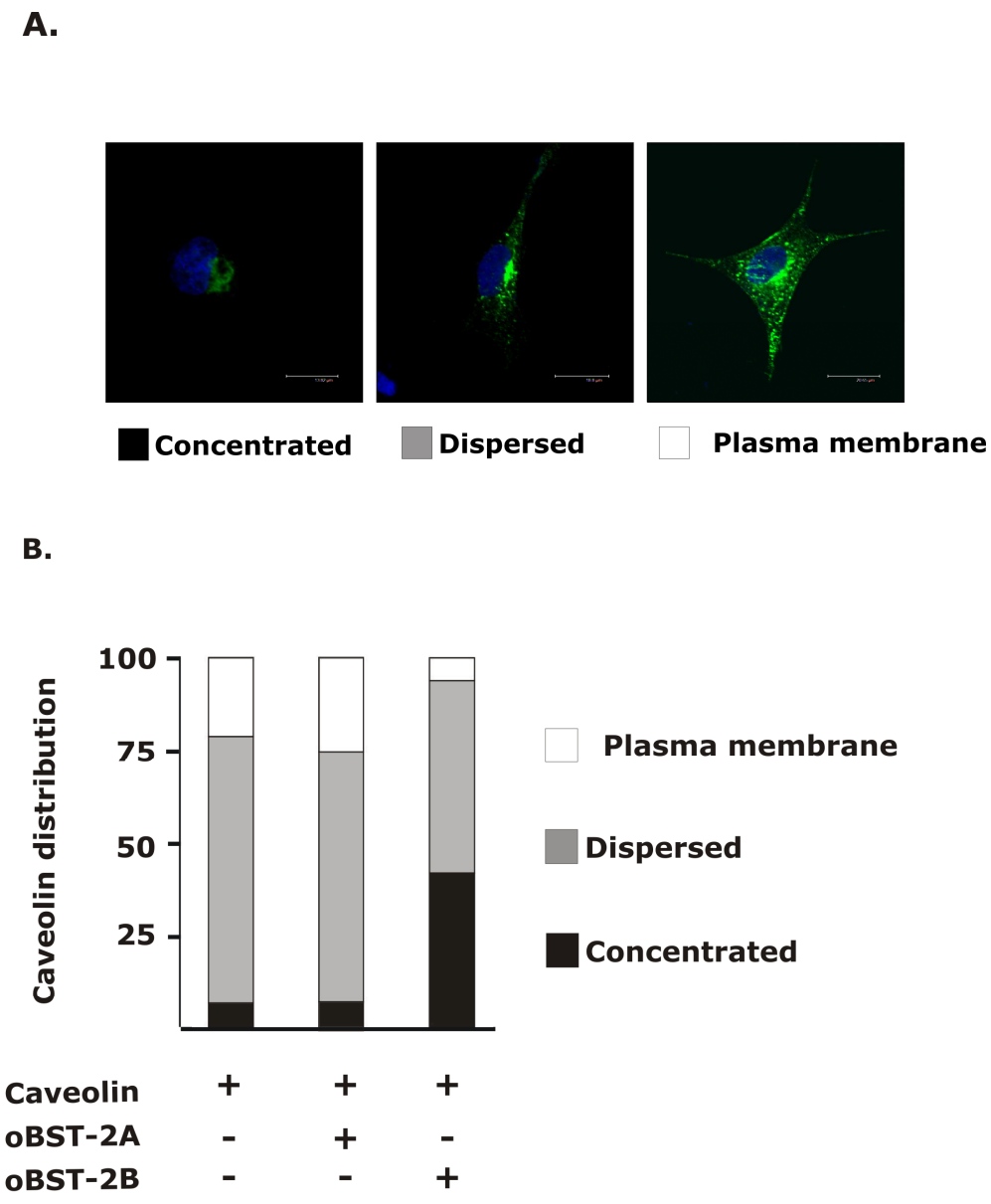


Figure 45. oBST-2B redistributes the intracellular localisation of non viral proteins.

CPT-tert cells were transfected with 1μg of a Caveolin-1-GFP expression plasmid in the presence and absence of oBST-2A/ -2B (0.25μg of each plasmid). A. Internalisation was classed and counted as 'concentrated', 'dispersed' or 'plasma membrane based'. B. No significant redistribution of Caveolin-1 in the presence of oBST-2A was observed. However in the presence of oBST-2B we found an increased proportion of Caveolin-1 to be in a concentrated form.

4.3.4 oBST-2B reduces the incorporation of Env into viral particles

oBST-2A was found to redistribute intracellular Gag to the plasma membrane, supporting the "tethering" restriction model. However, we found that oBST-2B blocked intracellular trafficking of both Betaretrovirus Gag and Env proteins by retaining them within a perinuclear area which is highly likely to impact viral particle assembly. Given this observation, we next investigated the ratio of Gag and Env being incorporated into the small number of viral particles released in the presence of the oBST-2A or -2B. We performed transient transfections of 293T cells with an expression plasmid for enJSRV-18 in the presence or absence of different amounts of oBST-2A or -2B expression plasmids. 24 hours post-transfection, cell supernatants were collected and concentrated by ultracentrifugation. Viral pellets were separated by SDS-PAGE and both Gag and Env were detected by western blotting using appropriate antibodies.

There are wide differences in the stoichiometry of Gag and Env in a retroviral particle. Each virion contains far more Gag than Env. Consequently, we took great care in optimising the dilutions of anti-Gag and Env antibodies to be used in western blotting so that we could be confident that these proteins were detected with similar sensitivity in our assays. Figure 46 (A) shows that serial dilutions of enJSRV-18 viral particles are detected with similar sensitivity by both anti-Gag and anti-Env antibodies. As expected, in the presence of oBST-2A enJSRV-18 viral particle release is greatly inhibited (Figure 46 (B)). On the other hand, inhibition of enJSRV-18 particle release appears to be only moderately affected by oBST-2B (Figure 46 (C)). However, each experiment was

performed independently three times and the blots shown are representative of the trend observed. A comparison of Env incorporation of into enJSRV-18 viral particles is based on a visual reading from the blots. It may be suggested in Figure 46 that, in the presence of oBST-2B, Env incorporation into viral particles is reduced compared to viral particles expressed in the absence of oBST-2B. Similar data were also obtained with exogenous JSRV in the presence or absence of oBST-2A and -2B and is shown in Figure 47. We attempted to confirm this data using standard DLS technology and Nanoparticle tracking analysis (NTA) to estimate the size (diameter) of virions released by JSRV/ enJSRV-18 in the presence and absence of oBST-2A/ -2B. To do this we first estimated the size of virions released following transfection of 293T cells with JSRV/ enJSRV-18 and JSRVΔEnv/ enJSRV-18ΔEnv. Each experiment was performed independently three times and an average result representative of the trend observed is shown in Figure 48. We found that standard DLS technology did not produce accurate or reproducible results for the size of JSRV/enJSRV-18 particles (data not shown). NTA was able to detect particles at the 100nm size predicted for JSRV (but not enJSRV-18). No difference was observed between 'complete' JSRV/enJSRV-18 virions and those devoid of Env.

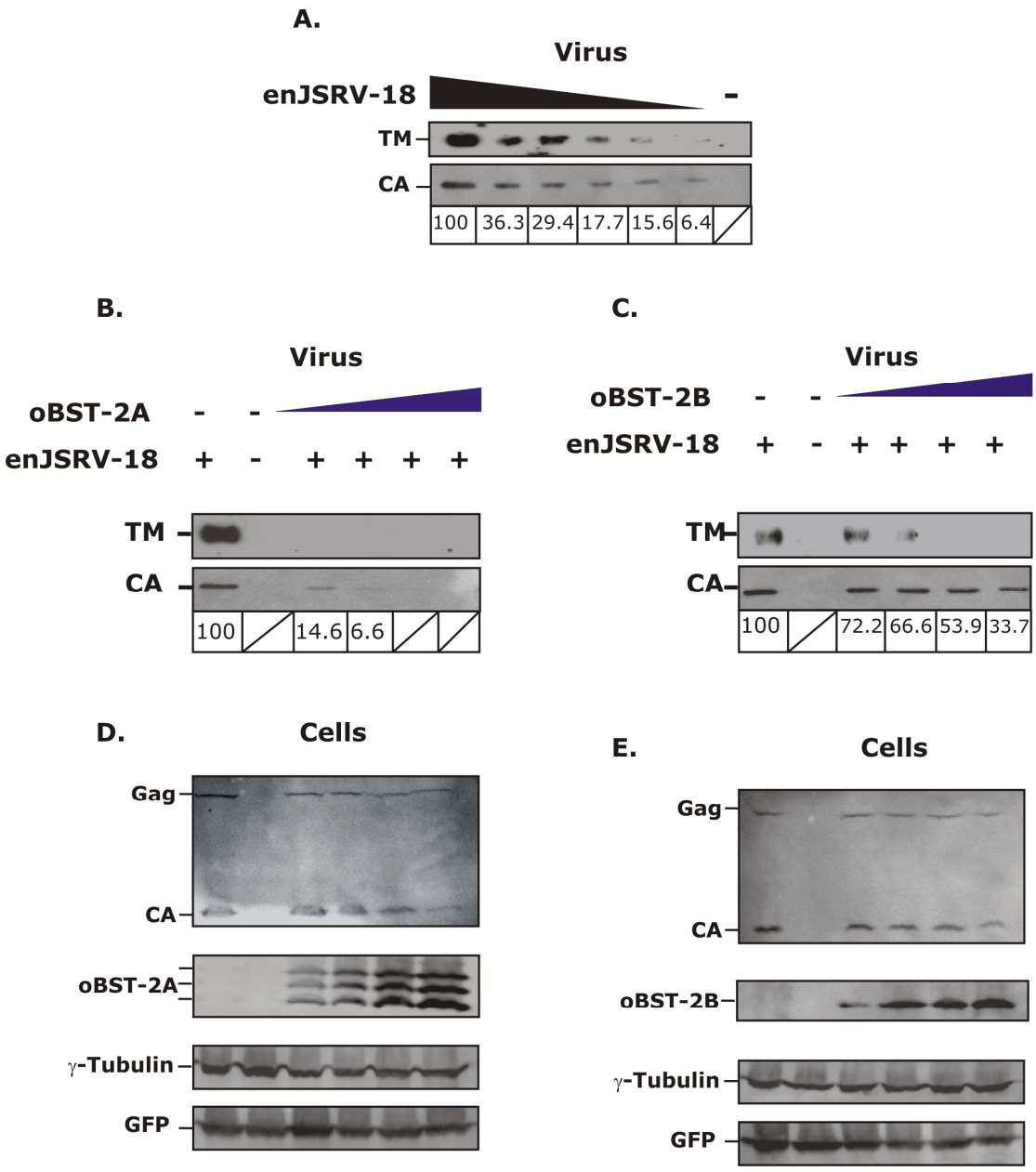


Figure 46. oBST-2B blocks Env incorporation by enJSRV-18 viral particles.

A. Western blotting of serial dilutions of concentrated supernatants of 293T cells transfected with an expression plasmid for enJSRV-18. Dilutions of anti-Gag (CA) and anti-Env (TM) sera used in this series of experiments were pre-determined in order to obtain approximately the same levels of sensitivity. B-C. Western blotting of ultracentrifuged supernatants (virus) from 293T cells cotransfected with 1 μg of an expression plasmid for enJSRV-18 and variable amounts (0.25 – 1 μg) of expression plasmids for either oBST-2A or oBST-2B. Blots were incubated with the appropriate

antisera as indicated beside each panel. Gag associated with the viral particles was quantified by chemifluorescence using a Molecular Dynamics Storm 840 imaging system using ImageQuant TL software (Molecular Dynamics). The numbers indicated below each panel quantify Gag release in the presence of oBST-2A/ -2B compared to the standard virus transfected alone (100). Each experiment was repeated at least three times. D-E The corresponding cell lysates are also shown γ-tubulin was used as loading control while a cotransfected plasmid expressing GFP was used as transfection control.

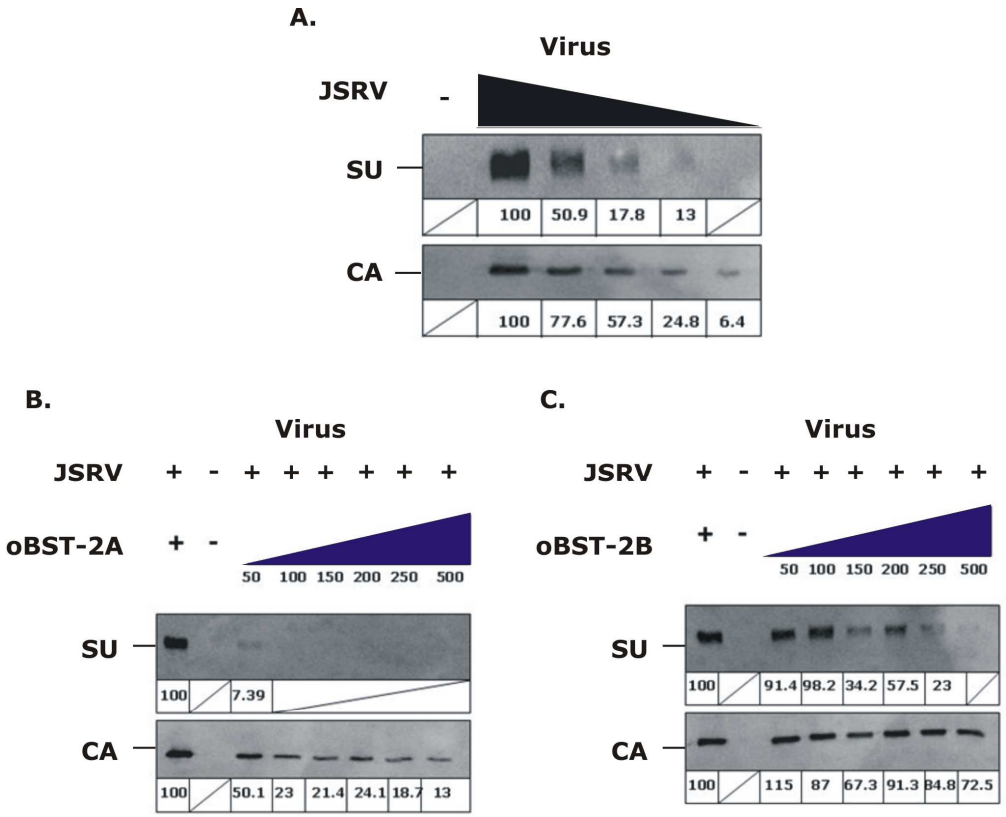


Figure 47. oBST-2B reduces envelope incorporation by JSRV viral particles.

A. Western blotting of serial dilutions of concentrated supernatants of 293T cells transfected with an expression plasmid for JSRV. Dilutions of anti-Gag (CA) and anti-Env (SU) sera used in these series of experiments where pre-determined in order to obtain approximately the same levels of sensitivity. B-C. Western blotting of ultracentrifuged supernatants (virus) of 293T cells co-transfected with 4 µg of an expression plasmid for JSRV, and variable amounts (0.05 – 0.5 µg) of expression plasmids for either oBST-2A or oBST-2B. Blots were incubated with the appropriate antisera as indicated beside each panel. Gag and Env associated with viral particles was quantified by chemifluorescence using a Molecular Dynamics Storm 840 imaging system using ImageQuant TL software (Molecular Dynamics) and numbers are indicated below each relevant panel. The

numbers indicated below each panel quantify Gag and Env release in the presence of oBST-2A/ -2B compared to the standard virus transfected alone (100).

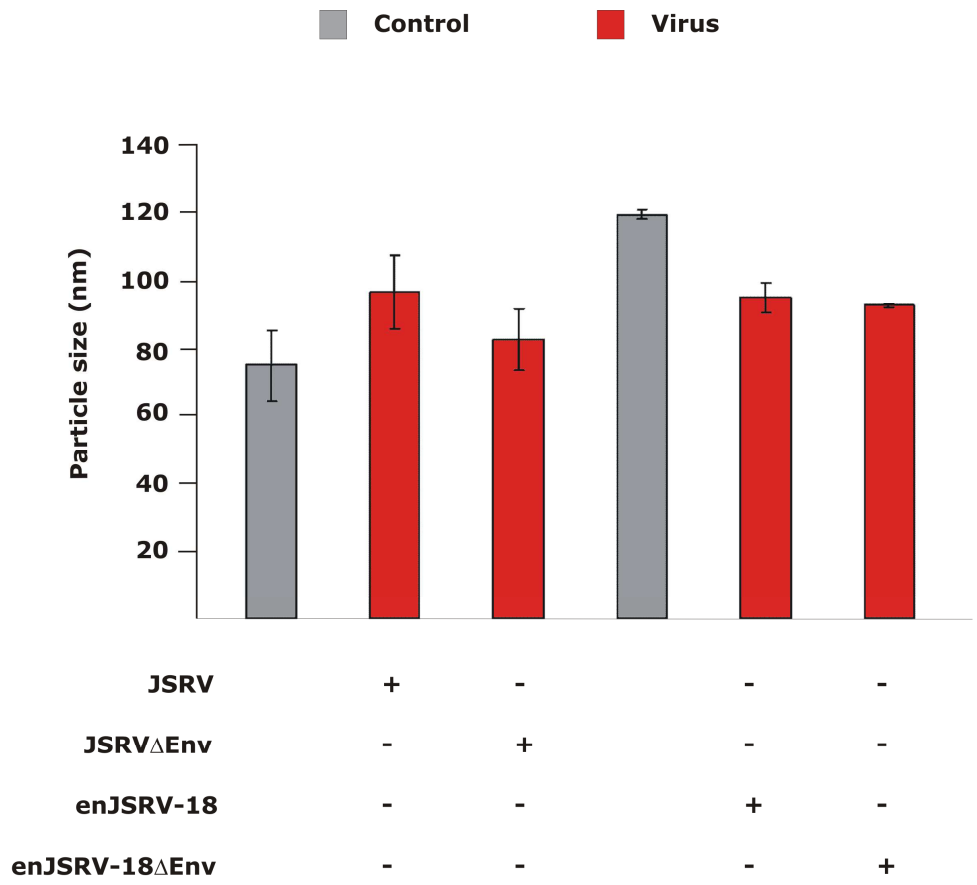


Figure 48. NTA analysis of exogenous and endogenous JSRV virions and those released devoid of Env.

Nanoparticle Tracking Analysis (NTA) was employed to measure the hydrophobic diameter of ultracentrifuged supernatants of 293T cells transfected with 1 μg of expression plasmid for enJSRV-18/enJSRVΔEnv, or 4 μg of JSRV/ JSRVΔEnv. NTA of JSRV virions revealed an average particle size of 96.7nm while JSRVΔEnv virions were smaller at 82.7nm (red) with a background reading of particles averaging at 75nm (grey). For enJSRV-18 the average particle size was larger at 95nm and enJSRVΔEnv virions were 92nm (red) with a background reading of particles averaging at 119nm (grey).

4.3.5 oBST-2B C-terminal truncated mutants do not affect the incorporation of Env into sheep Betaretrovirus viral particles.

Having confirmed the effects of oBST-2A/ -2B on Gag and Env, we then focused on determining whether the function of oBST-2B relied on its localisation in the Golgi. In order to experimentally address this point we used the previously described truncation mutant oBST-2BGPIc-28, which no longer retained the predominately concentrated localisation exclusive to oBST-2B and displayed instead a more dispersed localisation (Figure 25 from Chapter 3). We performed transient transfections of the enJSRV-18 expression plasmid in the presence of variable amounts of oBST-2BGPIc-28 as described above and once again assessed the incorporation of Gag and Env into viral particles by western blotting. We found that, unlike wild type oBST-2B, oBST-2GPIc-28 was not able to deplete Env incorporation into enJSRV-18 viral particles (Figure 49). We conclude that the C-terminal portion of oBST-2B and its characteristic concentrated perinuclear localisation appear to be essential for its antiviral function.

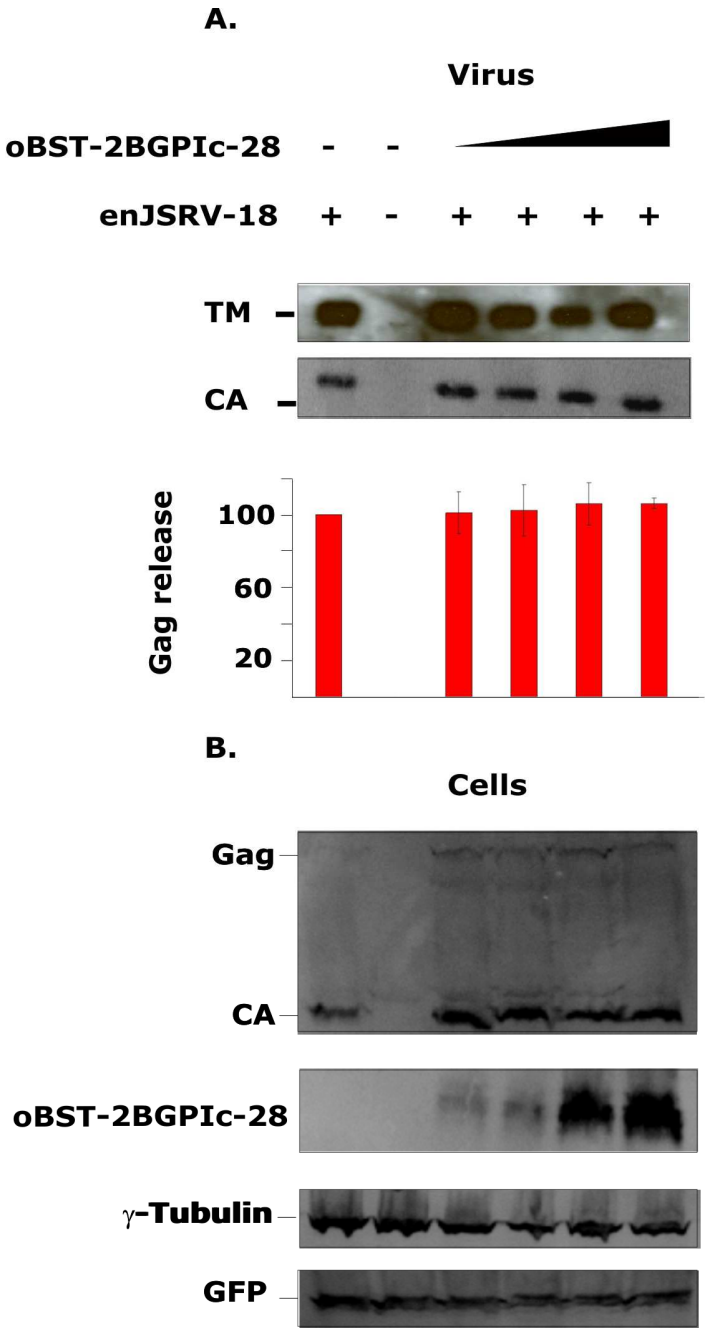


Figure 49. The C terminal portion of oBST-2B is essential for inhibiting Env incorporation.

A. Western blotting of ultracentrifuged supernatants (virus) and cell extracts (B) of 293T cells co-transfected with 1 μg of an expression plasmid for enJSRV-18 and variable amounts (0.25 – 1 μg) of oBST-2BGPIc-28. Blots were incubated with the appropriate antisera as indicated beside each panel. Data show that the C-terminal truncation mutant has no effect on either Gag (CA) or Env (TM) release.

4.4 Discussion

It is well established that BST-2 proteins directly antagonise the late phases of the retroviral lifecycle by engaging with viral particles as they exit from the cell and retain them by tethering them to the plasma membrane. BST-2 resides within the lipid rafts of the host membrane, which are known to be hotspots for viral release, and the various BST-2 orthologs isolated from different animal species seem to restrict viral replication in a similar manner.

Sheep (and other ruminants within the sub-family of the Bovinae) have a duplicated BST-2 gene (termed oBST-2B) that has been conserved throughout evolution. In a previous study, it has been shown that oBST-2B blocks viral exit less efficiently than oBST-2A as judged by the relative amount of Gag released in the supernatants of cells transfected with plasmids expressing retroviral constructs (Arnaud, Black et al. 2010). Indeed, in this current study we showed that oBST-2B localises mainly in the Golgi and possesses different structural characteristics from oBST2-A.

Within this Chapter, we found that oBST-2A and -2B both redistribute ovine Betaretrovirus structural proteins, although in a different manner. oBST-2A increases the concentration of Gag at the plasma membrane, similarly to what has been observed for the classic ‘tethering’ model with other BST-2 orthologs that retain virions to the cell surface. In contrast, oBST-2B induces Gag accumulation in a concentrated form in a perinucleolar region. Furthermore, oBST-2B also co-localises with Env and induces accumulation of this protein in the same perinuclear region.

These data strongly suggested that oBST-2A and -2B acted in substantially different ways. Previous studies have shown that the Gag of Betaretroviruses, including Mason-Pfizer monkey retrovirus (M-PMV) and sheep Betaretroviruses (Sfakianos and Hunter 2003; Sfakianos, LaCasse et al. 2003; Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007) concentrate in the pericentrosomal area in a dynein and microtubule-dependent fashion. The assembled viral particles then traffic to the cell membrane by a mechanism that is influenced by the pericentriolar recycling endosomes and by Env (Arnaud, Murcia et al. 2007). Thus, the data obtained in this chapter could be explained with a model whereby oBST-2B interacts (directly or indirectly) with the betaretroviral Env and inhibits its trafficking to the cell membrane. As the Env protein influences the assembly and trafficking of virions, inhibition of Env trafficking would in turn lead to the accumulation of Gag in a pericentrosomal area. Betaretroviral particles can exit transfected cells in the absence of viral Env although it is expected that this would occur in a slower manner than in the presence of Env.

Consistent with this model, western blotting suggested that in the presence of oBST-2B there may be a reduction of Env protein incorporation into viral particles.

The data obtained in this Chapter suggest that the antiviral action of oBST-2B might be based on a mechanism not previously reported for other BST-2 orthologs. In the presence of oBST-2B, Env appears to be sequestered in a cellular compartment (likely the Golgi, although we have not formally assessed this point) and does not reach the cell membrane and consequently reduces viral incorporation of Env. The retention of Caveolin-1 in a concentrated perinuclear region in the presence of oBST-2B suggests that BST-2B may have similar effects on other membrane proteins, suggesting that this paralog may have a global

effect on trafficking of certain proteins rather than target specifically the retroviral Env. oBST-2B may act indirectly by inhibiting a general cellular function which results in Env (and other proteins) being physically sequestered within the Golgi. These data suggest that oBST-2B interferes with a fundamental requirement for Golgi trafficking, rather than being based on any specific interaction/recognition event with viral proteins. However, in this Thesis we only tested Caveolin-1. Attempts were made with a Cadherin-GFP fused protein but the construct was considerably cytotoxic in CPT-tert cells. Further experiments are required with other host endogenous proteins which traffic and leave the Golgi.

Previously, we observed close association of oBST-2B with p115 and Giantin (Chapter 3). Both p115 and Giantin have been linked with cargo/vesicle movement between stacks (Sonnichsen, Lowe et al. 1998; Sengupta and Linstedt 2011). We may speculate that it is possible for oBST-2B to interact with and disrupt the flow of protein cargo passing between the Golgi stacks and exiting the TGN, which could also explain the retention of Env within the Golgi. Co-immunoprecipatation studies followed by proteomic screens would be required to confirm particular associations of oBST-2B with Golgi-based proteins which could suggest particular modes of function. Although the exact nature of the oBST-2 interaction within the Golgi was outside the scope of this study, we can conclude with confidence that the antiviral restriction induced by oBST-2B does not follow the pattern of the classic ‘tethering’ mechanism.

We tested the lack of Env incorporation into the sheep betaretroviral particles in the presence of oBST-2B by western blotting. As mentioned in the results section of this Chapter, we took care in optimising the dilutions of anti-Gag and anti-Env antibodies in order to equilibrate the sensitivity of detection of Gag and Env. We attempted to obtain more

detailed measurements of virion size and weight in the presence and absence of oBST-2B. Both dynamic light scattering and NTA were employed to measure virion size. Nanosight technology proved to be more sensitive at accurately measuring the virion size of JSRV/enJSRV-18 than DLS, but did not reveal any significant, reproducible differences between the size of JSRV/enJSRV-18 virions and the VLPs produced by transfection of enJSRV-18/JSRVΔEnv truncation mutants (Figure 48). This is likely due to the incorporation of plasma membrane proteins into VLPs during exit of viral particles from the cell (Hammarstedt, Wallengren et al. 2000; Hammarstedt and Garoff 2004; Cantin, Methot et al. 2005).

There is some debate as to whether BST-2 is able to effectively restrict viral infection *in vivo*. While human BST-2 actively reduces cell free virus transmission, it appears to have little influence over cell-to-cell virus transmission (Jolly, Booth et al. 2010). The latter has been evolved by retroviruses as an efficient way to evade host defences. Thus, the different mechanism of antiviral restriction employed by oBST-2B may have an impact on cell-to-cell viral spread. There are different mechanisms that have been envisaged for cell-to-cell virus spread including plasma membrane based fusion, use of filopodia, nanotubules or formation of the virological synapse (Jolly and Sattentau 2004; Sherer, Lehmann et al. 2007). All of these mechanisms share the common feature of viral transfer directly from an infected to an uninfected cell largely driven by Env-receptor(s) interaction. We suspect oBST-2B would prevent cell-to-cell transmission by retaining Env within the Golgi. Thus, the unique duplication of BST-2 in ruminants potentially induces a novel and more stringent antiviral mechanism for Betaretroviruses.

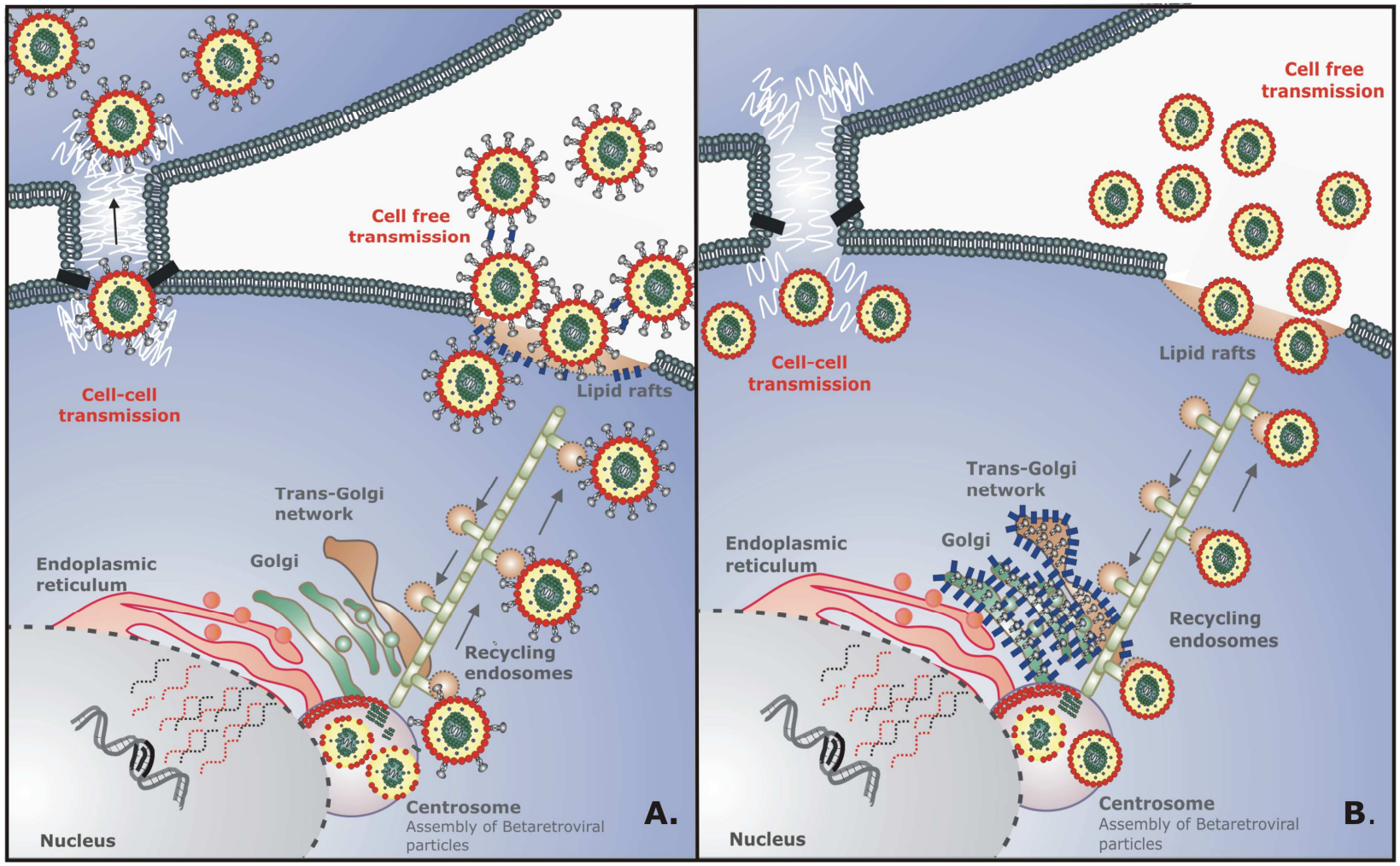


Figure 50. Models of oBST-2A and -2B viral restriction.

Figure 50. Models of oBST-2A (panel A) and oBST-2B viral restriction (panel B).

We suspect that oBST-2A restricts virion release by the ‘classical tethering mechanism’. However, we predict that a different strategy is employed by BST-2. Betaretroviral particles assemble in the pericentrosomal area and exit through the recycling endosomes by a mechanism that is influenced by the pericentriolar recycling endosomes and Env. We predict that oBST-2B inhibits trafficking of the betaretroviral Env the cell membrane. As the Env protein influences the assembly and trafficking of virions, inhibition of Env trafficking would in turn lead to the accumulation of Gag in a pericentrosomal area. Betaretroviral particles in the presence of oBST-2B exit the cell void of Env are deemed non infectious VLPs.

Chapter 5

5.1 General Discussion

There is a delicate evolutionary balance between viruses and their hosts. The hosts have evolved innate, intrinsic and adaptive immunity in order to fight viral (including retroviral) invasion. At the same time, viruses have evolved counteracting measures in order to evade such host defences in order to survive and propagate.

Betaretroviruses of sheep have represented a unique model to study virus-host over long evolutionary periods (Arnaud, Caporale et al. 2007; Arnaud, Murcia et al. 2007; Arnaud, Varela et al. 2008; Chessa, Pereira et al. 2009; Varela, Spencer et al. 2009). The sheep genome has been invaded by Betaretroviruses for at least the last 5-7 million years, before the divergence of the genera Ovis and Capra. During evolution, two enJSRV loci (enJS56A1 and enJSRV-20) have acquired a mutation in Gag (R21W) that renders them defective as they assemble viral particles that are not able to exit the cells (Mura, Murcia et al. 2004). Interestingly, it has been shown that this mutation has been positively selected and is transdominant over other endogenous and exogenous Betaretroviruses with a process termed “JLR” for JSRV late restriction, and estimates based upon evolutionary rates suggest that these transdominant enJSRV proviruses became fixed in the genome of the host around the time of domestication (Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007; Armezzani, Arnaud et al. 2011). However, viruses always appear to possess an answer to the host defences and a recently integrated provirus, enJSRV-26, was found to be able to escape viral restriction due to a single point mutation in the signal peptide of the enJSRV-26 viral Env protein. Thus, sheep Betaretroviruses have left “fossils” that have allowed us to trace the evolutionary arms race between viruses and hosts.

Cellular restriction factors have also been positively selected during evolution. In this thesis we have investigated the interplay between the two oBST-2 genes and sheep Betaretroviruses. BST-2 is an IFN-inducible restriction factor, with a broad antiviral activity targeting the late stages of viral replication. Curiously, in

ruminants BST-2 is duplicated, resulting in the paralogs oBST-2A and -2B (Arnaud, Black et al. 2010). Whilst oBST-2A possesses an antiviral activity similar to other BST-2 orthologs, we found oBST-2 B to possess distinct biological properties.

Gene duplication offers the possibility to produce a large number of "novel" genes to bacteria, archaebacteria and eukaryotes and it is linked to the development of species-specific functions. Duplication of a gene is most often generated by unequal cross over, retroposition or chromosomal duplication events within the genome (Zhang 2003). In the majority of cases duplication creates an identical copy of the gene. This process is beneficial to the host for strongly expressed genes, but where there is no requirement for additional gene expression duplicated genes may undergo "subfunctionalization" or "neofunctionalization". Subfunctionalization involves the original gene and its paralog sharing only small parts of the original gene function, resulting in two genes which perform individually refined functions in place of a previous gene. On the other hand, neofunctionalization involves the duplicated gene taking on an entirely novel function altogether. It is difficult to envisage how a gene could undergo rapid modification to adopt an entirely new function within the constraints of the host genome.

Two models are suggested to explain how this may have arisen within the host. The first model proposes that extensive mutations may accumulate within the duplicated gene over time without the pressure of positive selection as it is functionally redundant. The second model suggests that the duplicated genes have been subject to positive selection where even a few mutations induce a weak but novel function, which is then positively selected or that the original gene had dual action which mutations followed by selection result in this action being shared and refined between the current paralogs (Zhang 2003).

Recent analysis of the history of gene duplication and loss within fungi yielded important information with regard to the significance of duplication within the host (Wapinski, Pfeffer et al. 2007). It highlighted that there was little evidence of duplication of genes with a fundamental physiological importance to cell function. Those genes which were continually subjected to 'environmental stress' were found to be more frequently duplicated within the genome.

Interestingly, hosts which have undergone whole genome duplication appeared to favour the addition and loss of genes for the majority of gene duplications observed (Flintoft 2007). Gene duplication has been implicated in the development and function of major intracellular organelles such as the Golgi (Klute, Melancon et al. 2011). In addition it is considered to resolve intralocus sexually antagonistic variation. This is the presence of genes on the X chromosome shared in both males and females which are under selection in both species in opposing directions (Gibson, Chippindale et al. 2002; Gallach and Betran 2011). There is evidence to suggest that gene duplication has also contributed to the evolution of placental pregnancy (Hughes, Green et al. 2000).

We suggest that oBST-2B could be seen as a rare example of neofunctionalization, a gene duplication event which occurred prior to the speciation of the bovinae and which has resulted in an entirely novel function for oBST-2. This is novel in that oBST-2B appears to restrict Betaretroviruses with a mechanism entirely different from oBST-2A.

Genes involved in host immunity are among those under the most significant evolutionary pressure. We did not address in this thesis whether oBST-2B has or is under positive selection. The extensive lifespan of primates is at odds with the rapid turnover of retroviral populations and inevitably gives the virus the evolutionary advantage with more chances to generate ways to defeat the host immune system. Therefore, oBST-2B represents a rare example of the unique forms of positive selection occurring within the host in response to the continual challenge a particular retrovirus infecting a particular animal species. Betaretroviruses, unlike other retroviruses, assemble at the pericentrosomal area, a unique feature of the Betaretrovirus lifecycle. Viral particles then exit with a process that is influenced by Env and by the recycling endosomes. oBST-2B appears to co-localise strongly with Env and in turn reduce Env incorporation into the envelope of nascent virions. In light of the BST-2 gene duplication event having occurred prior to divergence of cattle and sheep, the challenge posed by ancient betaretroviral ancestors to enJSRVs may have lead to the positive selection of a second oBST-2 gene. The cattle genome has been shown to contain Betaretroviruses that are distinct from enJSRVs. The pattern of restriction observed for oBST-2B is unique amongst all the BST-2 orthologs known.

As mentioned before, oBST-2A is a glycosylated protein with a broad based internal/plasma membrane based distribution while we found oBST-2B appears to be unglycosylated with a concentrated perinuclear localisation. The perinuclear accumulation of oBST-2B was shown to localise to the Golgi stacks and TGN. In addition, functional experiments with Brefeldin-A confirmed that oBST-2B is localised with the Golgi and behaved like a Golgin. Interestingly, this is the first description of a perinuclear based location for an IFN induced BST-2 protein. Given the striking differences we observed between the oBST-2A and -2B localisation, it was difficult to envisage how both paralogs were capable of restricting viral replication by the same mechanism. Furthermore, the classical tethering mechanism is dependent upon the residence of BST-2 within the lipid rafts of the plasma membrane where it engages with viral particles exiting the cell. The unusual location for oBST-2B did not fit with this model of restriction and led us to question if another mode of restriction was utilised by oBST-2B.

In concurrence with the classical tethering model we found that oBST-2A redistributed Gag protein to the plasma membrane. This is consistent with the fundamental idea that viral particles are tethered to the plasma membrane in order to prevent exit (Neil, Zang et al. 2008). oBST-2A had no effect over Env distribution. However, oBST-2B redistributed both Gag and Env proteins in a concentrated perinuclear pattern. Previously, it was observed that oBST-2A was more efficient at blocking virion exit than oBST-2B based on viral Gag exit levels (Arnaud, Black et al. 2010). During this thesis we found that that oBST-2A is a highly efficient restriction factor for both Gag and Env. oBST-2B in contrast appears inefficient at blocking Gag exit but we obtained data suggesting that oBST-2B substantially reduced the presence of Env in the virions released into the supernatants. We suspect in the presence of oBST-2B uninfectious VLPs are released.

Thus, in this thesis we suggest that oBST-2B is in fact, an efficient restriction factor which blocks Betaretroviruses by a unique method of restriction, different from oBST-2A and one not previously described for any other BST-2 protein. oBST-2B appears to exert its restriction by sequestering Env in a perinuclear compartment, most likely the Golgi, preventing trafficking and reducing the incorporation of Env into assembling virions. The virions released are devoid of Env and therefore we suspect are non-infectious VLPs. We speculate that oBST-

2B, unlike oBST-2A, may provide an efficient restriction mechanism by preventing cell-to-cell transmission which is driven by the interaction between Env and the host receptor. This interaction would be unable to occur due the unique pattern of Env perinuclear localisation in the presence of oBST-2B.

Unfortunately there are inherent difficulties in our experimental system that makes it difficult to firmly establish that indeed oBST-2B blocks Env incorporation. Because there is less Env than Gag in viral particles, western blotting could provide misleading results although (i) we carefully titrated anti-Gag and anti-Env antibodies in order to obtain a similar sensitivity in this test and (ii) confocal microscopy also showed an accumulation of Env in the Golgi.

It was assumed that viral particles devoid of Env would be a different diameter to wild type particles with a full complement of Env. We therefore attempted to measure the size of the viral particles in the presence or absence of oBST-2B by dynamic light-scattering methods. Our results revealed no difference in the size of sheep betaretroviral particles in the presence or absence of oBST-2B. However, using the same technique we could not detect a difference in size between our positive controls (wild type JSRV/enJSRV-18) and the respective Env truncation mutants. The reason for these data is that betaretoviral particles formed by Gag alone are still enveloped by the cell membrane. Consequently it is very difficult to resolve the size of a betaretroviral particle with Env from one without Env. An alternative approach would be to visualise intracellular or budding particles by transmission electron microscopy with immunogold labelling for Env detection.

There is no tissue culture system for the propagation of JSRV/enJSRVs. During this thesis we also tried to develop infectious assays for JSRV/enJSRVs but without success and therefore it has proven difficult to corroborate oBST-2B restriction by infectious assays.

Overall, we suggest that oBST-2B provides another example of a host restriction factor. Future experiments need to be directed to prove lack of Env incorporation with other techniques. Use of other Betaretroviruses, such as Mason-Pfizer monkey virus may help to develop infectious assays that could further corroborate our studies.

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