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Clayton Hattlmann, *The University of Western Ontario*

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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RESTRICTION OF HIV-1 REPLICATION BY UNIQUE TRIM22 ISOFORMS

(Spine title: Restriction of HIV-1 replication by unique TRIM22 isoforms)

(Thesis format: Monograph)

by

Clayton Hattlmann

Graduate program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

CERTIFICATE OF EXAMINATION

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Restriction of HIV-1 replication by unique TRIM22 isoforms.

is accepted in partial fulfilment of the
requirements for the degree of
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Date______________________________

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Dr. David Heinrichs
Chair of the Thesis Examination Board

ABSTRACT

An estimated 34 million people worldwide are infected with human immunodeficiency virus (HIV). Understanding how the immune system reacts to HIV infection and why normal antiviral defenses are insufficient to fight infection is a key step towards creating better therapies. Several interferon-induced proteins, such as the tripartite motif protein TRIM22, are capable of restricting HIV-1 replication in vitro; however the contribution of these antiviral factors to HIV-1 pathogenesis is unclear. Previous studies have observed that single nucleotide polymorphisms (SNPs) can dramatically impact the actions of these proteins and influence the severity of HIV-1 infection. While numerous SNPs have been reported in the *trim22* gene, no study has addressed how these may affect TRIM22 functions. Here we used U2OS cells to provide the first direct comparison of two TRIM22 isoforms. Through confocal microscopy we observed these isoforms to exhibit different patterns of localization, was dependent on the TRIM22 B30.2 domain. In vitro studies revealed that both isoforms restricted release of infectious HIV-1 particles, though to different extents. Furthermore, both isoforms restricted transcription from the HIV-1 and cytomegalovirus promoters to varying degrees, as determined by qRT-PCR analysis. Collectively, these data suggest that TRIM22 antiviral activity is variable between isoforms, and that SNPs may alter its biological characteristics.

KEYWORDS

Human immunodeficiency virus, TRIM22, tripartite motif proteins, antiviral, innate immunity, interferon, restriction factor, single nucleotide polymorphism

DEDICATION

I dedicate this thesis to my father, Peter, and my step-mother, Sharon. Your unwavering love and support has helped guide me to where I am today, and your encouragement to always pursue my dreams means the world to me. Thank you for always believing in me.

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I would also like to thank my Advisory Committee members, Dr. Joe Mymryk, Dr. Rodney DeKoter, and Dr. Joaquín Madrenas, for their unique input, thoughtful insight, and constructive criticism, all of which have benefited my project.

Finally, I am indebted to the faculty, staff, and my colleagues within the MNI department. To anyone who loaned me a reagent, helped with an experiment, provided advice or an open ear – and even those who were a welcome distraction during late nights in the lab, thank you for everything. You have made my time here memorable.

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LIST OF ABBREVIATIONS

A3	APOBEC3
AIDS	Acquired immune deficiency syndrome
APOBEC	Apolipoprotein B mRNA-editing catalytic polypeptide-like
cDNA	Complimentary DNA
CMV	Cytomegalovirus
ddNTP	Dideoxy nucleoside triphosphates
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HIC	HIV controllers
HIV-1	Human immunodeficiency virus type 1
IFN	Interferon
IRF-9	Interferon regulatory factor 9
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
Jak/STAT	Janus Kinases/Signal Transducers and Activators of Transcription
LTNP	Long-term nonprogressors
LTR	Long terminal repeat
MDM	Monocyte derived macrophages
mRNA	Messenger RNA
NaCl	Sodium Chloride
NFκB	Nuclear factor κ B
NLS	Nuclear localization signal
OAS1	2´,5´-oligoadenylatesynthetase 1
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PKR	Protein Kinase R
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBCC	Ring, B-Box, Coiled-coil
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
Tat	Trans-Activator of Transcription protein
TRIM	Tripartite motif protein
VLP	Virus-like particle
VMD	Visual Molecular Dynamics

CHAPTER 1: INTRODUCTION

1.1 Human Immunodeficiency Virus Type 1

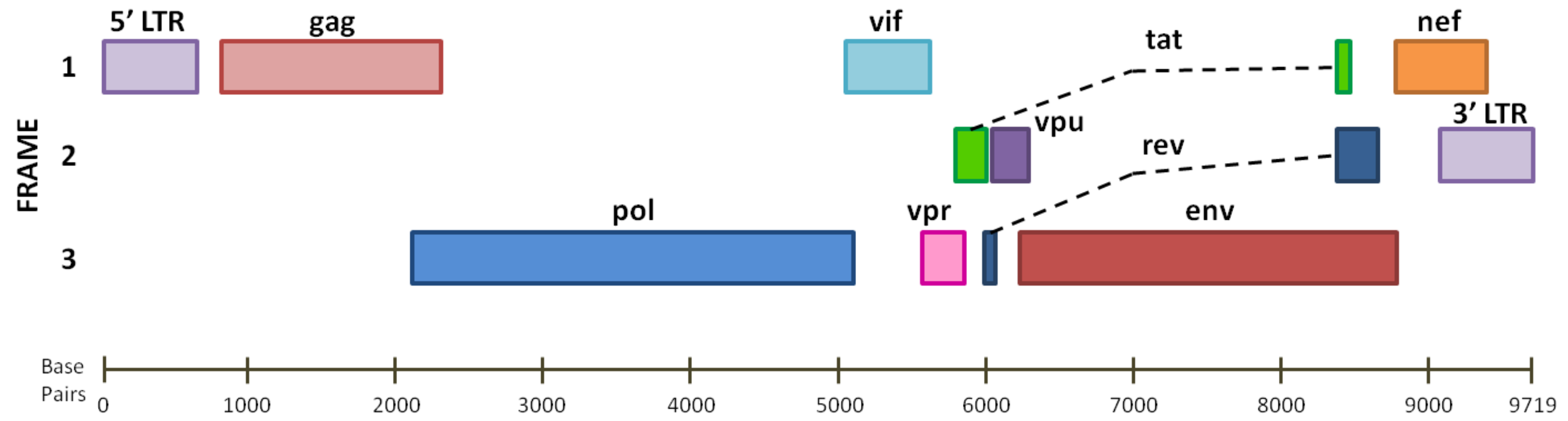
Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that primarily infects CD4+ T lymphocytes (T cells), leading to gradual destruction of the immune system and the eventual progression to Acquired Immune Deficiency Syndrome (AIDS). The HIV-1 genome is approximately 9.7 kilobase pairs in length, and is composed of 9 genes that are flanked by two long terminal repeats (LTR) (Figure 1). The HIV-1 LTRs have central roles in integration of the viral genome into the host cell genome, and subsequent transcription of the integrated viral genes. The *gag*, *pol*, and *env* genes are conserved among all retroviruses, and code for structural proteins, enzymes, and envelope glycoproteins, respectively. Two regulatory proteins, Tat and Rev, also have significant roles in HIV replication. Tat is important for efficient transcription from the 5’ LTR and Rev is required for the transport of viral RNAs from the nucleus to the cytoplasm. Finally, HIV-1 encodes 4 accessory proteins: Vif, Vpu, Vpr, and Nef. These proteins are not essential for *in vitro* replication; however they possess a range of important and interesting functions for productive infections, such as immune evasion and counter-measures (reviewed in [1, 2]).

1.1.1 HIV-1 lifecycle

The HIV-1 lifecycle can be divided into early and late stages (Figure 2). In the early stages, infection begins with adsorption of mature virions to CD4 receptors on the target cell, which is mediated through the HIV surface glycoprotein gp120. Entry also

Figure 1: Schematic of the HIV-1 genome.

The HIV-1 genome is approximately 9.7 kilobase pairs (kb) in length, and encodes 9 genes. Three genes (gag, pol, env) are common among all retroviruses, and are synthesized as polyprotein precursors. HIV-1 also encodes 6 accessory proteins (tat, rev, vif, vpr, vpu, nef), which are the primary translation products of spliced mRNA. Two of these genes (tat and rev) contain spliced exons, as indicated by the dotted black lines. Two long terminal repeats (LTR) boarder the genome, and have roles in integration, replication, and transcription.

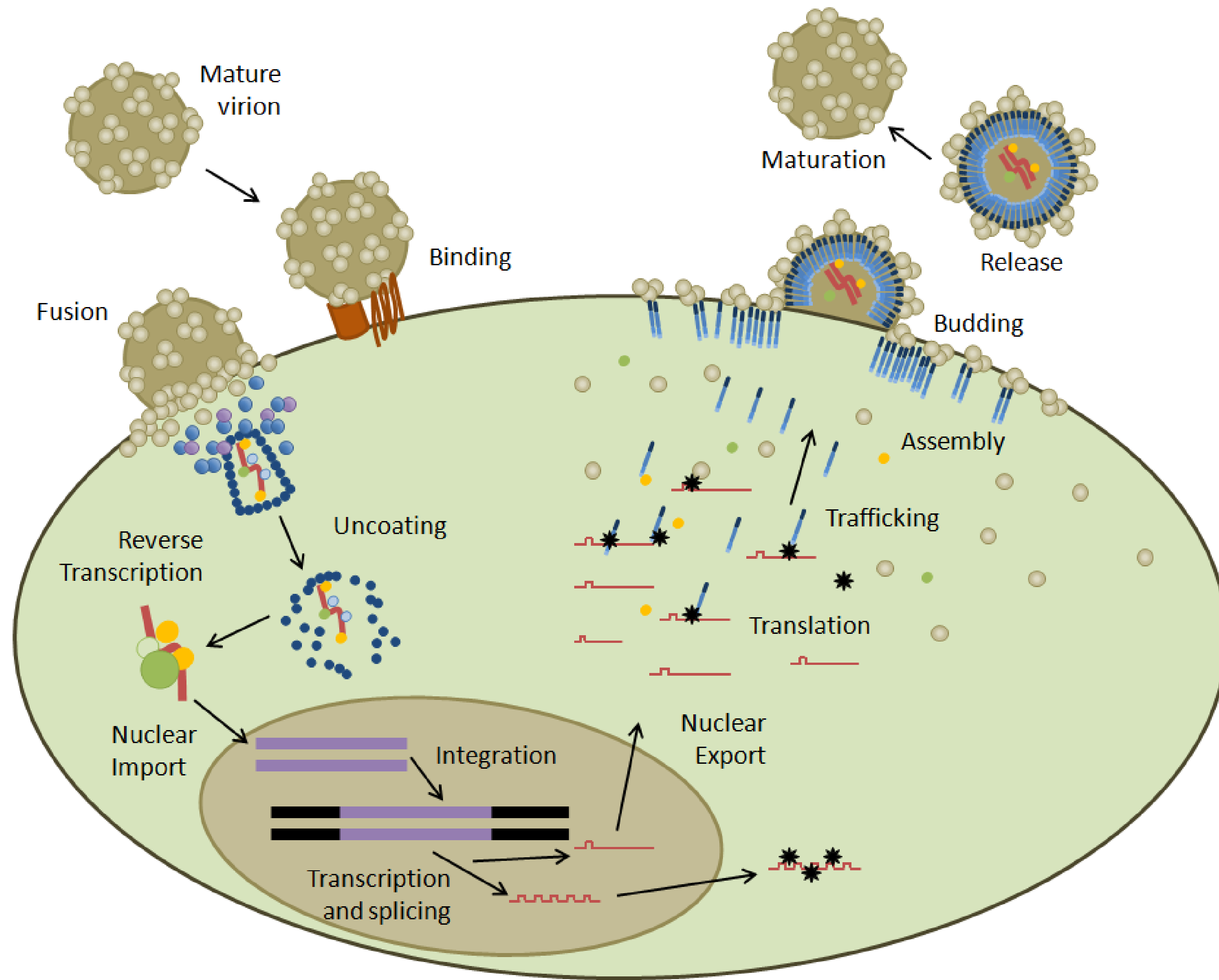

5′ LTR
gag
vif
tat
nef
1
2
3
FRAME
vpu
3′ LTR
rev
pol
vpr
env
Base
Pairs
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
9719

requires binding to a chemokine co-receptor, which also determines viral tropism, and which cell types it can infect. The two most common co-receptors are CCR5 and CXCR4. CCR5 is expressed by macrophages and primary lymphocytes, and is important during early stages of infection, such as HIV-1 transmission (reviewed in [1-3]). This observation is highlighted by the resistance to HIV-1 infection in individuals homozygous for the CCR5/Δ32 mutation, which encodes a non-functional CCR5 co-receptor [4-6]. Although CXCR4 is also expressed by primary lymphocytes, in many patients the emergence of CXCR4- and dual-tropic (CXCR4 and CCR5) viral variants are not observed until late stages of infection, typically around the onset of AIDS [7]. Binding of gp120 induces conformational changes in the HIV transmembrane protein gp41, which in turn mediates fusion of the viral and host cell membranes [8]. Upon capsid uncoating, several viral proteins remain associated with the negative-sense ssRNA genome, including matrix and nucleocapsid structural components, the reverse transcriptase and integrase enzymes, and Vpu. These proteins form the reverse transcription complex and mediate reverse transcription of the genome into dsDNA. From here the newly synthesized DNA associates with several viral and host proteins, forming the preintegration complex, which is subsequently imported into the nucleus. Once in the nucleus the viral integrase protein mediates integration of the viral dsDNA into the host cell genome, completing the early stages of HIV-1 infection (reviewed in [1, 2]).

Late stages of the viral lifecycle begin with transcription of viral genes. Transcription of the integrated provirus is directed from the viral 5’ LTR, which contains several promoter and regulatory elements, and requires both host and viral proteins

Figure 2: Schematic outline of the HIV-1 lifecycle.

Infection begins with adsorption of mature viral particles to the host cell (primarily CD4+ T cells), mediated through binding of the viral envelope protein to a cellular CD4 receptor and chemokine co-receptor (most often CXCR4 or CCR5). Viral and cell membranes fuse, releasing the virus capsid into the cytoplasm. The capsid is broken down (uncoating), releasing the viral genome, two single strands of negative sense RNA, and associated proteins (i.e. reverse transcriptase and integrase). The RNA is reversed transcribed into double stranded cDNA, which is transported into the nucleus as part of the pre-integration complex, and integrated into the host genomic DNA. Stages up to and including integration encompass the early stages of the HIV-1 lifecycle. Late stages of the HIV-1 lifecycle begin with transcription of the integrated viral DNA, which is directed from the HIV-1 5' long terminal repeat (LTR) and enhanced by the viral Tat protein. Viral messenger RNAs (mRNA), some of which are spliced, are exported into the cytoplasm in a manner dependent on the viral Rev protein, and translated into viral proteins. Envelope proteins are transported from the Golgi complex to the cell surface, where they embed in the plasma membrane. Gag polyproteins, some associated with genomic RNA, are targeted to the membrane where they oligomerize and direct budding of nascent particles through the membrane. After release, the Gag polyprotein is subsequently cleaved into its domains (matrix, capsid, nucleocapsid and p6) by the virion-encoded protease. This allows structural and morphologic rearrangement, such as condensation of the core into a cone-shaped structure, and generates mature, infectious particles.


Mature
virion
Binding
Fusion
Uncoating
Reverse
Transcription
Nuclear
Import
Integration
Transcription
and splicing
Nuclear
Export
Translation
Trafficking
Assembly
Budding
Release
Maturation

(reviewed in [9]). The core promoter contains a TATA element, and three tandem Sp1 binding sites which are critical to viral transcription [10, 11]. Upstream of the core promoter is the enhancer, which contains binding sites for three transcription factors (NFκB, NFAT, AP-1) that are involved in viral transcription in T lymphocytes following activation [12-14]. The NFκB binding motif is conserved in all HIV-1 isolates, and is vital to viral transcription [15-17]. Likewise, the HIV-1 Trans-Activator of Transcription protein (Tat) is indispensible for HIV-1 infection, and enhances LTR-directed transcription by hundreds to thousands fold [18, 19]. Tat functions by binding the TAR element, a secondary RNA structure formed by the 5' end of all nascent HIV-1 transcripts. Once bound, Tat is involved in recruiting cellular cofactors and stabilizing RNA polymerase II [20]. Notably, without a functional Tat protein, transcripts are randomly and prematurely terminated, and progeny virions are not produced [18, 19].

Nascent viral transcripts are exported from the nucleus via the viral Rev protein, and are translated in the cytoplasm using normal host machinery. Envelope glycoproteins are targeted to, and assemble on the outside of the host cell membrane, awaiting viral assembly – a process that is driven via the Gag polyprotein (Pr55Gag). Interestingly, expression of Gag alone is sufficient for the formation of noninfectious virus-like particles (VLPs) [21]. Pr55Gag contains four major domains, each of which have important roles in HIV assembly and release. In brief, the N-terminal matrix domain targets Pr55Gag to the site of assembly at the plasma membrane, the capsid domain facilitates multimerization of Pr55Gag polyproteins, the nucleocapsid domain binds the ssRNA genome, and the p6 domain recruits cellular proteins important for budding and release (reviewed in [22-24]). Upon release the viral protease cleaves the Pr55Gag

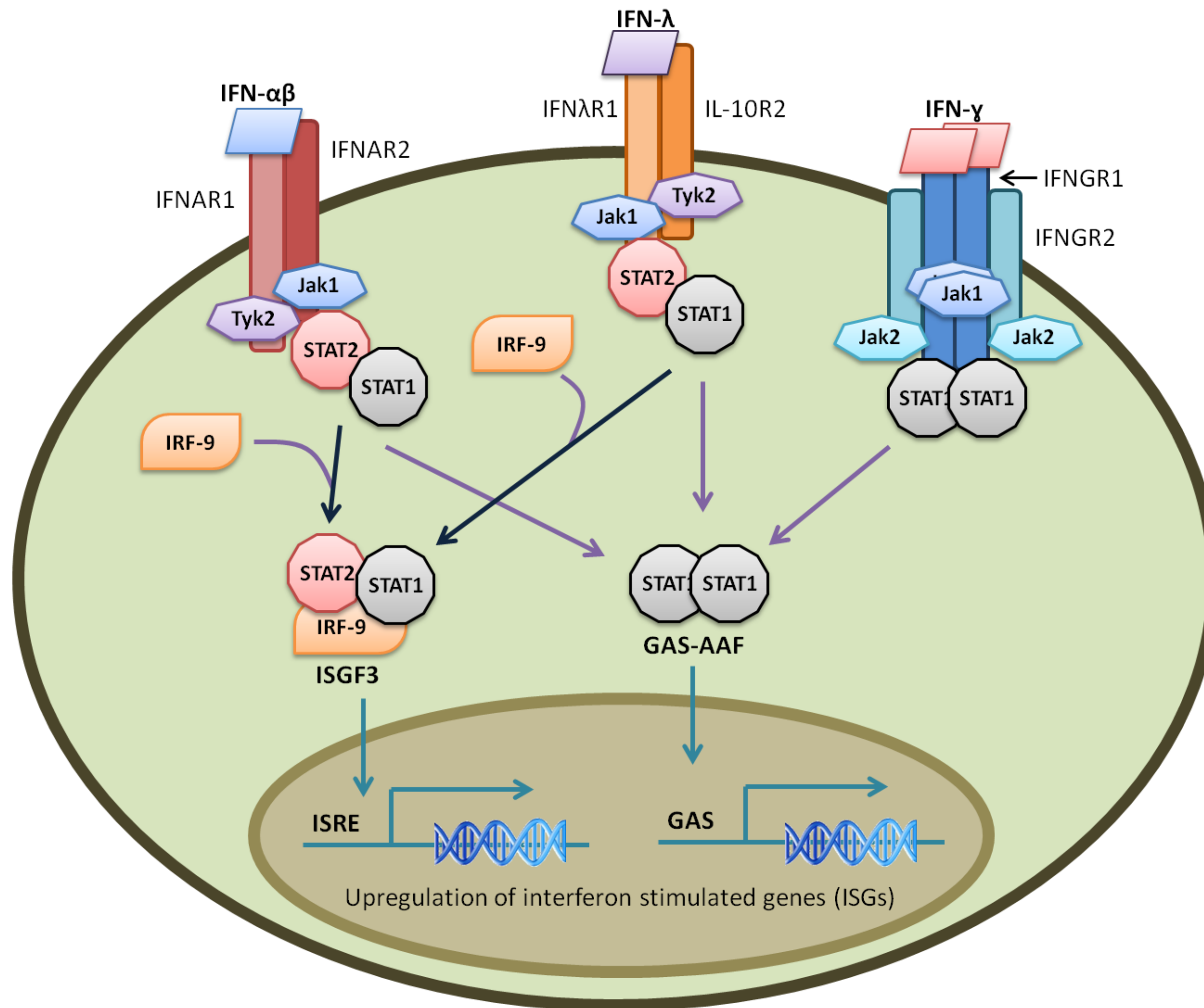
polyprotein into its respective domains, forming the structural components of the HIV virion [25]. This process is called maturation, and is the final step in creating infectious HIV particles [26].

1.2 The interferon response

The interferon (IFN) system is a key mediator of the innate immune response, and the first line of defense against viral infections. IFNs are a class of cytokines produced and secreted in response to external stimuli, such as viral infection, and signal neighbouring cells to initiate an antiviral state. The three major types of IFNs (type I-III) are differentiated based on the receptors they bind (Figure 3), with each type also having slightly different sets of functions. Type I IFNs (α, β, ε, κ, ω) are indispensible for defence against many viruses, and the two main subtypes, IFN-α and IFN-β, are produced by almost every cell in the body. All type I IFNs signal through a common, ubiquitously expressed interferon alpha receptor (IFNAR) – a heterodimeric receptor composed of the IFNAR1 and IFNAR2 subunits. Similarly, type II IFN (IFN-ɣ) bind to an interferon gamma receptor (IFNGR) composed of the IFNGR1 and IFNGR2 subunits. In contrast to type I IFNs, type II IFNs have a larger role in immune regulation as opposed to direct antiviral actions, and is primarily released by immune cells, such as NK or effector T cells (reviewed in [27-31]. Type III IFNs (IFN-λ) signal through a third heterodimeric receptor, which is composed of an IFNλR1 chain and an IL-10R2 chain, and is primarily expressed on epithelial cells. Although type III IFN is a relatively new and distinct member of the IFN family, it appears to share many similarities to type I IFNs, including expression patterns, induction mechanisms, and biological activities (reviewed in [28-31]).

Figure 3: Schematic of interferon signaling pathways and induction of ISGs.

The three major types of IFNs (type I-III) are separated based on the receptors they bind. Type I IFNs (α, β) signal through the interferon alpha receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits. Type III IFNs (IFN-λ) bind a heterodimeric receptor composed of an IFNλR1 chain and an IL-10R2 chain. Both type I and III IFN receptors associate with the Tyk2 and Jak1 kinases, resulting in the tyrosine phosphorylation and activation of STAT2 and STAT1. STAT1 and STAT2 combine with IFN regulatory factor 9 (IRF-9) to form the transcription factor complex IFN stimulated gene factor 3 (ISGF3), which translocates to the nucleus and interacts with IFN-stimulated response elements (ISRE) to regulate transcription of interferon stimulated genes (ISGs). In contrast, type II IFN (IFN-ɣ) binds as a dimer to the interferon gamma receptor (IFNGR) composed of two IFNGR1 and IFNGR2 subunits. The IFNGR1 and IFNGR2 receptors associate with Jak1 and Jak2 and result in the phosphorylation and activation of STAT1 alone. STAT1 homodimers translocate to the nucleus and are capable of binding alternative promoter elements, such as gamma activated sites (GAS), and regulating transcription of other IFN-responsive genes.


IFN-αβ
IFNAR2
IFNAR1
Jak1
Tyk2
STAT2
STAT1
IFN-λ
IFNλR1
IL-10R2
Tyk2
Jak1
STAT2
STAT1
IFN-γ
IFNGR1
IFNGR2
Jak1
Jak2
Jak2
STAT1
STAT1
IRF-9
IRF-9
STAT2
STAT1
IRF-9
ISGF3
STAT1
STAT1
GAS-AAF
ISRE
GAS
Upregulation of interferon stimulated genes (ISGs)

Binding of IFNs to their associated receptors activates a Janus Kinases/Signal Transducers and Activators of Transcription (Jak/STAT) signalling cascade that ultimately results in the upregulation of a vast array of interferon stimulated genes (ISGs) (Figure 3). Both type I and III IFN receptors associate with the Tyk2 and Jak1 kinases through the IFNAR1/IL-10R2 and IFNAR2/IFNλR1 receptor subunits, respectively. This association results in the tyrosine phosphorylation and activation of STAT2 and STAT1, which together with IFN regulatory factor 9 (IRF-9), form the transcription factor complex IFN stimulated gene factor 3 (ISGF3). Upon formation, ISGF3 translocates to the nucleus and regulates transcription though binding to IFN-stimulated response elements present in the promoters of certain ISGs. Alternatively, the IFNGR1 and IFNGR2 receptors associate with Jak1 and Jak2, respectively, leading to the phosphorylation and activation of STAT1 alone. In addition, all IFN signalling pathways can lead to the formation of STAT1–STAT1 homodimers capable of binding alternative promoter elements (Gamma activated sites) and regulating transcription of other IFN-responsive genes (reviewed in [27-31].

Many interferon-induced proteins, termed cellular restriction factors, have been identified to have specific antiviral functions that target various stages of the viral lifecycle. For instance, the ubiquitin-like molecule ISG15 is noted to be one of the most upregulated genes in response to IFN. Interestingly, several proteins with important roles in the type I IFN response have been identified as putative targets for modification with ISG15 (termed ISGylation) [32]. ISG15 has also been reported to help prevent viral counteraction of the IFNβ response [33], to possess immune modulatory capabilities [34], and several studies in mice have shown that ISG15 deficiency corresponds to increased

susceptibility to multiple viruses (reviewed in [31]). Furthermore, ISG15 has been shown to restrict the replication of a range of viruses [35-42], including Influenza A [35-38], Ebola [40], and HIV-1 [41, 42].

Similarly, other well-known IFN-induced antiviral proteins, such as Protein Kinase R (PKR) and 2´,5´-oligoadenylatesynthetase 1 (OAS1)/RNaseL, are expressed at basal levels in addition to being highly upregulated in response to IFN, allowing them to serve as both viral sensors and antiviral effectors. These proteins sense viral infection and are activated by the presence of dsRNA, which is not normally present in uninfected cells. This activation signals inactive OAS1 monomers to oligomerize and synthesize 2',5'-oligoadenylates, which in turn activate RNaseL, a ribonuclease that degrades viral and cellular RNA. Similarly, activation of PKR results in the dimerization of inactive monomers, forming a functional protein capable of inhibiting translation (reviewed in [31, 43, 44]). Interestingly, both of these proteins have been shown to be activated by, and to possess antiviral activity against HIV-1 infection [45-48].

Not surprisingly, the actions of type I IFNs on HIV-1 replication have been extensively studied. Type I IFN treatment of cells *in vitro* interrupts both early [49-51] and late stages of the viral lifecycle [52-55]. The use of type I IFN to treat HIV-1 patients has also had success [56-60], but has come under scrutiny due to adverse effects [61]. Similarly, antiretroviral drugs have failed to provide a cure due to the emergence of drug-resistant strains [62] and toxicity-induced patient noncompliance [63]. In addition, an effective HIV-1 vaccine has yet to come to fruition (reviewed in [64]). In an attempt to develop new strategies against HIV infection, much research has been conducted on the mechanisms of action for different cellular HIV-1 restriction factors (reviewed in [43]).

1.3 HIV-1 restriction factors

1.3.1 APOBEC3

One of the best characterized HIV-1 restriction factor families is the human apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) family. There are seven members of APOBEC3 proteins (A-H), all of which are cytidine deaminases capable of converting cytosine to uracil in RNA or DNA, and all have some degree of activity to mutate and restrict HIV-1 (reviewed in [65]). APOBEC3G (A3G) was the first member identified to block HIV-1 infection, and also appears to be the most potent family member against HIV-1 [66]. In the absence of HIV-1 Vif, A3G is packaged into newly formed virions and subsequently imparts its antiviral action upon infection of a new cell. During reverse transcription, A3G induces cytidine deamination (C→U mutations) in the negative strand of newly synthesized viral cDNA. This results in G→A hyper-mutation of the viral genome, and consequently the possibility for the production of premature stop codons or mutated, non-functional viral proteins [67-74]. In addition, A3G is known to function in a deaminase-independent manner by interfering with reverse transcription [75-78], and has been linked to decreased accumulation of viral cDNA [74, 79-84]. Nevertheless, HIV-1 possesses an A3G counter-measure in the Vif protein, which is capable of restoring infectivity by inducing the degradation of multiple APOBEC3 proteins [70, 85-89].

1.3.2 Tetherin

In contrast to APOBECs, the restriction factor tetherin (Bone Marrow Stromal Cell Antigen 2; CD317) blocks late stages of HIV-1 replication [90]. Tetherin is a transmembrane protein capable of binding the host cell and viral membranes during the

viral assembly/release stage. Binding results in the accumulation of HIV-1 particles at the cell membrane in a chain-like fashion [91], and ultimately induces the reinternalization of released particles and their subsequent degradation within the cell [92]. Although the exact mechanisms behind tetherin-mediated restriction are still unclear, certain aspects have been elucidated, including the fact that dimerization of tetherin is required for HIV-1 restriction [93]. Nevertheless, HIV-1 also encodes a tetherin counter-measure in the *vpu* gene, limiting tetherin's effectiveness to HIVΔVpu strains [90]. Similar to Vif-induced degradation of A3G, the Vpu protein is capable of targeting tetherin for degradation, restoring effective release of mature HIV-1 virions from the host cell [90, 94]. The details of Vpu-mediated degradation are still uncharacterized, however two major hypotheses currently exist. One theory involves the post-translational ubiquitination of tetherin [95-97], leading to subsequent endocytosis from the cell membrane and degradation [92, 94, 98]. The second theory states that tetherin trafficking to the plasma membrane is blocked, and is instead delivered to late endosomal compartments [99, 100].

1.4 Tripartite Motif (TRIM) Proteins

Some restriction factors, such as members of the Tripartite Motif (TRIM) family (TRIM5α and TRIM22), can target multiple stages of the HIV-1 lifecycle. The TRIM family is a class of innate immune proteins with widespread antiviral activity. There are currently 75 identified members, all containing a highly conserved "RBCC" motif (RING domain, one or two B-box domains, and a predicted Coiled-Coil region) (reviewed in [101]) (Figure 4). The N-terminal RING (Really interesting new gene) domain contains a specialized zinc finger that coordinates two zinc atoms, and many RING proteins have been shown to have E3 ubiquitin ligase activity [102, 103]. Considerably less is known

about the other domains of TRIM proteins. The B-box domain is unique to TRIM proteins and also contains a zinc finger. Although the function of these domains is not yet known, the B-box 2 of TRIM5α is important in forming higher-order associations among TRIM5α oligomers, and mutations result in reduced binding to the HIV-1 capsid protein [104]. The B-box domain is followed by a predicted coiled-coil region, which is believed to be involved in oligomerization of at least some TRIM proteins, such as TRIM22 [105]. The RBCC motif is often followed by a C-terminal domain, which for 60% of TRIM proteins, including TRIM22, is a B30.2/SPRY domain [101]. Although the exact function of the SPRY domain is still unclear, it is believed to be involved in RNA binding [106] and/or protein-protein interaction [107]. This domain also appears to have essential links to antiviral activity, as observed in studies of HIV-1 restriction by TRIM5α [108].

1.4.1 TRIM5α

TRIM5α is the earliest acting HIV-1 restriction factor currently known. Before TRIM5α was identified as an HIV-1 restriction factor, it was observed that HIV-1 could enter the cells of Old World monkeys, such as the rhesus macaque; however the virus was blocked from producing a productive infection [109-111]. This introduced the idea of species-specific restriction of HIV-1 replication, and from here it was discovered that the rhesus macaque TRIM5α (RhTRIM5α) was capable of potently restricting HIV-1 replication [112]. Further research has indicated that restriction is believed to be due to specific recognition of the HIV-1 capsid protein through the C-terminal B30.2 domain of RhTRIM5α, resulting in premature disassembly of the capsid during infection [113]. In addition, RhTRIM5α appears to interfere with HIV-1 reverse transcription and nuclear import of the viral cDNA [114-116]. Although controversial, RhTRIM5α also blocks late

Figure 4: Schematic representation of TRIM22 isoforms used in literature.

All tripartite motif (TRIM) proteins contain a highly conserved “RBCC” (Ring, one or two B-Boxs, Coiled-Coil) motif, followed by a C-terminal domain, the most common of which is the B30.2/SPRY domain. The start/end amino acid positions of each domain are indicated below each isoform. The locations of SNPs are reported in relation to the TRIM22β/BC035582 sequence, and are depicted by yellow bands. The bi-partite and ‘KRK’ nuclear localization signals (NLS) located at amino acids 257/265 and 380 are depicted by purple and blue bands, respectively. A) TRIM22β is commercially available, and matches the consensus sequence for *trim22*. B) HQ_842635 was cloned from U937 cells, and contains SNPs at nucleotides 463 and 725, resulting in two amino substitutions: D155N, and R242T. C) The first *trim22* clone, X82200, was created from a splice variant missing nucleotides 519-531, resulting in a 4 amino acid deletion from the coiled-coil domain. It contains an SNP at nucleotide 725, resulting in the R242T substitution. A single nucleotide deletion at nucleotide 1316 causes a frameshift mutation and the production of a premature stop codon at nucleotide 1326. The resulting protein is 442 amino acids, and contains the unique C-terminal sequence ‘LPVVLGFS’. D) TRIM22α was cloned using primers based on X82200. It also lack nucleotides 519-531, and contains the R242T substitution, however there is no deletion at nucleotide 1316. As a result, there is no premature stop codon at nucleotide 1326, and the clone runs 18 nucleotides into the pcDNA3.1 backbone, creating the unique C-terminal sequence ‘ARACI’.

A) TRIM22β (BC035582) – (498 amino acids)

B) HQ_842635 – (498 amino acids)

C) X82200 – (442 amino acids)

D) TRIM22α – (448 amino acids)

stages of HIV-1 infection by targeting the Gag polyprotein for degradation and interfering with viral assembly [117-119]. Interestingly, the human TRIM5α homologue (HuTRIM5α) possesses little to no antiviral activity against HIV-1 replication [112]. Furthermore, the lack of restriction by HuTRIM5α has been mapped to a single amino acid mutation (R332P) in the B30.2 domain [108], and restoration of a proline at this position restores capsid binding capabilities and greatly improves HIV-1 restriction by HuTRIM5α [120].

1.4.2 TRIM22

Human TRIM22 (also known as Stimulated trans-acting factor 50, Staf-50) was originally isolated in 1995 during a search for IFN-induced genes in Daudi cells, a well characterized B lymphoblast cell line [121]. The *trim22* gene is located at chromosomal position 11p15, immediately adjacent to the *TRIM5α* gene [122]. TRIM22, along with TRIM5α, have been under positive selection episodically for approximately 23 million years; however these two genes have evolved in a mutually exclusive manner, with only one being selected for in a given primate lineage [123]. Although relatively little is known about the function of TRIM22 within the cell, it may play a role in cellular processes such as cell differentiation/proliferation [124, 125], and in diseases such as Wilms tumor [126, 127] and systemic lupus erythematosus [128]. TRIM22 is constitutively expressed in resting T cells [129], is a known p53 target gene [124] and NFκB activator [130], and is upregulated in response to type I and II IFNs [121, 122, 125, 131-136]. In addition, its expression is altered in response to a variety of stimuli, including T-cell activation/co-stimulation [129, 135], multiple cytokines [135, 137], and multiple viral antigens/infections [138-143]. Furthermore, TRIM22 has been shown to

have antiviral activity against HIV-1 [131, 134, 144, 145], Hepatitis B virus (HBV) [132], and encephalomyocarditis virus [146].

1.5 TRIM22 inhibits HIV-1 Replication

Despite being identified as a potential HIV-1 restriction factor over a decade ago, relatively little is known about the effect of TRIM22 on HIV-1 replication. TRIM22 was first discovered by Tissot and Mechti in 1995 during a search for IFN-induced genes in Daudi cells [121]. It was also noted that TRIM22 displayed high homology to the mouse Rpt-1 gene, which had previously been shown to down-regulate expression from the HIV-1 LTR [147]. Similarly, exogenous expression of TRIM22 was observed to down-regulate transcription from the HIV-1 LTR in the COS7 cell lines [121]. Although this was performed using a luciferase reporter gene under the control of the LTR as opposed to an HIV-1 proviral genome, it provided the first evidence suggesting that TRIM22 may block HIV-1 transcription and ultimately replication.

In 2006, TRIM22 was shown to be highly upregulated in primary monocyte-derived macrophage (MDM) in response to HIV-1 infection or IFNα treatment. Exogenous expression of TRIM22 was subsequently shown to inhibit HIV-1 infection up to 50% in 293T cells modified to express the CD4 and CCR5 receptors. Furthermore, co-transfection of TRIM22 with plasmids encoding a lentiviral packaging system based on the HIV-1 structure (M107, pMD-G and pCMV-ΔR8.9) resulted in reduced titres of pseudotyped virus compared to an empty vector control. Interestingly, in this pseudotyped virus system, expression of HIV-1 genes is directed from a cytomegalovirus (CMV) promoter (pCMV-ΔR8.9) as opposed to an actual HIV-1 LTR [131]. Since TRIM22 has been shown not to restrict transcription from the CMV promoter in 293T

cells [144], it serves to show that the potent restriction observed in 293T cells must be a result of TRIM22 acting at a separate late stage of the HIV-1 lifecycle. In addition, the over-expression of TRIM22 in primary MDM was also shown to restrict HIV-1 infection by 70-90%, and was capable of preventing the formation of syncytia [131]. Together, these experiments provided the first evidence that TRIM22 can restrict HIV-1 replication *in vitro*, and suggested that TRIM22 may possess transcription-independent antiviral activity.

In 2008, Barr, *et al.* provided the first mechanistic data linking TRIM22 to restriction of HIV-1 replication. TRIM22 was shown to be highly upregulated in response to IFNβ treatment of HOS cells modified to express the CD4 and CXCR4 receptors (HOS-CD4/CXCR4). These cells support robust HIV-1 replication, which can be attenuated by IFNβ. Moreover, TRIM22 was shown to be an integral part of the IFNβ response against HIV-1 infection, noting that IFNβ-induced restriction of HIV-1 replication was abolished after shRNA knockdown of TRIM22. In addition, exogenous over-expression of TRIM22 was shown to inhibit HIV-1 replication in several other cell lines. Interestingly, in the HOS and HeLa cell lines, TRIM22 expression repressed release of HIV-1 particles into the supernatant, but had no effect on the intracellular levels of HIV-1 Gag. Conversely, in the U2OS and 143b cell lines, both the release of HIV-1 particles into the supernatant as well as intracellular levels of Gag were decreased in the presence of TRIM22 [134].

Restriction in HOS-CD4/CXCR4 cells also appeared to be independent of any effect on the HIV-1 LTR, as TRIM22 was also shown to restrict the release of virus-like particles containing only the Gag protein expressed from the CMV promoter.

Furthermore, this restriction was determined to be a result of altered Gag trafficking to the plasma membrane. Although no mechanism of action was ever studied in U2OS or 143b cells, several possibilities could explain the observed decrease of intracellular Gag, including inhibition of transcription or degradation of the Gag polyprotein. It is notable that the antiviral actions of TRIM22 were E3 ligase-dependent, and TRIM22 was shown to interact with HIV-1 Gag specifically [134]. This could suggest that TRIM22 mediates the ubiquitination of Gag, resulting in altered trafficking or proteasomal degradation, depending on the position and number of ubiquitin molecules [148]. Nevertheless, TRIM22 appears to have several distinct activities depending on the cell-line being used for investigation.

It appears that TRIM22 is capable of restricting HIV-1 replication through at least two mechanisms: by targeting trafficking of the Gag polyprotein to the plasma membrane, as well as by down-regulating transcription from the HIV-1 LTR. Interestingly, clones of the U937 promonocytic cell line have been previously described as either permissive or nonpermissive, based on their efficient or inefficient support of HIV-1 replication [149]. Investigation of these clones revealed that *trim22* expression could only be detected in nonpermissive clones, whereas other IFN-induced restriction factors were readily detected in both subsets. In addition, use of a luciferase reporter plasmid under the control of the HIV-1 LTR revealed that LTR-mediated transcription was decreased 7-10 fold in nonpermissive clones, which was recoverable to levels observed in permissive cells via shRNA knockdown of *trim22* expression. Furthermore, exogenous expression of TRIM22 in permissive clones resulted in decreased LTR transcription comparable to that observed in nonpermissive clones. Similar results were

observed in the A3.01 T cell line, further supporting the effects of TRIM22 on HIV-1 infection in critical cell targets [144].

The first clinically relevant evidence to support a role for TRIM22 as an anti-HIV effector *in vivo* was provided in 2011. A study monitoring gene expression in high-risk HIV-1 negative individuals detected a positive correlation between TRIM22 expression and increased control of HIV-1 infection. It was observed that IFNβ and TRIM22 levels in peripheral blood mononuclear cells (PBMCs) were increased in patients after HIV-1 infection. In addition, infected patients expressing higher TRIM22 levels exhibited significantly lower viral loads and significantly higher CD4+ T cell counts, suggesting that TRIM22 may play a role in controlling HIV-1 infection. Furthermore, knockdown of TRIM22 in the Jurkat T cell line resulted in increased HIV-1 particle release and replication *in vitro*. Surprisingly, a significant inverse correlation was observed between the closely related IFN-inducible TRIM5α protein and IFNβ expression [145]. Nevertheless, these results suggest that human TRIM22 may be an important protein in controlling HIV-1 and/or other retrovirus infections, and additional studies will be required to determine the prevalence of TRIM22 forms and their relation to antiviral capability *in vivo*.

1.6 Rationale for studying innate viral restriction factors

As of 2010, the World Health Organization estimates that approximately 34 million people worldwide are infected with HIV. Although the majority of infected individuals eventually progress to AIDS, especially in the absence of highly active anti-retroviral therapy (HAART), a small percentage appear to possess levels of natural resistance to infection. Two general phenotypes are observed among these resistant

individuals: Long-term nonprogressors (LTNP) and HIV controllers (HIC). Approximately 5% of infected individuals are classified as LTNP, and are defined by the ability to maintain high CD4+ T-cell counts in the absence of HAART for 10 years or more. Levels of viral RNA in the blood and viral DNA in PBMCs is quite variable between individuals, and the majority of LTNP eventually experience a decline in their CD4+ T cell counts. In comparison, less than 1% of infected individuals are classified as HIC, as defined by having extremely low viral DNA in PBMCs, undetectable levels of viral RNA in the blood, and rarely showing signs of disease progression. Conversely, approximately 5% of individuals also experience accelerated infection kinetics, progressing to AIDS within 1-3 years of infection (Reviewed in [150-152]).

Interestingly, the phenomenon of viral control during HIV infection appears to be spontaneous and multifactorial, with variable causes. Although the exact determinants responsible for rate of progression are largely unknown, several elements have been identified as contributing factors to prolonged control during HIV-1 infection, including strong CD4+ and CD8+ T cell responses [153]. Nevertheless, adaptive immune responses require time to develop, thus it is also believed that a number of innate immune mechanisms are important in limiting replication during early infection, allowing for the later development of strong T-cell responses [154, 155].

The role of innate cellular restriction factors in viral control is both controversial and insufficiently studied. Thus far, the primary focus has been on potential effects of APOBEC3 (A3) proteins, for which there are reports both supporting [156-158] and refuting [159, 160] potential involvement in control of HIV-1 infection. However, A3 activity may also be misrepresented due to some reports not accounting for potential A3

deaminase-independent mechanisms [79], and the fact that some A3 proteins can be counteracted by the HIV-1 Vif protein [161]. Interestingly, loss of functional A3B was found to be associated with an increased risk of HIV-1 acquisition, higher viral setpoints, and accelerated disease progression [162]. Other reports have argued against the involvement of cellular restriction factors based on the observation that CD4+ T cells from HIC are susceptible to HIV-1 infection *in vitro* [163]. Alternatively, other reports have noted that HIC CD4+ T cells exhibit decreased susceptibility to HIV-1 infection, which was associated with decreased viral reverse transcription, integration, and mRNA transcription [164, 165]. Furthermore, the involvement of cellular factors was suggested, as knockdown of p21, a factor previously implicated in control of HIV-1 replication [166, 167], resulted in increased viral reverse transcripts and mRNA production in CD4+ T cells from HIC. It was also noted that resistance to infection could be overcome with high viral inocula [164, 165]. Regardless, this narrow focus and lack of research has resulted in the under-appreciation of HIV-1 restriction factors as potential contributors to control of HIV-1 infection.

A number of genetic factors are also believed to have a role in some cases of HIV control. It has been observed that individuals homozygous for the aforementioned CCR5/Δ32 mutation are resistant to HIV-1 infection [4-6], and a heterozygous genotype is associated with a number of LTNP [6, 168, 169]. Alternatively, it has been observed that mutations in the *CCR5* promoter resulting in increased CCR5 expression are associated with rapid progression to AIDS [170]. In addition, certain human leukocyte antigen (HLA) haplotypes appear to be associated with disease progression, such as HLA-B57 and HLA-B27, which are consistently overrepresented in HICs [163, 171-

174]. Interestingly, single-nucleotide polymorphisms (SNPs) have been identified as a contributing factor to variation in viral load set-points during asymptomatic early infection [175] – a stage that holds important implications for rate of disease progression [176].

A SNP is defined as a single nucleotide variation in a given genomic DNA sequence between an individual and other members of that species. Biological consequences of SNPs can vary from benign synonymous mutations, and missense mutations resulting in single amino acid substitutions, to more severe nonsense and frameshift mutations. These mutations result in the production of a premature stop codons and vastly altered amino acid sequences, respectively, which in turn can potentially lead to inactivation of the normal biological activity of the affected protein. Interestingly, it has been observed that SNPs in the *TRIM5α* gene may have an impact on both the susceptibility to HIV-1 infection, as well as the clinical course of HIV-1 infection [177, 178]. Similarly, certain SNPs in the *APOBEC3H* gene have been shown to have effects on the stability and subcellular localization of A3H, which subsequently corresponded to variable degrees of HIV-1 restriction. Furthermore, A3H variants were also resistant to Vif, the HIV-1 protein responsible for the degradation of A3F/G [179-181]. In addition, an association of certain A3H haplotypes with natural resistance to HIV-1 infection was observed, highlighting the fact that SNPs and restriction may have implications on disease progression to AIDS [182].

1.7 Hypothesis and specific aims

Preliminary data obtained in the Barr laboratory has identified a long and short isoform of TRIM22, resulting from one of many SNPs in the *trim22* gene. These naturally occurring differences in the *trim22* gene can be exploited to further investigate the biological role(s) of TRIM22, and to help elucidate specific domains and amino acids that are important for its functions. The overall objective of my thesis project was to characterize a long and short isoform of TRIM22 and compare their ability to inhibit HIV-1 replication. I hypothesized that these long and short isoforms of TRIM22 differ in their ability to restrict HIV-1 replication, and that this difference is attributed to different mechanisms of restriction.

To address this hypothesis, my specific aims are:

(i) To identify and associate currently published TRIM22 isoforms to known TRIM22 functions.

(ii) To determine the pattern of localization of the long and short TRIM22 isoforms.

(iii) To compare the restrictive capabilities of the long and short TRIM22 isoforms.

(iv) To compare the ability of the long and short TRIM22 isoforms to restrict viral transcription.

(v) To identify the allele present at a known *trim22* SNP in commonly used cell lines.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cells and cell lines

Cells were maintained in standard growth medium (Dulbecco's Modified Eagle's Medium for adherent cells and RPMI-1640 for suspension cells), supplemented with 10% heat-inactivated Fetal Bovine Serum, 100 U/mL Penicillin and 100 μg/mL Streptomycin at 37°C with 5% CO2. Cell lines were obtained from American Type Culture Collection unless otherwise stated. HOS-CD4/CXCR4 was provided by Dr. F. Bushman (University of Pennsylvania, USA). The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (GHOST (3) R3/X4/R5; Cat. 3943) from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman [183]. PBMCs were isolated from whole blood from healthy volunteers using a Ficoll Hypaque (Sigma) gradient according to the manufacturer's instructions. Informed consent was obtained from all subjects according to the ethics protocol #16682E, approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) (Appendix 3).

2.2 Plasmids, transfections, and antibodies

The plasmid encoding TRIM22α (pTRIM22α) was previously described by Barr, et al. [134], and the TRIM22β plasmid (pTRIM22β) was purchased from Open Biosystems. Both plasmids were previously modified by our lab to express N-terminal HA-FLAG tags. The plasmid encoding TRIM22β containing a deleted B30.2 domain (TRIM22β-ΔB30.2) was previously generated in our lab, and is also N-terminally FLAG-tagged. The promoterless empty vector plasmid pGL3 was purchased from Promega. The plasmid encoding codon-optimized Gag (pGag) was obtained through the NIH AIDS

Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Yingying Li, Feng Gao and Beatrice H. Hahn (p96ZM651gag-opt) [184]. The plasmid encoding the replication-competent provirus HIV-1 R9 was obtained from Dr. F. Bushman (University of Pennsylvania, USA). All plasmid transfections were performed using Lipofectamine 2000 (Invitrogen), except for the Gag release western blot, which was performed using FuGene HD. Co-transfections were performed at a 5:1 ratio (pGL3, pTRIM22α, or TRIM22β: pR9 or pGag-opt respectively). Antibodies: anti-TRIM22 was obtained from Abnova, anti-FLAG from Sigma, and anti-β-actin from Rockland. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly [185-187].

2.3 Quantification of infectious virus

Clarified supernatants containing virus particles were pelleted over a 20% sucrose cushion for 2 hours at 21,000 × g. Pellets were resuspended in 300 μL fresh medium with polybrene (20μg/mL), and used to infect GHOST(3) indicator cells at approximately 50% confluency in a 12-well plate. Infection was allowed to proceed for 2-3 hours, after which the virus media was removed and replaced with 1 mL of fresh media. Approximately 36-48 hours later media was removed, and cells were harvested in 800 μL of 1x phosphate buffered saline (PBS)/10 mM EDTA. Samples were added to 5 mL round bottom tubes containing 200 μL of 10% formaldehyde in PBS (final concentration of 2% formaldehyde), and allowed to fix for at least 10 minutes before samples were analyzed for GFP expression by flow cytometry.

2.4 Western blotting

Clarified supernatants containing Gag-only particles were pelleted over a 20% sucrose cushion for 2 hours at 21,000 × g. Cells were detached, centrifuged at 350 × g for 5 minutes, and washed twice with PBS. Supernatant or cell pellets were lysed with 1× RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1× Complete Protease Inhibitor (Roche), 1% Triton X-100, 0.1% SDS). For quantitative Western blotting, samples were mixed with 4× loading buffer (40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, and 5% beta-mercaptoethanol) to a final 1× concentration and separated on a 10% SDS-PAGE gel. Protein was transferred to FluorTransW (Pall) membrane by semi-dry transfer. Western blotting was carried out by blocking the membrane for 1 hour in Li-cor Blocking Buffer (Li-cor Biosciences) followed by an overnight incubation with 1:1000 dilution of primary antibody at 4°C. Detection was carried out using IR dye-labelled secondary antibody (1:20,000 for 30 minutes at room temperature) and the Li-cor Odyssey Detection System (Li-cor Biosciences). Densitometric analysis was performed using ImageJ 1.43 u 64-bit version software (NIH, USA).

2.5 Reverse transcription polymerase chain reaction

Total RNA was isolated from transfected cell lysates using the PureLink RNA Mini Kit (Invitrogen) and reverse transcribed using M-MLV reverse transcriptase and poly dT primers according to manufacturer's instructions (Invitrogen). Quantitative real-time PCR was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) and primer pairs specific for HIV-1 Gag (fwd: 5' AAT GAT GAC AGC ATG TCA GGG 3'; rev: 5' TAC AGT TCC TTG TCT ATC GGC 3'), or β-actin (fwd: 5'

GGT CAT CAC CAT TGG CAA TGA GCG G 3'; rev: 5' GGA CTC GTC ATA CTC CTG CTT GCT G 3'). Results were expressed as the relative fold-difference between control cells and cells expressing TRIM22.

2.6 SNaPshot PCR

Total genomic DNA was isolated from cell lysates using the PureLink Genomic DNA Mini Kit (Invitrogen), and a 597 base pair region of the *trim22* B30.2 domain was amplified by PCR using the following primers: TRIM22 forward- 5' GGA TCA GAG ACA AGT GAA AAC TTT TGG TGT CTT CGG CTG CC 3'; TRIM22 reverse- 5' ACG TTC TAG ATC AGG AGC TCG GTG GGC ACA CAG 3'. Samples were sent for SNaPshot PCR analysis using the primer: TRIM22 SNP primer 5'- AGG AAA ACC CCA ATA CGA CAG GG -3'. This unique primer binds directly upstream of the SNP of interest, and a PCR extension is performed using fluorescence labelled dideoxynucleotides (ddNTP), with each of the four nucleotides (A, T, C, G) conjugated to a different wavelength molecule. The use of ddNTPs ensures only one base is added during the extension, providing a specific fluorescent signal corresponding to the incorporated nucleotide, representing the allele present at the SNP of interest.

2.7 Confocal Immunofluorescence Microscopy

Adherent cells were cultured overnight in 12-well plates on 18 mm coverslips to approximately 80% confluency. For suspension cells, approximately 1 x 10^6 cells/well were seeded into 12-well plates immediately prior to interferon treatment. For interferon stimulation, media was replaced with fresh media containing recombinant human interferon β-1b (Pestka Biomedical Laboratories) at a final concentration of 500 U/mL. For suspension cells, following a 24 hour treatment with IFN-β, cells were centrifuged at

350 × g for 5 minutes, resuspended in 300 μL of 1x PBS, and allowed to settle on poly-L-lysine coated coverslips for at least 1 hour at 37°C. For transfections, cells were transfected with pTRIM22α, pTRIM22β, or pTRIM22β-ΔB30.2 using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Twenty-four hours post-transfection/stimulation, the coverslips containing the cells were washed twice with PF buffer (1× PBS + 1% FBS), and fixed for 10 minutes in 1× PBS containing 5% formaldehyde and 2% sucrose, permeabilized in 1× PBS containing 5% NP-40 and then washed twice more with PF buffer. The coverslips were incubated with primary antibodies for one hour, washed 6× with PF buffer, incubated with secondary antibodies (Alexa Fluor 546 anti-mouse or AlexaFluor 488 anti-mouse, Invitrogen) for one hour and then washed 6× with PF buffer. Coverslips were mounted onto glass slides with ~10 μL of Vectashield mounting media with DAPI (Vector Laboratories) and then sealed with nail polish. Slides were examined using a Zeiss LSM 510 confocal fluorescence microscope and images were obtained with sequential imaging.

2.8 Molecular Modelling

Three-dimensional models of the B30.2 domain from each TRIM22 isoform were built based on homologues of known structures using the web-based server 3D-JIGSAW, version 2.0 (http://bmm.cancerresearchuk.org/~3djigsaw/) [188, 189] using the following query sequences: TRIM22α (YWVDVMLNPGSATSNVAISVDQRQVKTVRTCTFKNSNPCDFSAFGVFGCQYFS SGKYYWEVDVSGKIAWILGVHSKISSLNKRKSSGFAFDPSVNYSKVYSRYRPQY GYWVIGLQNTCEYNAFEDSSSSDPKVLTLFMAVPPCRIGVFLDYEAGIVSFFNVT NHGALIYKFSGCRFSRPAYPYFNPWNCLVPMTVCPPSS); and TRIM22β

(YWVDVMLNPGSATSNVAISVDQRQVKTVRTCTFKNSNPCDFSAFGVFGCQYFS SGKYYWEVDVSGKIAWILGVHSKISSLNKRKSSGFAFDPSVNYSKVYSRYRPQY GYWVIGLQNTCEYNAFEDSSSSDPKVLTLFMAVPPCRIGVFLDYEAGIVSFFNVT NHGALIYKFSGCRFSRPAYPYFNPWNCLVPMTVCPPSS). Models were visualized using the program Visual Molecular Dynamics (VMD), version 1.9, developed by the Theoretical and Computational Biophysics Group at the Beckman Institute for Advanced Science and Technology of the University of Illinois at Urbana-Champaign [190], using the MultiSeq extension.

CHAPTER 3: RESULTS

3.1 Summary of the general biological characteristics of the current published TRIM22 isoforms

There are currently 36 known SNPs in the *trim22* gene, including multiple frameshift or nonsense mutations that result in the production of different truncated isoforms of TRIM22 (Table 1). There are currently 19 publications characterizing the biological function of TRIM22; however, not a single report has suitably discussed the potential impact that SNPs may have on these findings. Furthermore, the TRIM22 field appears to relate all biological functions identified to a single TRIM22 isoform. In an attempt to associate known TRIM22 functions with specific *trim22* SNPs, I mined the literature to identify which TRIM22 isoforms have previously been reported. At least 4 different isoforms of TRIM22 have been studied (Figure 4), and at least 5 additional *trim22* clones have been developed without recorded nucleotide sequences (Table 2).

Retrospective analysis has revealed that the first identified *trim22* clone (accession number X82200) [121] was derived from an mRNA splice variant with a 4 amino acid deletion in the coiled-coil domain. In addition, this gene, cloned from the Daudi cell line, appears to contain a single nucleotide deletion resulting in the production of a premature stop codon, and subsequently a 52 amino acid truncation of the C-terminus. This 442 amino acid protein was suggested to restrict transcription from the HIV-1 LTR, however no further investigation was performed [121]. Although no accession numbers are given, it appears that this clone has been used in 4 additional studies (Table 2), including a 2006 study that demonstrated TRIM22 can restrict HIV-1 replication in monocyte-derived macrophages [172].

Table 1: Summary of known single nucleotide polymorphisms (SNPs) in the *trim22* gene.

Nucleotide Position	Codon Position	Amino Acid Position	Type of Mutation	SNP Allele	Resulting Amino Acid
140	2	47	Missense	A	Glu [E]
			Contig reference	T	Val [V]
182	2	61	Missense	A	Asn [N]
			Contig reference	C	Thr [T]
206	2	69	Missense	A	Gln [Q]
			Contig reference	G	Arg [R]
268	1	90	Missense	A	Lys [K]
			Contig reference	G	Glu [E]
300	3	100	Synonymous	C	His [H]
			Contig reference	T	His [H]
313	1	105	Missense	A	Lys [K]
			Contig reference	C	Gln [Q]
318	3	106	Synonymous	T	Ile [I]
			Contig reference	C	Ile [I]
372	3	124	Synonymous	G	Glu [E]
			Contig reference	A	Glu [E]
463	1	155	Missense	A	Asn [N]
			Contig reference	G	Asp [D]
510	3	170	Synonymous	T	Thr [T]
			Contig reference	C	Thr [T]
537	3	179	Frameshift (Insertion)	G	Glu [E]
			Contig reference		Glu [E]
624	3	208	Synonymous	C	Gly [G]
			Contig reference	T	Gly [G]
642	3	214	Synonymous	C	Asp [D]
			Contig reference	T	Asp [D]
694	1	232	Missense	G	Ala [A]
			Contig reference	A	Thr [T]
725	2	242	Missense	C	Thr [T]
			Contig reference	G	Arg [R]
731	2	244	Missense	T	Leu [L]
			Contig reference	C	Ser [S]
763	1	255	Missense	A	Ile [I]
			Contig reference	G	Val [V]
790	1	264	Missense	A	Met [M]
			Synonymous	C	Leu [L]
			Contig reference	T	Leu [L]
836	2	279	Missense	A	Gln [Q]
			Contig reference	G	Arg [R]

Nucleotide Position	Codon Position	Amino Acid Position	Type of Mutation	SNP Allele	Resulting Amino Acid
881	2	294	Missense	A	Lys [K]
			Contig reference	C	Thr [T]
913	1	305	Synonymous	T	Leu [L]
			Contig reference	C	Leu [L]
936	3	312	Synonymous	A	Ser [S]
			Contig reference	G	Ser [S]
962	2	321	Missense	A	Lys [K]
			Contig reference	G	Arg [R]
980	2	327	Missense	A	His [H]
			Missense	T	Leu [L]
			Contig reference	G	Arg [R]
1035	3	345	Synonymous	T	Phe [F]
			Contig reference	C	Phe [F]
1056	3	352	Synonymous	A	Ser [S]
			Contig reference	G	Ser [S]
1092	3	364	Missense	T	Asn [N]
			Contig reference	G	Lys [K]
1134	3	378	Synonymous	T	Leu [L]
			Contig reference	G	Leu [L]
1203	3	401	Synonymous	C	Tyr [Y]
			Contig reference	T	Tyr [Y]
1244	2	415	Missense	T	Ile [I]
			Contig reference	C	Thr [T]
1316	2	439	Frameshift (Deletion)		Leu [L]
			Contig reference	C	Pro [P]
1320	3	440	Frameshift (Insertion)	C	Pro [P]
			Contig reference		Pro [P]
1324	1	442	Missense	T	Cys [C]
			Contig reference	C	Arg [R]
1364	2	455	Nonsense	A	Stop [X]
			Contig reference	C	Ser [S]
1414	1	472	Missense	A	Ser [S]
			Contig reference	T	Cys [C]
1473	3	491	Missense	A	Ile [I]
			Contig reference	G	Met [M]

Table 2: Summary of *trim22* clones used in literature.

Accession Number	First Published	Length (bp; aa)	Reported Source	Notes/Unique Features [a]	Reported Use [b] (By reference)
X82200	1995	1329; 442	cDNA from Daudi cell line	a) Splice variant: - Missing NT 519-531 (AA 174-177) b) SNP at NT 725 (AA 242); R-->T c) SNP at NT 1316; Deletion - Premature stop codon at NT 1326 - Unique C-term ("LPVVLGFS")	[105, 121, 125, 131, 135]
BC035582 [c]	2002	1497; 498	cDNA from testis	Available through Open Biosystems: TRIM22-pCMV-SPORT6 (ID: 5583800)	[144, 146, 191-194]
N/A	2006	N/A	cDNA from human placenta	Sequence unknown.	[195, 196]
NM_006074	2007	1497; 498	Open Biosystems	SNP at NT 642; Synonymous	[197]
N/A	2008	N/A	CDS from human PBMCs	Sequence unknown.	[130, 132, 198]
N/A [d]	2008	1347; 448	Coding region subcloned from X82200 [e]	a) Splice variant: - Missing NT 519-531; AA 174-177 b) SNP at NT 725; R-->T (AA 242) c) Primers based off X82200: - No SNP at NT 1316, therefore no stop codon at NT 1326 - NT 1330-1347 from pcDNA3.1 backbone - Unique C-term ("ARACI")	[134]
N/A	2009	N/A	cDNA sequence	Sequence unknown.	[199]
HQ_842635	2011	1497; 498	Gene from U937 nonpermissive cells	a) SNP at NT 463; D-->N (AA 155) b) SNP at NT 725; R-->T (AA 242)	[144]
N/A	2011	N/A	CDS from monocyte-derived dendritic cells/macrophages [f]	Sequence unknown.	[144]

[a] Variations listed in relation to the BC035582 reference sequence.
[b] Due to no accession number listed, use is only suspected in some references listed, based on publication year and source listed as N. Mechti.
[c] Denoted as TRIM22β throughout this thesis.
[d] Denoted as TRIM22α throughout this thesis.
[e] Reported source may not be accurate, due to lack of SNP at nucleotide 1316.
[f] Cells were stimulated with IFNβ and lipopolysaccharide.

In 2008, Barr, et al. observed that a similarly truncated, 448 amino acid TRIM22 protein (from here referred to as TRIM22α) (Figure 4) (Table 2) was also capable of restricting HIV-1 replication. In contrast to the effects on transcription observed by Tissot and Mechti (1995), TRIM22α was shown to interfere with Gag trafficking to the plasma membrane of HOS cells [134]. Furthermore, TRIM22α was observed to restrict HIV-1 replication in multiple cell lines. Of note, intracellular Gag levels in the HOS and HeLa cell lines appeared unaffected by TRIM22α expression, yet were dramatically reduced in the U2OS and 143b cell lines, suggesting that certain TRIM22 functions may be cell-type specific [134].

A full-length (498 amino acid) protein was more recently reported to also block HIV-1 replication and LTR-mediated transcription [144]. However, a deeper look into the TRIM22 clones used revealed that this study used three different clones throughout the study, at least two of which contain unique SNPs (Table 2). One clone, which had been described in earlier studies, is a 498 amino acid protein, with a nucleotide sequence matching the *trim22* consensus sequence (BC035582, from here referred to as TRIM22β). In addition, a novel clone was created from the *trim22* gene in non-permissive U937 cells, for which the sequence is published (HQ_842635). Although the new clone is also 498 amino acid in length, it contains SNPs at nucleotides 463 and 725, which result in amino acid substitutions at positions 155 and 242, respective to TRIM22β. A third *trim22* coding sequence was also cloned from a mix of stimulated monocyte-derived macrophages and dendritic cells, for which no sequence is provided. Furthermore, it is somewhat unclear which *trim22* clones are used for which experiments,

resulting in additional uncertainty surrounding the potential effects of SNPs on TRIM22 function.

It appears that at least three other *trim22* clones have been independently produced and used for various studies (Table 2). No sequences have been published for these clones, nor have any accession numbers been given. In addition, several other groups have studied TRIM22 during in vivo studies without any indication of which isoform was studied. As a result, it is unclear which TRIM22 isoforms are associated with which biological functions of TRIM22. Taken together, these findings show that a number of different TRIM22 isoforms have been used in the literature, and multiple isoforms have been shown to restrict HIV-1 replication.

3.2 The TRIM22α and TRMI22β isoforms exhibit different patterns of localization

According to published reports, the subcellular localization of TRIM22 appears to be variable and dynamic. Some reports show TRIM22 to be cytoplasmic [105, 199], whereas others show it to be nuclear [130, 132, 193, 198] or both [144, 191, 192, 197] (Table 3). Furthermore, TRIM22 has been shown to localize to Cajal bodies [191], the centrosome, or vimentin containing aggresome-like structures next to the endoplasmic reticulum [192]. These reports do not discuss the discrepancies observed between the various TRIM22 localization patterns. Possible explanations for these observed discrepancies in subcellular localization include cell type differences, endogenous versus exogenous expression of TRIM22, different TRIM22 isoforms (long versus short), and genetic variability in the TRIM22 isoforms studied (SNPs).

Table 3: Summary of the localization patterns observed for TRIM22 in literature.

Localization	Pattern	Cell Type	Epitope Tag	Reference
Cytoplasm	Diffuse	293T	GFP or V5/His	[199]
	Diffuse	COS7	GFP or V5/His	[199]
	Diffuse	HeLa	Endogenous	[199]
	Diffuse with speckles/bodies	HeLa	GFP	[105]
	Diffuse	HeLa	GFP or V5/His	[199]
	Diffuse	PBMCs	Endogenous	[199]
	Diffuse with speckles/bodies	U2OS	GFP	[105]
Cytoplasm & Nucleus	Nucleoplasmic, with nuclear bodies[1]	ABC28	Endogenous	[191]
	Nuclear and cytoplasmic bodies	293T	HA	[144] [7]
	Diffuse throughout, or nuclear bodies[2]	HeLa	EGFP	[191] [7]
	Nucleoplasmic, with nuclear bodies	HeLa	Endogenous	[191]
	Diffuse, with cytoplasmic bodies[3]	HeLa	FLAG	[197]
	Nucleoplasmic with NB[4]	MCF7	EGFP, EYFP, or FLAG	[191] [7]
	Nucleoplasmic, with nuclear bodies	MCF7	Endogenous	[191]
	Nucleoplasmic and cytoplasmic	T47D	Endogenous	[191]
	Diffuse with speckles[5,6]	U2OS	Endogenous	[192]
Nucleus	Aggregates/bodies	293T	Myc	[130]
	Aggregates/bodies	COS7	Myc	[198]
	Diffuse with speckles/bodies	HepG2	Endogenous	[132]
	Diffuse with speckles/bodies	HepG2	Myc	[132]
	Diffuse with bodies	MCF7	FLAG	[193] [7]

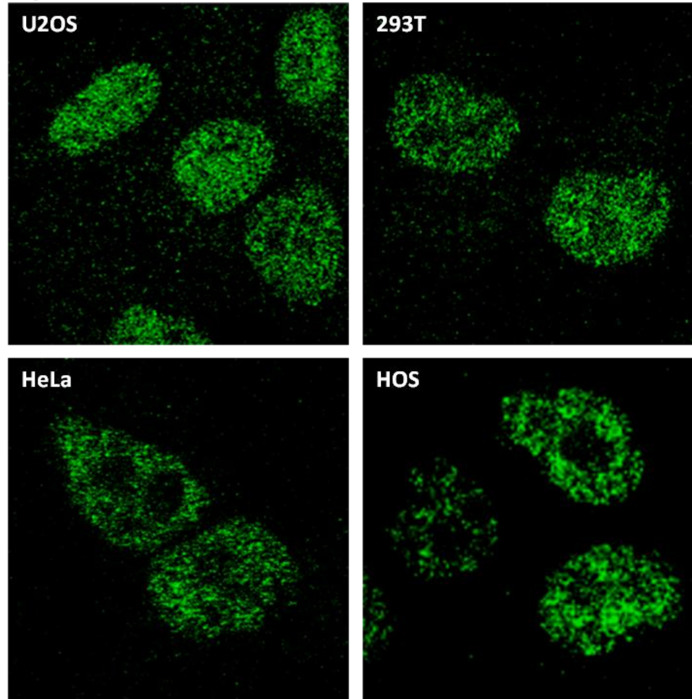
[1] Some co-localization with fibrillarin (Nucleoli).
[2] Pattern changes with cell cycle phase;
(G0/G1 = Nuclear Bodies; S-Phase = Nuclear speckles & cytoplasmic; Mitosis = Diffuse throughout cell).
[3] TRIM22 plasmid was co-expressed with Rhesus TRIM5α.
[4] Partial co-localization with Cajal bodies.
[5] Potential co-localization with calnexin (Endoplasmic reticulum).
[6] Partial co-localization with the centrosome.
[7] Same clone as TRIM22β isoform.

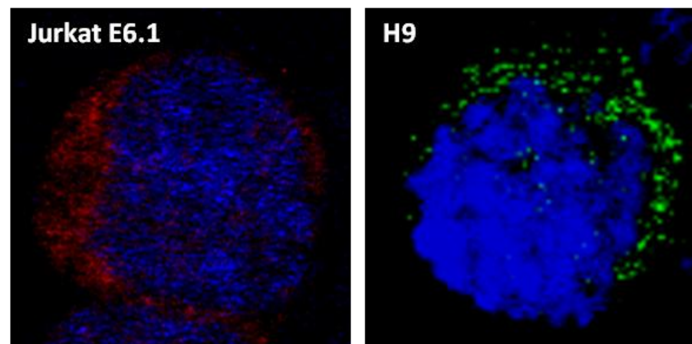
To determine if the localization of endogenous TRIM22 varied between cell types, several cell lines were treated with IFNβ to induce TRIM22 expression and analyzed by confocal immunofluorescence microscopy (Figure 5). Cells were harvested 24 h later, and expression was detected using a TRIM22 monoclonal antibody. Interestingly, TRIM22 exhibited a variety of localization patterns, depending on the cell type observed. In the U2OS, HOS, 293T, and HeLa cell lines, TRIM22 localized primarily in the nucleus in a diffuse to punctate pattern, however some TRIM22 also localized in the cytoplasm (Figure 5a). In the T cell lines Jurkat E6.1 and H9, TRIM22 localized in a diffuse to punctuate pattern throughout the cytoplasm (Figure 5b). PBMCs from two different donors were isolated and treated with IFNβ to induce TRIM22 expression. Interestingly, TRIM22 localized exclusively in clusters in the nucleus of cells from one donor, whereas in the second donor, TRIM22 localized predominantly in the cytoplasm (Figure 5c).

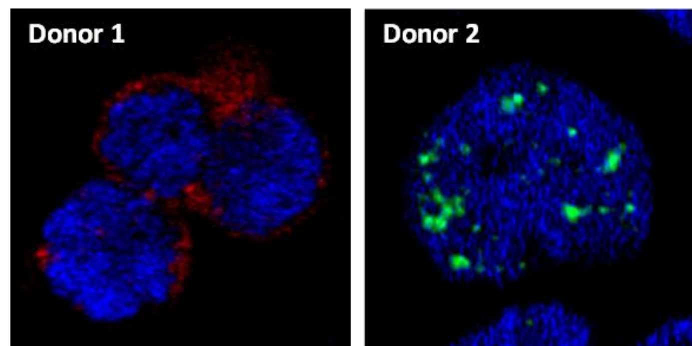
To eliminate potential cell-type differences, which may in turn impact TRIM22 function and localization, a single cell line was chosen to study the TRIM22α and TRIM22β isoforms. U2OS cells were chosen based on previous observations by Barr, et al. demonstrating that TRIM22α expression not only restricted release of HIV-1 from U2OS cells, but also resulted in decreased levels of intracellular Gag. Conversely, TRIM22α expression in HOS cells was only capable of restricting release of HIV-1, and had no effect on intracellular Gag levels. This is particularly interesting because it was observed that TRIM22α expression resulted in altered Gag trafficking in HOS cells, but no mechanism was ever investigated in U2OS cells [134]. As TRIM22β expression has

Figure 5: Subcellular localization of endogenous TRIM22 in multiple cell lines.

All cells were treated with 500 U/ml of recombinant IFN-β overnight to induce TRIM22 expression. Cells were fixed and stained with mouse anti-human TRIM22 antibody, and with secondary anti-mouse AlexaFluor 488 or AlexaFluor 546. A) TRIM22 primarily localized to the nucleus of several non-lymphoid cell lines (U2OS, 293T, HeLa, HOS). B) TRIM22 primarily localized to the cytoplasm of the T-cell lines (Jurkat E6.1, H9). C) TRIM22 exhibited different patterns of localization in PBMCs from two different donors. In donor 1, TRIM22 primarily localized to the cytoplasm in a diffuse/punctate pattern, whereas in donor 2, TRIM22 primarily localized to the nucleus and appeared to organize into nuclear bodies.


A)
U2OS
293T
HeLa
HOS


B)
Jurkat E6.1
H9


C)
Donor 1
Donor 2

been shown to block transcription from the HIV-1 LTR [144], we sought to determine which mechanism of action was active in U2OS cells.

To determine the localization of TRIM22α and TRIM22β, plasmids encoding each isoform with an N-terminal FLAG-tag were individually transfected into U2OS cells. Cells were harvested 24 hours after transfection, stained with FLAG antibodies, and analyzed using confocal immunofluorescence microscopy (Figure 6). We observed that TRIM22α localized predominantly in the cytoplasm, and exhibited a diffuse pattern of localization. Conversely, TRIM22β localized predominantly in the nucleus and often appeared to localize in clusters. Taken together, these findings reveal that TRIM22 exhibits a range of localization patterns, which is likely influenced by several factors. It appears that differences in cell lines, cell types, TRIM22 isoforms, genetics, and endogenous vs. exogenous expression may play a role in determining the localization of TRIM22.

3.3 The B30.2/SPRY domain of TRIM22β is important for nuclear localization

Given that the nuclear TRIM22β isoform has a B30.2 domain that is 50 amino acids longer than the cytoplasmic TRIM22α isoform, we hypothesized that this domain helps dictate the subcellular localization of TRIM22. To determine if the B30.2/SPRY domain is required for nuclear localization, a plasmid encoding a FLAG-tagged TRIM22β isoform with the B30.2/SPRY domain deleted (TRIM22β-ΔSPRY, previously made in our laboratory) was transfected into U2OS cells and analyzed using confocal immunofluorescence microscopy (Figure 6). We observed that deletion of the B30.2/SPRY domain abolished the nuclear localization of TRIM22β, resulting in a diffuse cytoplasmic pattern that closely resembled the localization pattern of TRIM22α.

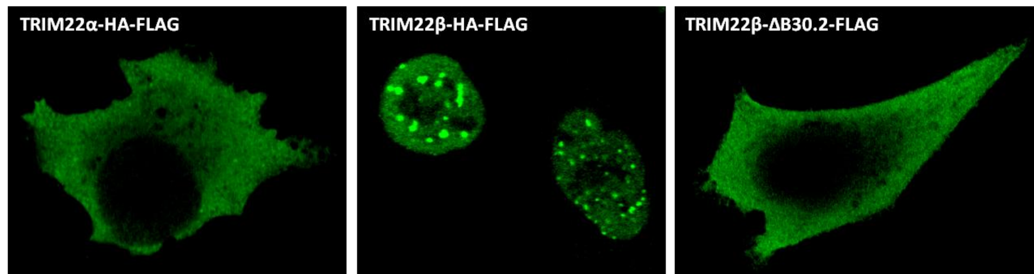


Figure 6: Subcellular localization of different TRIM22 isoforms in U2OS cells.

U2OS cells were transfected with plasmids encoding one of the TRIM22 isoforms. Twenty-four hours post-transfection, cells were fixed and stained with mouse anti-FLAG antibody, and with secondary anti-mouse AlexaFluor 488. TRIM22α exhibited diffuse localization throughout the cytoplasm, whereas TRIM22β predominantly localized to the nucleus in a nuclear body pattern. Deletion of the B30.2 domain of TRIM22β abolished body formation and nuclear localization.

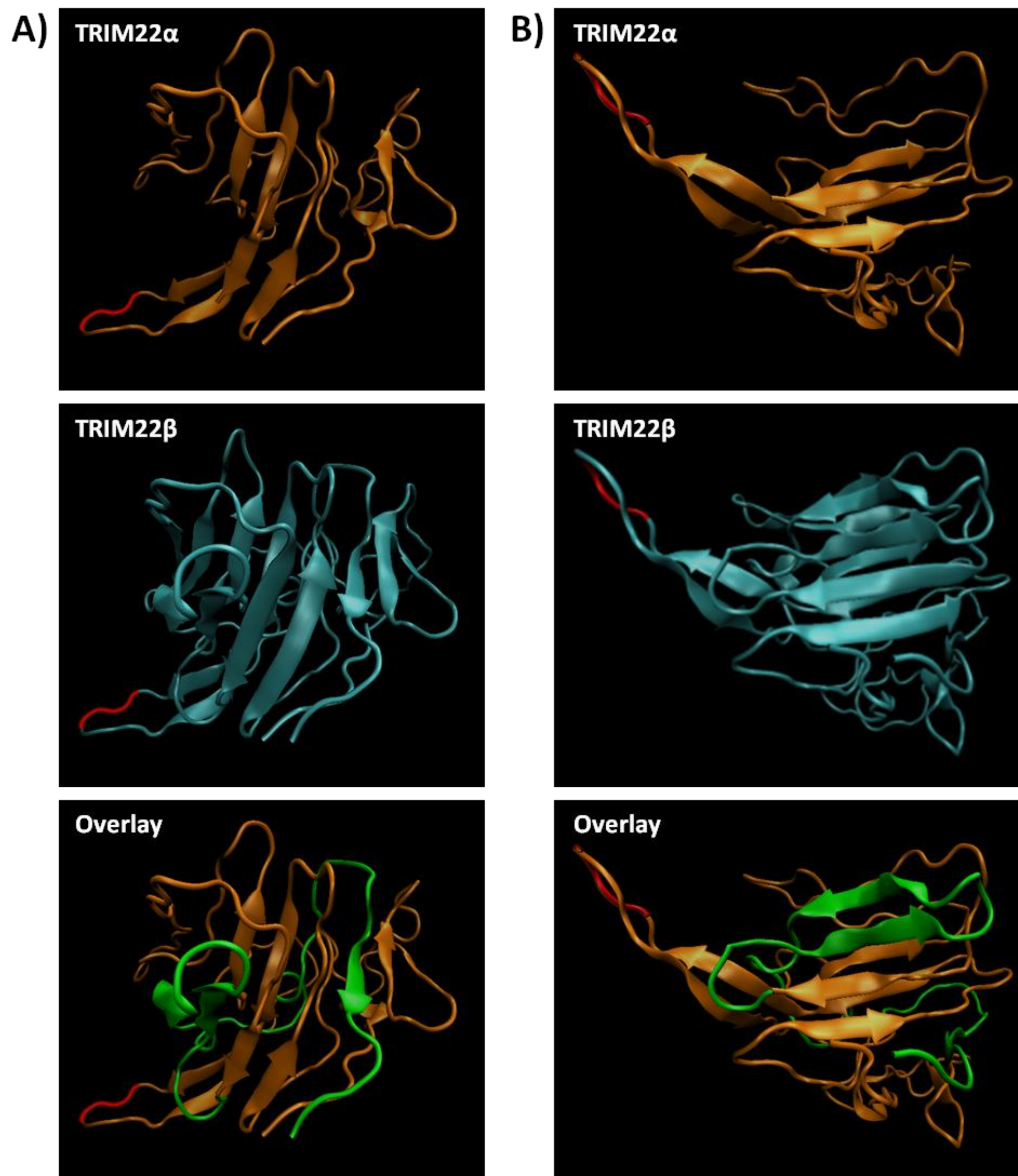
In addition, multiple reports have noted similar findings [132, 193, 199], supporting the conclusion that the B30.2/SPRY domain is required for the nuclear localization of TRIM22β.

It has been previously reported that the Spacer 2 domain of TRIM22 contains a predicted bi-partite nuclear localization signal (NLS) at amino acids 265 to 269 [121] (Figure 4). Conversely, there are no predicted NLS consensus sequences in the B30.2 domain; however, a KRK sequence is present at amino acid 380 (Figure 4). While this does not match the Lys-Arg/Lys-X-Arg/Lys consensus NLS sequence described by Chelsky, et al. [200], other groups have shown that the KRK sequence is sufficient to direct nuclear localization of several proteins, such as SHP-1 [201, 202]. As a result, we hypothesized that the presence of amino acid substitutions and/or deletions in the B30.2 domain may alter its structure and hide this KRK sequence, resulting in altered localization patterns.

Although the crystal structure of TRIM22 is yet to be solved, molecular modeling of the B30.2 domain from TRIM22α/β was performed using the programs 3D-JIGSAW and Visual Molecular Dynamics (Figure 7). The TRIM22α and TRIM22β B30.2 domain models were assigned accuracy scores of 5.40 and 5.69, respectively, indicating over 95% probability that the query and template sequences alignments are accurate. Although the position of the KRK sequence is not directly affected by the truncation, we did note the appearance of a large pocket in the TRIM22α B30.2 domain that is partially filled by the additional amino acids present in TRIM22β (Figure 7a). In addition, the TRIM22β B30.2 domain contains four anti-parallel β-sheets, two of which are lost in the TRIM22α

Figure 7: Molecular modelling of the B30.2 domain from different TRIM22 isoforms.

Molecular modelling was used to predict the structure of the B30.2 domain from different TRIM22 isoforms. The B30.2 domain from TRIM22α is depicted in orange, and that from TRIM22β is depicted in blue. The KRK NLS sequence is highlighted in red. The overlay frames illustrate the TRIM22α B30.2 domain with the C-terminal 50 amino acids from TRIM22β overlaid in green to accentuate the differences between the two isoforms. A) A large pocket is visible in the TRIM22α B30.2 domain that is partially filled by the additional amino acids possessed by TRIM22β, as indicated by the white arrow. B) A plane of four anti-parallel β-sheets present in the TRIM22β B30.2 domain is disrupted in the TRIM22α structure, as indicated by the white arrow.


A)
TRIM22α
TRIM22β
Overlay
B)
TRIM22α
TRIM22β
Overlay

structure (Figure 7b). While it is clear that B30.2 domain is important, the role that these features play in controlling localization requires further investigation.

3.4 The TRIM22α and TRIM22β isoforms possess different degrees of HIV-1 restriction in the same cell type

Although different short and long TRIM22 isoforms have independently been shown to restrict HIV-1 replication in vitro [131, 134, 144, 145], the degree to which these isoforms restrict HIV-1 in comparison to each other is unknown. To determine and compare the restriction capabilities of TRIM22α and TRIM22β in vitro, we performed HIV-1 release assays. In brief, plasmids encoding a TRIM22 isoform (or empty vector control) and a replication-competent HIV-1 provirus (pR9) were co-transfected into either HOS-CD4/CXCR4 or U2OS cells. After 48 hours, supernatants containing virus were collected, clarified via low-speed centrifugation, and used to infect the HIV reporter cell line GHOST (3) X4/R5. This reporter cell line supports HIV-1 replication and contains a green fluorescent protein (GFP) construct under the transcriptional control of the HIV-2 LTR promoter, thus cells that become infected will express GFP. Infections were allowed to proceed for 48 hours, after which the level of infection was quantified by determining the percentage of GFP-expressing cells using flow cytometry.

As shown in Figure 8, both TRIM22 isoforms substantially inhibited the release of infectious HIV-1 particles from HOS-CD4/CXCR4 cells and U2OS cells. HOS-CD4/CXCR4 cells expressing the TRIM22α isoform exhibited a 1.3-fold reduction in virus release, whereas the TRIM22β isoform resulted in a 2.8-fold reduction in virus release, compared to cells transfected with a vector control. Notably, the effects of both isoforms appeared to be much more potent in U2OS cells. Expression of TRIM22α and

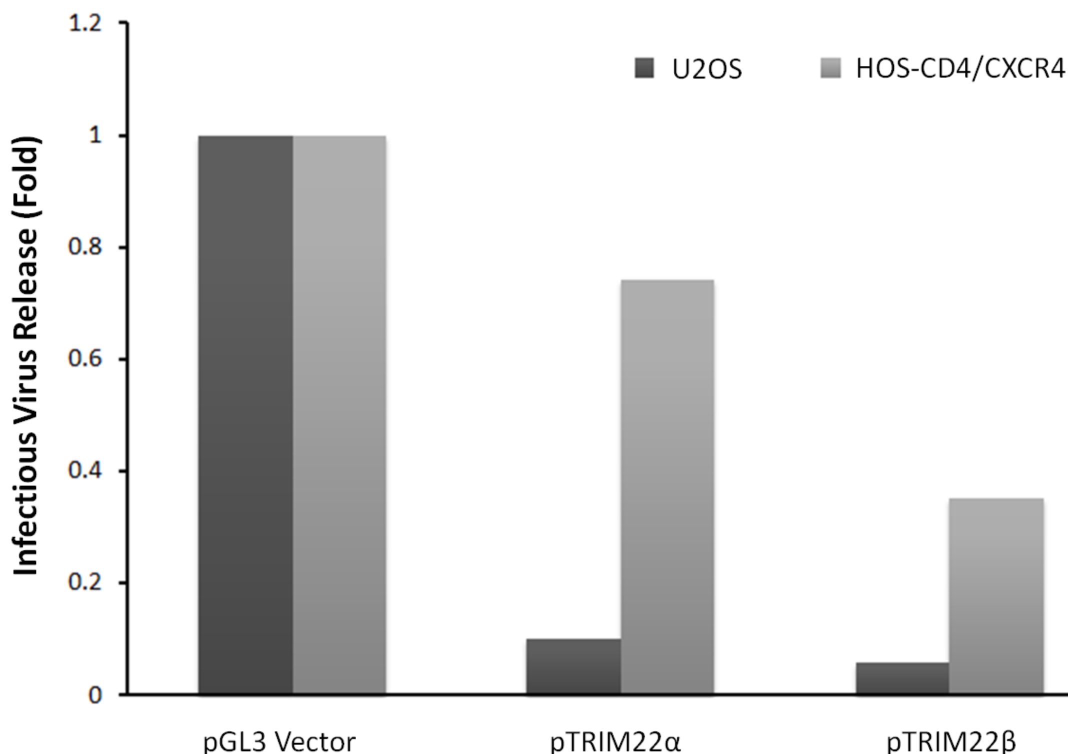


Figure 8: The TRIM22α and TRIM22β isoforms vary in their ability to restrict release of infectious HIV-1 particles.

HOS-CD4/CXCR4 and U2OS cells were co-transfected with plasmids encoding a replication-competent HIV-1 provirus (pR9), and TRIM22α, TRIM22β, or the control empty expression vector pGL3. Virus supernatants were collected 48 hours post-transfection, and used to infect the reporter cell line GHOST(3) X4/R5. Infection was allowed to proceed for 48 hours, after which GHOST(3) X4/R5 cells were harvested, fixed, and analyzed for GFP expression by flow cytometry. Results (percentage of cells fluorescing) are presented as fold difference compared to the pGL3 vector control, and represent the amount of infectious virus released from the transfected HOS-CD4/CXCR4 and U2OS cells.

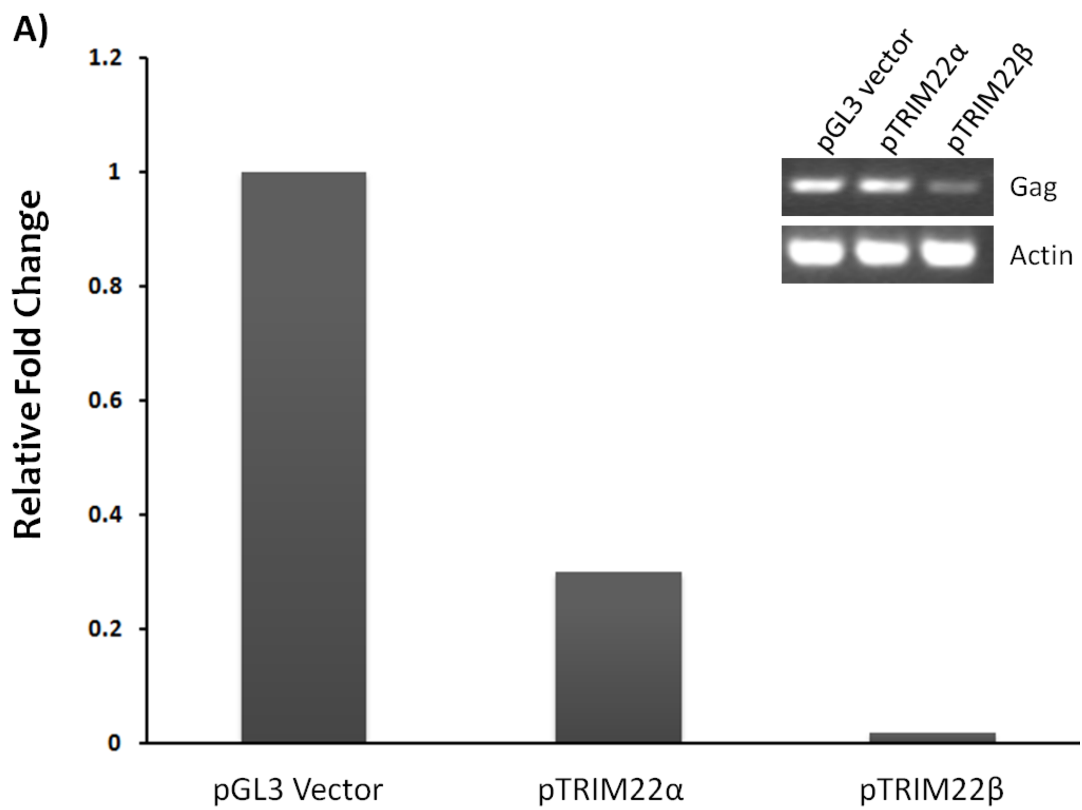
TRIM22β in U2OS cells resulted in approximately a 10-fold and 20-fold reduction in virus release, respectively, compared to cells transfected with a vector control. Taken together, these data show that TRIM22α and TRIM22β both effectively inhibit release of infectious HIV-1 particles; however the actions mediated by TRIM22β appear to be more dominant.

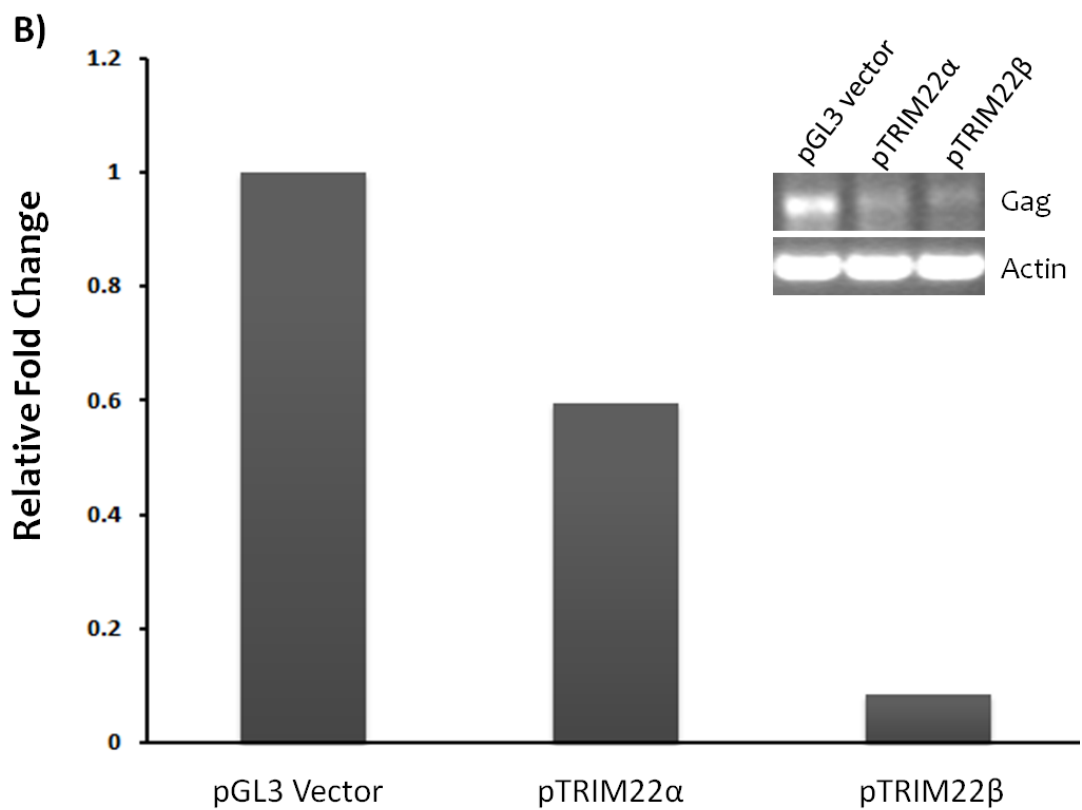
3.5 The TRIM22α and TRIM22β isoforms restrict transcription from viral promoters to varying degrees

Previous reports have shown that TRIM22α alter trafficking of the Gag polyprotein to the plasma membrane [134], and TRIM22β can inhibit transcription from the HIV-1 LTR [144]. Interestingly, TRIM22α also prevents the accumulation of intracellular Gag polyprotein when co-expressed with an HIV-1 provirus in U2OS cells [134], suggesting it may also block LTR-mediated transcription. We therefore sought to compare the ability of the TRIM22α and TRIM22β isoforms to block HIV-1 LTR transcription during a productive infection in vitro. Plasmids encoding a TRIM22 isoform (or empty vector control) and a replication-competent HIV-1 provirus (pR9) were co-transfected into U2OS cells. Cells were harvested 48 hours post-transfection and total mRNA was harvested and reverse transcribed using poly d(T) primers. HIV-1 LTR transcription was assessed via quantitative real-time PCR (qPCR) using primers specific to a region of the HIV-1 gag gene. The β-actin gene was amplified as a loading control, and CT values for gag were normalized to β-actin levels prior to analysis. We observed that both TRIM22α and TRIM22β inhibited transcription from the HIV-1 LTR, resulting in approximately a 3-fold and 20-fold reduction in gag cDNA levels, respectively, compared to cells transfected with a vector control (Figure 9a).

Figure 9: The TRIM22α and TRIM22β isoforms vary in their ability to restrict transcription from viral promoters.

U2OS cells were co-transfected with plasmids encoding TRIM22α, TRIM22β, or the control empty expression vector pGL3, and (A) a replication-competent HIV-1 provirus (pR9) or (B) a plasmid encoding Gag under the control of the CMV promoter (pGag-opt). Total cellular mRNA was reverse transcribed and cDNA was analyzed by quantitative real-time PCR (qPCR) using primers specific to a region of the gag gene, and primers amplifying β-actin as a loading control. Results were normalized to β-actin levels, and are expressed as relative fold change in expression compared to the pGL3 vector control.


A)
1.2
1
0.8
0.6
0.4
0.2
0
Relative Fold Change
pGL3 vector
pTRIM22α
pTRIM22β
Gag
Actin
pGL3 Vector
pTRIM22α
pTRIM22β


B)
1.2
1
0.8
0.6
0.4
0.2
0
Relative Fold Change
pGL3 vector
pTRIM22α
pTRIM22β
Gag
Actin
pGL3 Vector
pTRIM22α
pTRIM22β

To determine if these effects on transcription were unique to the HIV-1 LTR, the experiment was repeated using a plasmid encoding a codon optimised Gag polyprotein under control of a CMV promoter (pGag-opt), instead of an HIV-1 provirus. TRIM22α and TRIM22β inhibited transcription from the CMV promoter in a similar manner to the LTR, resulting in approximately a 2-fold and 11-fold reduction in gag cDNA levels, respectively, compared to cells transfected with a vector control (Figure 9b). To confirm this effect at the protein level, the TRIM22/pGag-opt co-transfection was repeated, and samples were harvested 48 hours post-transfection. Clarified supernatants were centrifuged over a sucrose cushion to pellet Gag VLP, and a western blot was performed on the cell lysate and supernatant fractions (Figure 10). Densitometric quantification revealed that TRIM22α and TRIM22β reduced the amount of intracellular Gag by approximately 1.76-fold and 1.57-fold, respectively, in addition to restricting the amount of Gag VLP released by 1.75-fold and 1.51-fold, respectively.

3.6 Several commonly used cell lines are negative for an SNP insertion in the *trim22* gene

The level of impact that SNPs have on controlling HIV-1 infection is yet to be determined; however, it is clear that they are an important contributing factor [175]. This concept is easily observed in individuals homozygous for the CCR5/Δ32 mutation, which confers robust resistance to HIV-1 infection [4-6]. Polymorphisms in host innate immunity genes that inhibit HIV-1 replication have also been shown to impact HIV-1 infection and disease progression. For example, certain SNP profiles for the *APOBEC3H* gene have been shown to not only affect the stability and subcellular localization of A3H, but also its capacity to restrict HIV-1 replication [179-181] and slow disease progression [182]. Recent observations have shown that *trim22* expression may be associated with

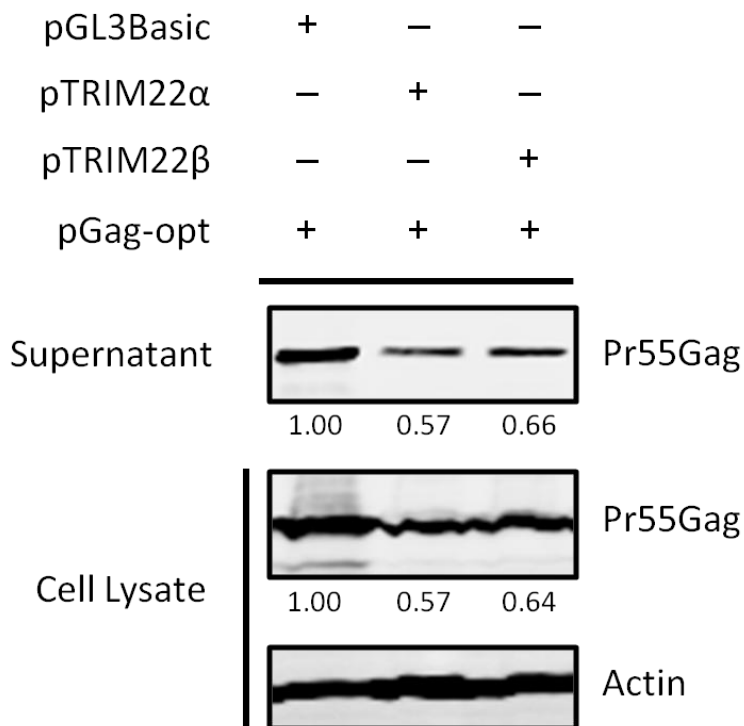


Figure 10: The TRIM22α and TRIM22β isoforms block release of HIV-1 Gag-only particles.

U2OS cells were co-transfected with plasmids encoding TRIM22α, TRIM22β, or the control empty expression vector pGL3, and a plasmid encoding Gag under the control of the CMV promoter (pGag-opt). Cells and supernatants were harvested 48 hours post-transfection, and Gag particles were pelleted by centrifugation. A western blot was performed on the cell and supernatant fractions using p24CA (anti-Gag) antibodies. Densitometric analysis was performed using ImageJ software, and is indicated by the numbers.

viral control during primary HIV-1 infection [145]. In addition, we have shown here that the localization patterns and potency of antiviral activity appear to vary between TRIM22 isoforms. The TRIM22α isoform localized to the cytoplasm and exhibited less antiviral activity than the nuclear localized TRIM22β. These isoforms may also possess different antiviral mechanisms, as demonstrated by the potent transcriptional repression exhibited by TRIM22β (Figure 9), and the ability for TRIM22α to alter trafficking of the HIV-1 Gag protein [134].

Although the TRIM22α isoform is not the direct result of an SNP, at least 3 *trim22* SNPs result in the production of isoforms very similar to TRIM22α. As similar sized TRIM22 isoforms likely have similar characteristics, we were interested in determining if any of these SNPs are present in the *trim22* gene from several commonly used cell lines. Due to financial constraints, we were only able to investigate a single SNP at the time of this study. We chose to determine the allele located at NT position 1320, as this position is subject to a known SNP insertion, resulting in a frameshift mutation, and subsequently the creation of a premature stop codon. The corresponding truncated protein is of similar length and sequence to the TRIM22α protein used in our studies, thus this SNP holds interesting implications for the localization and function of endogenous TRIM22 observed in these cell lines. Genomic DNA was first extracted from each cell line, the full B30.2 domain was amplified for each sample, and all samples were sent for SNaPshot PCR analysis. This technique is a quick and economical approach to rapidly identify specific SNPs of interest (see Methods for details). As seen in Table 4, all samples were identified to contain the consensus allele, indicating that they do not contain an insertion at position 1320. It will be important to investigate the presence of

other SNPs in these genes, as a frameshift mutation at position 1316, as well as a nonsense mutation at position 1364, also result in similarly sized TRIM22 isoforms.

Table 4: List of alleles present at nucleotide 1320 in commonly used cell lines, as reported by SNaPshot PCR.

Cell Line	**Allele**
143b	A
293T	A
CEM-SS	A
HeLa	A
HOS	A
Jurkat	A
THP-1	A
U2OS	A
U937	A

CHAPTER 4: DISCUSSION

The data detailed in this thesis clearly show that a short and a long TRIM22 isoform (TRIM22α and TRIM22β, respectively) possess different biological characteristics and antiviral capabilities. We have observed that these isoforms possess different patterns of localization, which molecular modelling suggests may be due to a change in the structure of the B30.2/SPRY domain. Although both isoforms have independently been reported to restrict HIV-1 replication [134, 144], this is the first direct comparison of two TRIM22 isoforms. Furthermore, we have shown that these isoforms exhibit different degrees of HIV-1 restriction, which is, at least in part, due to inhibition of transcription from the 5' LTR. Nevertheless, this transcriptional block is not specific to the HIV-1 LTR, as both isoforms were also capable of restricting transcription from the CMV promoter.

Several complications arise when studying TRIM22 due to i) contradictory reports on biological function, ii) potential cell type differences, iii) the existence of numerous reported SNPs in the *trim22* gene, and iv) the failure to identify the specific TRIM22 isoform used in certain published studies. To better understand the potential impact of *trim22* SNPs, we mined the literature and summarized the information currently known about TRIM22 functions. We have presented a list of 36 known SNPs in the *trim22* gene (Table 1). This list includes 4 SNPs known to result in the production of premature stop codons, and thus shorter TRIM22 proteins, similar to the TRIM22α isoform used in this study. We have also shown that no fewer than four unique TRIM22 isoforms have been used in past literature, along with at least four additional *trim22*

clones for which no sequence information has been published (Table 2). Taken together, these data help to highlight the importance of studying the effects of *trim22* SNPs and demonstrate that different TRIM22 isoforms possess varying degrees of activity.

The importance of reporting SNPs/isoforms is abundantly clear in the case of APOBEC3H, as its stability, localization and ability to restrict HIV-1 replication and slow disease progression have all been linked to SNPs [179-182]. While we are only beginning to uncover the impact that SNPs may have on TRIM22 function, the recent observation that *trim22* expression may be associated with viral control during primary HIV-1 infection [145] stresses the importance of proper documentation and understanding of *trim22* SNPs in the literature. Here we used SNaPshot PCR to show that several commonly used cell lines are negative for a single nucleotide insertion at position 1320, which is one of the SNPs resulting in a TRIM22 truncation. Unfortunately due to financial constraints, we have been unable to investigate any other SNPs in the *trim22* gene; however, an alternative project in our laboratory will be using this approach to investigate a number of SNPs in the *trim22* gene from primary donors.

While the biological impact of *trim22* SNPs is still largely unknown, there is no doubt that some of the variation between TRIM22 reports is a result of different isoforms being studied. This point is highlighted by the fact that TRIM22α and TRIM22β have been previously shown to restrict HIV-1 replication by separate mechanisms [134, 144], and several reports have observed a range of different localization patterns for TRIM22 (Table 3). In addition, numerous studies have investigated the functions of endogenous TRIM22 in different samples/cell lines with unsequenced *trim22* genes. Unfortunately, without information about the possible SNPs or isoforms present, we are unable to fully

associate and compare these findings to other information known about TRIM22 in the literature.

Several other factors have also been suggested to affect the localization of TRIM22, including cell type, method of fixation, and the epitope tag used for detection. To gain further insight into these factors, we began by investigating the localization pattern of IFNβ-induced endogenous TRIM22 in several common cell lines (Figure 5). Interestingly, endogenous TRIM22 was primarily localized to the nucleus in several non-lymphoid cell lines (HOS, U2OS, HeLa, 293T), compared to the predominantly cytoplasmic localization in two T-cell lines (Jurkat E6.1, H9), suggesting that cell type differences may be a factor contributing to localization. Similar localization patterns have previously been described for IFN-induced TRIM22 expression in U2OS and HeLa cells [191, 192]; however another report found endogenous TRIM22 to only be present in the cytoplasm of HeLa cells [199]. Nevertheless, this report did not appear to induce TRIM22 expression by any means, which may account for the observed differences. In contrast, IFNβ-treated PBMCs isolated from two different donors exhibited contrasting patterns of localization (Figure 5c), suggesting that host genetics may also impact the biological characteristics of TRIM22.

To further explore the potential effects of *trim22* SNPs on localization pattern, U2OS cells were used to investigate two different TRIM22 isoforms previously described in the literature. We observed that our TRIM22α isoform, which contains a 50 amino acid truncation of the B30.2 domain, exhibited a predominantly diffuse pattern throughout the cytoplasm (Figure 6). Although many of the reports on TRIM22 localization have no sequence information available, a similar short form (X82200) was also observed to

localize predominantly to the cytoplasm of U2OS cells [105]. Interestingly, our full-length isoform, TRIM22β, localized almost exclusively to the nucleus, and exhibited a diffuse pattern with nuclear bodies (Figure 6).

We also observed that deletion of the B30.2 domain in TRIM22β resulted in a localization pattern nearly identical to that of TRIM22α (Figure 6). This result was not surprising, as multiple reports have also noted the importance of the B30.2 domain for TRIM22 localization [132, 193, 199]. Molecular modeling of the B30.2 domain predicted the presence of a large pocket (Figure 7a), as well as the loss of several β-sheets in the TRIM22α model (Figure 7b), although the KRK NLS sequence remains in the same position in both isoforms. It is possible that the TRIM22α truncation disrupts a binding groove/interface in the B30.2 domain that is required for interaction with proteins which dictate the localization of TRIM22. Nevertheless, as we are currently limited to modeling programs, it is also possible that the loss of amino acids in the TRIM22α B30.2 domain disrupts the stabilization of this domain, resulting in unfolding and complete loss of structure. Regardless, it appears that the C-terminal 50 amino acids are required for nuclear localization, thus it will be interesting to examine if the other 4 SNPs known to exist in this region also have an impact on subcellular localization and potentially function. Future experiments using more targeted mutations within the TRIM22β B30.2 domain will help to elucidate the specific amino acids determinants for nuclear localization, and protein binding assays may be useful in discovering potential proteins that interact with this domain.

Although TRIM22α and TRIM22β have both been shown to restrict HIV-1 replication [134, 144], this was performed in separate studies and different cell lines, thus

it was important to directly compare the potency of their anti-HIV-1 activity in relation to each other. We co-transfected HOS and U2OS cells with TRIM22/HIV-1 provirus, and used the resulting viral supernatants to infect the GHOST(3) X4/R5 reporter cell line (Figure 8). This provides a measurement of the infectious virus released, allowing a direct comparison of antiviral activity, regardless of the mechanism of action. As a result, this method holds an advantage over western blot, which measures total Gag protein, and cannot discriminate between infectious and non-infectious material. Importantly, expression of either isoform resulted in a dramatic decrease in the amount of virus released, regardless of cell-type. Furthermore, TRIM22β mediated restriction of virus release was approximately twice as effective as compared to TRIM22α, suggesting that their relative antiviral activity may be consistent between cell lines.

Of note, the overall antiviral effect was much stronger in U2OS cells, suggesting that certain cell line specific differences may affect TRIM22 function. Similarly, Barr, *et al.* observed that TRIM22α expression in HOS cells had differential effects on intracellular Gag levels during proviral replication in HOS and U2OS cells. While intracellular Gag levels were unaffected by TRIM22α expression in HOS cells, they were dramatically reduced in U2OS cells [134]. It is possible this difference in intracellular Gag levels is merely a result of the reduced antiviral activity we observed in HOS cells, which may reflect cell type differences, such as the abundance of unknown cofactors, or an impact on some rate limiting step. On the other hand, we observed TRIM22α to restrict LTR transcription in U2OS cells, whereas Barr, *et al.* showed that TRIM22α alter trafficking of the Gag polyprotein in HOS cells. Therefore, it is possible that the difference in overall antiviral activity observed between HOS and U2OS cells may be the

result of separate mechanisms being utilized, which will provide an interesting avenue to follow up in future experiments.

Both the TRIM22α and TRIM22β isoforms were capable of restricting transcription from the HIV-1 LTR in U2OS cells (Figure 9a). Although TRIM22β and the X82200 TRIM22 isoform have been shown to restrict LTR-mediated transcription in 293T and Daudi cells, respectively [121, 144], this is the first evidence that TRIM22α can affect viral transcription. Interestingly, the effects on transcription are not unique to the HIV-1 LTR, as both isoforms also showed potent restriction of the CMV promoter in U2OS cells (Figure 9b). This result is in stark contrast to a previous report that observed TRIM22β to have no effect on a luciferase reporter gene under control of a CMV promoter in 293T cells [144]. An unknown TRIM22 isoform has also been shown to inhibit the activity of the hepatitis B virus core promoter in the hepatocellular carcinoma cell line HepG2, thus it is likely that TRIM22 is a broadly acting repressor of viral transcription, and potentially targets similar features among several promoters. As a result, we believe that the contradictory results obtained in U2OS and 293T cells may be a result of certain cell-specific differences, as discussed above.

How TRIM22 restricts transcription from viral promoters is unknown but a fascinating problem for further study. It is intriguing that both isoforms can effectively repress transcription, despite the fact that TRIM22α is predominantly located in the cytoplasm, and TRIM22β is predominantly nuclear. It is possible that the two isoforms target the same protein/pathway or have entirely different targets, thus it will be important to further investigate and characterize the mechanisms of action. While we have yet to rule out that TRIM22 may also target HIV-1 RNA somehow, such as Rev-

dependent RNA export, is important to note that the *gag* gene under control of the CMV promoter is both codon-optimized and Rev-independent. Taken together with the ability to restrict transcription from multiple viral promoters, it seems unlikely that RNA is a primary target.

A recent report observed a significant decrease in LTR transcription was observed in TRIM22β-expressing cells during stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin [144]. This stimulation activates the transcription factors AP-1 and NFAT [203, 204], which are both important cis-acting elements present in the enhancer region of the HIV-1 LTR [12, 14]. Furthermore, AP-1 is important for optimal transcription from the HBV [205] and CMV [206] promoters, and NFAT was recently shown to be required for optimal reactivation of latent HIV-1 in CD4+ T cells [13]. One candidate model would be that TRIM22 interferes with one or more of the transcription factors required for optimal expression, such as AP-1 of NFAT. Furthermore, certain transcription factors, such as NFAT, reside in the cytoplasm until activated, which may explain why the cytoplasmic TRIM22α isoform is also capable of restricting transcription. It will be useful to identify if either TRIM22 isoform is capable of interacting with or altering the function of these transcription factors. PMA + ionomycin stimulation is also known to result in chromatin remodelling in the 5′ LTR of bovine leukemia virus [207], thus it is also possible that TRIM22 interferes with chromatin organization surrounding the HIV-1 LTR of the integrated provirus.

It remains likely that different TRIM22 isoforms possess unique mechanisms of restriction in addition to a common ability to repress LTR dependent transcription. It is interesting to note that TRIM22β expression resulted in approximately twice the

restriction of TRIM22α in the virus release assay (Figure 8), and even more so in the qPCR assays (Figure 9); however, the two isoforms exhibited similar restriction profiles for the pGag-opt western blot (Figure 10). Only TRIM22α has previously been shown to alter Gag trafficking to the plasma membrane, thus it is possible TRIM22β possesses stronger effects on transcription and TRIM22α activity is stronger at the protein level. Surprisingly, TRIM22β restriction of the HIV-1 LTR was shown to be independent of its E3 ligase activity [144]. As this activity has been shown to be important for other TRIM22 antiviral activities, including restriction of the HBV core promoter [132, 134, 146], it will be interesting to determine if E3 ligase activity is required for the antiviral activity that we observed. Additional studies will be useful to identify the mechanisms employed by these two TRIM22 isoforms, and to provide further insight into the potential impact of SNPs on TRIM22 functions.

4.1 Limitations of this study

One limitation to consider is that the majority of our experiments were primarily performed in the U2OS cell line. There is strong evidence to suggest that TRIM22 may possess different levels of activity or perform different actions, depending on which cell line is being investigated. While we observed both isoforms to restrict transcription from the HIV-1 LTR and CMV promoter in U2OS cells, previous studies observed TRIM22α to alter trafficking of the Gag polyprotein [134], and TRIM22β did not restrict transcription from the CMV promoter in 293T cells [144]. At this point it is unclear which factors may contribute to these differences, thus it will be important to repeat these experiments in additional cells lines. Furthermore, while these cell lines are useful study

tools, they are not biologically relevant to HIV-1 infection. Future experiments should include more relevant models, such as the Jurkat E6.1 T cell line or primary cells.

While there appears to be striking differences between the TRIM22α and TRIM22β isoforms, this study was also limited by our use of only two TRIM22 isoforms. A total of 36 SNPs are currently known to exist in the *trim22* gene, including 21 missense mutations, and 4 SNPs which result in different length proteins. Currently none of these SNPs have been investigated, thus it will be important to examine additional isoforms to better understand what effect these SNPs have on the functions of TRIM22. In addition, due to financial limitations, we were only able to investigate the prevalence of one SNP in a small number of cell lines; however, it will be essential to determine the prevalence of these SNPs in the population.

4.2 Future directions

The present study demonstrated that two unique TRIM22 isoforms are capable of restricting HIV-1 transcription and replication in the U2OS cell line. While we provided the first evidence that the TRIM22α isoform can restrict viral transcription, TRIM22α has previously been shown to alter Gag trafficking in HOS cells. Nevertheless, it is currently unknown if TRIM22α can also alter Gag trafficking in U2OS cells, or restrict LTR transcription in HOS cells. Determining which antiviral mechanisms are active in these cell lines may help to elucidate additional factors required for TRIM22-mediated restriction and explain the dramatic difference in antiviral potency between HOS and U2OS cells.

In addition, all previous experiments demonstrating that TRIM22 can restrict LTR transcription have been performed using components from HIV-1 subtype B viruses.

While this is the best characterized form, subtype B only accounts for 12% of global infections, and observations obtained with this subtype may not be transferrable to other subtypes due to considerable differences in LTR composition, such as the number of NFκB response elements [10, 16]. It will be important to determine the capacity of TRIM22 to restrict transcription from the LTR of other HIV-1 subtypes. Furthermore, TRIM22 has also been shown to restrict transcription from the hepatitis B virus core promoter, thus it will be interesting to test the effect of TRIM22 on other viral promoters. This will not only help determine the breadth of TRIM22 activity, but these experiments may also help elucidate possible targets for TRIM22 based on common elements between viral promoters.

In order to better understand the relationship between SNPs and TRIM22 function, it will also be important to identify the TRIM22 isoforms used in previous studies, where possible, and to report all TRIM22 isoforms used in future experiments. It will also be useful to develop and characterize a panel of TRIM22 isoforms to determine which SNPs influence TRIM22 features, such as localization. Furthermore, experiments examining the *trim22* haplotypes of both healthy and infected individuals will help determine the prevalence of each SNP and reveal any correlation between specific SNPs and HIV-1 disease progression.

4.3 Conclusions

In general, the study of how our immune system responds to HIV-1 infection has been a focus of researchers worldwide since AIDS was first recognized over 30 years ago. Understanding how a small percentage of individuals are naturally capable of

controlling HIV-1 replication remains one of the most sought after objectives towards the development of more effective therapies. While the contribution of cellular restriction factors in controlling HIV-1 infection is still controversial, evidence continues to build suggesting they do play a role. Furthermore, the effect that SNPs have on HIV-1 disease progression is becoming increasingly clear, and will be a necessary factor to consider in future investigations.

Although findings on TRIM22 continue to suggest it is an important antiviral protein capable of restricting HIV-1 replication, much work remains to be done in order to fully ascertain how this protein functions. A balance between laboratory and clinical studies will help to elucidate the mechanisms responsible for these antiviral actions, as well as any potential effect TRIM22 may have on the progression of HIV-1 infection *in vivo*. Regardless, routine reporting of the TRIM22 isoforms used, along with the execution of studies directly comparing the actions of multiple isoforms will be a crucial step towards better understanding how SNPs may impact the function of TRIM22. Overall, increasing our knowledge of these host-pathogen interactions will allow for increased understanding of HIV-1 pathogenesis and the continuation of breakthroughs in HIV/AIDS treatment and prevention.

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APPENDICES

Appendix 1: Sequence information for the TRIM22α isoform.

NUCLEOTIDE [CODING SEQUENCE: 1347 bp]

atggatttctcagtaaaggtagacatagagaaggaggtgacctgccccatctgcctggagctcctgacagaacctctgagccta
gattgtggccacagcttctgccaagcctgcatcactgcaaagatcaaggagtcagtgatcatctcaagaggggaaagcagctgt
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gatctgagtgggatgctgcaagttcttaaagagctgacagatgtccagtactactgggtggacgtgatgctgaatccaggcagtg
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tctgcttttggtgtcttcggctgccaatatttctcttcggggaaatattactgggaagtagatgtgtctggaaagattgcctggatcct
gggcgtacacagtaaaataagtagtctgaataaaaggaagagctctgggtttgcttttgatccaagtgtaaattattcaaaagtttac
tccagatatagacctcaatatggctactgggttataggattacagaatacatgtgaatataatgcttttgaggactcctcctcttctgat
cccaaggttttgactctctttatggctgtgcctccctgtcgtattggggttttcctagctcgagcatgcatctag

PROTEIN (448 aa)

MDFSVKVDIEKEVTCPICLELLTEPLSLDCGHSFCQACITAKIKESVIISRGESSCPV
CQTRFQPGNLRPNRHLANIVERVKEVKMSPQEGQKRDVCEHHGKKLQIFCKEDG
KVICWVCELSQEHQGHQTFRINEVVKECQEKLQVALQRLIKEDQEAEKLEDDIR
QERTAWKIERQKILKGFNEMRVILDNEEQRELQKLEEGEVNVLDNLAAATDQLV
QQRQDASTLISDLQRRLTGSSVEMLQDVIDVMKRSESWTLKKPKSVSKKLKSVF
RVPDLSGMLQVLKELTDVQYYWVDVMLNPGSATSNVAISVDQRQVKTVRTCTF
KNSNPCDFSAFGVFGCQYFSSGKYYWEVDVSGKIAWILGVHSKISSLNKRKSSGF
AFDPSVNYSKVYSRYRPQYGYWVIGLQNTCEYNAFEDSSSSDPKVLTLFMAVPP
CRIGVFLARACI

NOTE

Underlined sequence is from the pcDNA3.1 plasmid.

Appendix 2: Sequence information for the TRIM22β isoform.

ACCESSION BC035582

NUCLEOTIDE [CODING SEQUENCE: 1497 bp]

atggatttctcagtaaaggtagacatagagaaggaggtgacctgccccatctgcctggagctcctgacagaacctctgagccta
gattgtggccacagcttctgccaagcctgcatcactgcaaagatcaaggagtcagtgatcatctcaagaggggaaagcagctgt
cctgtgtgtcagaccagattccagcctgggaacctccgacctaatcggcatctggccaacatagttgagagagtcaaagaggtc
aagatgagcccacaggaggggcagaagagagatgtctgtgagcaccatggaaaaaaactccagatcttctgtaaggaggatg
gaaaagtcatttgctgggtttgtgaactgtctcaggaacaccaaggtcaccaaacattccgcataaacgaggtggtcaaggaatg
tcaggaaaagctgcaggtagccctgcagaggctgataaaggaggatcaagaggctgagaagctggaagatgacatcagaca
agagagaaccgcctggaagaattatatccagatcgagagacagaagattctgaaagggttcaatgaaatgagagtcatcttgga
caatgaggagcagagagagctgcaaaagctggaggaaggtgaggtgaatgtgctggacaacctggcagcagctacagacca
gctggtccagcagaggcaggatgccagcacgctcatctcagatctccagcggaggttgaggggatcgtcagtagagatgctgc
aggatgtgattgacgtcatgaaaaggagtgaaagctggacattgaagaagccaaaatctgtttccaagaaactaaagagtgtatt
ccgagtaccagatctgagtgggatgctgcaagttcttaaagagctgacagatgtccagtactactgggtggacgtgatgctgaat
ccaggcagtgccacttcgaatgttgctatttctgtggatcagagacaagtgaaaactgtacgcacctgcacatttaagaattcaaat
ccatgtgatttttctgcttttggtgtcttcggctgccaatatttctcttcggggaaatattactgggaagtagatgtgtctggaaagattg
cctggatcctgggcgtacacagtaaaataagtagtctgaataaaaggaagagctctgggtttgcttttgatccaagtgtaaattattc
aaaagtttactccagatatagacctcaatatggctactgggttataggattacagaatacatgtgaatataatgcttttgaggactcct
cctcttctgatcccaaggttttgactctctttatggctgtgcctccctgtcgtattggggttttcctagactatgaggcaggcattgtctc
atttttcaatgtcacaaaccacggagcactcatctacaagttctctggatgtcgcttttctcgacctgcttatccgtatttcaatccttgg
aactgcctagtccccatgactgtgtgcccaccgagctcctga

PROTEIN (498 aa)

MDFSVKVDIEKEVTCPICLELLTEPLSLDCGHSFCQACITAKIKESVIISRGESSCPV
CQTRFQPGNLRPNRHLANIVERVKEVKMSPQEGQKRDVCEHHGKKLQIFCKEDG
KVICWVCELSQEHQGHQTFRINEVVKECQEKLQVALQRLIKEDQEAEKLEDDIR
QERTAWKNYIQIERQKILKGFNEMRVILDNEEQRELQKLEEGEVNVLDNLAAAT
DQLVQQRQDASTLISDLQRRLRGSSVEMLQDVIDVMKRSESWTLKKPKSVSKKL
KSVFRVPDLSGMLQVLKELTDVQYYWVDVMLNPGSATSNVAISVDQRQVKTVR
TCTFKNSNPCDFSAFGVFGCQYFSSGKYYWEVDVSGKIAWILGVHSKISSLNKRK
SSGFAFDPSVNYSKVYSRYRPQYGYWVIGLQNTCEYNAFEDSSSSDPKVLTLFM
AVPPCRIGVFLDYEAGIVSFFNVTNHGALIYKFSGCRFSRPAYPYFNPWNCLVPM
TVCPPSS

Appendix 3: Ethics approval notice for use of human subjects.

Determining the impact of cellular restriction factor variants on HIV evolution and disease progression.
BARR, Stephen D. Research Proposal

Office of Research Ethics

The University of Western Ontario

(Contact information removed for publication)

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Mr. S. Barr
Review Number: 16682E **Review Level:** Expedited
Review Date: December 16, 2009 **Approved Local # of Participants:** 125
Protocol Title: Characterization of interferon-induced genes and their role in blocking HIV replication.
Department and Institution: Microbiology & Immunology, University of Western Ontario
Sponsor: CANADIAN INSTITUTE OF HEALTH RESEARCH-ONT HIV
Ethics Approval Date: January 05, 2010 **Expiry Date:** April 30, 2015
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:
a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

(Signature removed for publication)

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
☐ Janice Sutherland	☐ Elizabeth Wambolt	☑ Grace Kelly	☐ Denise Grafton

(Contact information removed for publication)

This is an official document. Please retain the original in your files. cc: ORE File

UWO HSREB Ethics Approval - Initial
V.2008-07-01 (rptApprovalNoticeHSREB_Initial) 16682E Page 1 of 1

CURRICULUM VITAE

Name:	Clayton Hattlmann	
Post-secondary Education and Degrees:	The University of Western Ontario, MSc. London, Ontario, Canada	2009-2012
	The University of Western Ontario, BMSc. London, Ontario, Canada	2004-2009
Honours and Awards:	Schulich Graduate Scholarship	2009-2011
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	Dean's Honor List	2008-2009
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Publications:

Woods, M.W., Kelly[+], J.N., **Hattlmann[+], C.J.**, Xu, L.S., Tong, J.G.K., Smiley, J.R., Barr, S.D. 2011. Human HERC5 restricts an early stage of HIV-1 assembly by a mechanism correlating with the ISGylation of Gag. Retrovirology. 8: 95. ([+] contributed equally)

Ha, S., Park, S., **Hattlmann, C.J.**, Barr, S.D., Kim, S.O. 2011. Inhibition or deficiency of cathepsin B leads to defects in HIV-1 Gag pseudoparticle release in macrophages and HEK293T cells. Antiviral Research. 93(1):175-84.

Kelly, J., Tong, J., **Hattlmann, C.**, Woods, M., Barr, S.D. (2011) ISBN 978-953-307-665-2. Book Title: HIV and AIDS – Updates on Biology, Immunology, Epidemiology and Treatment Strategies. Chapter Title: "Cellular Restriction Factors: Can We Exploit the Body's Natural Antiviral Proteins to Combat HIV/AIDS?" (Invited Book Chapter)