2013

Identifying and Characterizing the Degradative Pathway of the Retroviral Restriction Factor RhTRIM5α

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Recommended Citation


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IDENTIFYING AND CHARACTERIZING THE DEGRADATIVE PATHWAY OF
THE RETROVIRAL RESTRICTION FACTOR RHTRIM5α

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
RACHEL NELSON
CHICAGO, IL
DECEMBER 2013
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Edward Campbell for his guidance and encouragement throughout my thesis project. He has been a wonderful teacher and role model, and I have learned a lot during my time in his lab. I would also like to thank my committee members, Dr. Chris Wiethoff and Dr. Karen Visick for their helpful suggestions and input on my project. Lastly, I would like to thank my family and friends for supporting me during my time in graduate school. I couldn’t have done it without all of these valuable people.
TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF FIGURES vi

ABSTRACT viii

CHAPTER ONE: INTRODUCTION 1
  Human immunodeficiency virus 1
  Tripartite motif containing proteins 3
    TRIM5α: Species specific retroviral restriction factor 7
    Possible mechanisms of retroviral restriction by rhTRIM5α 9
  Cellular degradation pathways 11
    Ubiquitin-proteasome system 11
    Autophagic-lysosomal degradation 13
    Cross-talk between the UPS and autophagy-lysosomal pathways 21
  Degradation of TRIM proteins 23
    TRIM79α: Flavivirus restriction factor 23
    TRIM50: Implicated in Williams Beuren syndrome 24
    TRIM5α: Association with proteins important for both degradative pathways 25
  Summary and conclusions 28

CHAPTER TWO: MATERIALS AND METHODS 30
  Cell lines, viruses and pharmaceuticals 30
  Recombinant DNA constructs and generation of stable cell lines 31
  Steady state protein analysis 32
  Protein turnover assay 32
  Flow cytometry 33
  Detection of endogenous huTRIM5α 33
  Immunofluorescence microscopy 34
  Image Analysis 34
  siRNA transfections 35
  Quantitative Real-Time PCR for viral RT products 36
  Statistical Analysis 38
CHAPTER THREE: RESULTS
Identifying the cellular degradation pathway responsible for
TRIM5α turnover
TRIM5α is degraded via chaperone mediated autophagy
rhTRIM5α protein degradation is important for retroviral
restriction

CHAPTER FOUR: DISCUSSION
Degradation of rhTRIM5α and huTRIM5α by the autophagy-
lysosomal pathway
Degradation of rhTRIM5α by chaperone mediated autophagy
rhTRIM5α degradation alters the retroviral restriction profile
Conclusion

REFERENCES

VITA
LIST OF FIGURES

Figure                                Page
1. Steps in the retroviral life cycle   4
2. TRIM proteins are characterized by the C-terminal domain 6
3. Degradation of cellular proteins by the proteasome and autophagy-lysosomal systems 22
4. Subcellular localization of YFP-rhTRIM5α changes in the presence of BafA1 and MG132 41
5. rhTRIM5α protein is sensitive to the autophagy inhibitor BafA1 43
6. Altered rhTRIM5α protein turnover in the presence of BafA1 or MG132 45
7. Altered endogenous huTRIM5α protein degradation in the presence of BafA1 47
8. rhTRIM5α co-localizes with the autophagy marker LC3b 49
9. rhTRIM5α co-localizes with the autophagy marker LC3b independent of p62 51
10. rhTRIM5α co-localizes with the autophagy marker LAMP2 53
11. rhTRIM5α protein is sensitive to BafA1 in the presence of Atg5 knockdown 55
12. Reduced LAMP2 leads to increased rhTRIM5α protein 57
13. Reduced Hsc70 and LAMP2 leads to increased rhTRIM5α Protein 58
14. Identification and characterization of putative CMA vi
sequences in rhTRIM5α

15. rhTRIM5α mediated restriction of viral late RT products is not affected by lysosomal inhibition

16. rhTRIM5α mediated restriction of B-MLV is enhanced by LAMP2 siRNA

17. Model of CMA-mediated degradation of TRIM5α
ABSTRACT

Human immunodeficiency virus 1 (HIV-1) is a lentivirus belonging to the retroviridae family that leads to the development of acquired immunodeficiency syndrome (AIDS) via the destruction of CD4+ T cells. The cellular host protein TRIM5α found in rhesus macaques (rhTRIM5α) can prevent HIV-1 infection via an early block to infection after entry of the virion into target cells. The mechanism of restriction is poorly understood; however, treatment of rhTRIM5α expressing cells with inhibitors to a cellular degradation pathway, the proteasome, relieves an intermediate step of the viral life cycle, although infection remains restricted by TRIM5α. Interestingly, treatment of rhTRIM5α expressing cells with proteasomal inhibitors does not alter rhTRIM5α protein turnover. The role of a second cellular degradation pathway, the autophagy-lysosomal pathway, in TRIM5α mediated restriction has not been explored. Lysosomal degradation occurs within double membrane vesicles, derived from the endoplasmic reticulum. Delivery of proteins to lysosomes can occur via diverse mechanisms, such as macroautophagy, microautophagy or chaperone mediated autophagy (CMA). Previously, TRIM5α was shown to interact with two proteins, p62 and Hsc70, necessary for autophagic degradation via selective macroautophagy and chaperone mediated autophagy, respectively.

In the present study, we provide evidence that rhTRIM5α is degraded via chaperone mediated autophagy. Using inhibitors of lysosomal degradation, we show that rhTRIM5α protein turnover decreases and rhTRIM5α subcellular localization is altered. Additionally, we show that human TRIM5α protein degradation is inhibited when
lyosomal degradation is inhibited. These data indicate that lysosomal degradation is responsible for TRIM5α protein turnover. We found that treatment with lysosomal inhibitors increased the co-localization between TRIM5α and proteins important for autophagy-lysosomal degradation. We show that when macroautophagy is inhibited, TRIM5α protein degradation remains sensitive to lysosomal inhibition. We found that genetic inhibition of CMA prevented rhTRIM5α turnover, indicating that CMA may be the pathway responsible for rhTRIM5α degradation. Furthermore, we identify a CMA targeting motif within the coiled-coil domain of rhTRIM5α that decreases rhTRIM5α sensitivity to lysosomal inhibitors, indicating that rhTRIM5α is degraded by CMA.

Our data suggests that rhTRIM5α degraded by the lysosome, specifically chaperone mediated autophagy. Therefore, we hypothesized that the lysosomal degradation of TRIM5α is important for viral restriction. When rhTRIM5α expressing cells were treated with lysosomal inhibitors, there was no difference in the level of viral intermediates, while proteasomal inhibitors could restore these intermediates to similar levels as the unrestricted control cells. We also used siRNA specific for a lysosomal protein important for CMA. Under these conditions, we observed a slight, but statistically significant, decrease in viral intermediates. Additionally, we observed increased restriction of B tropic murine leukemia virus (B-MLV) infectivity, which is usually unaffected by TRIM5α. Therefore, we conclude that TRIM5α is degraded by chaperone mediated autophagy and that inhibition of chaperone mediated autophagy can enhance TRIM5α mediated restriction.
CHAPTER ONE

INTRODUCTION

**Human immunodeficiency virus**

Human immunodeficiency virus 1 (HIV-1) is a lentivirus belonging to the retroviridae family. HIV-1 is the infectious agent that leads to acquired immunodeficiency syndrome (AIDS), which emerged in human populations in the early 1980s. Since its discovery, HIV-1 and AIDS have been identified as a major epidemic in the 20th and 21st centuries. As of 2011, it was estimated that 34.2 million people were living with HIV, with 2.5 million people newly infected that year. In 2011 alone, 1.7 million people died from AIDS (Piot and Quinn 2013).

Patients infected with HIV-1 initially suffer from non-specific symptoms such as fever, sore throat and rash (Schacker, Collier et al. 1996). Patients with AIDS often succumb to fatal opportunistic infections and malignancies, which is due to a deficiency in CD4+ T cells (Gottlieb, Schroff et al. 1981; Masur, Michelis et al. 1981). Specifically, activated CD4+ CCR5+ T cells were soon identified as the major cell type infected by HIV-1 (Swanstrom and Coffin 2012).

Initial infection with HIV-1 leads to the development of non-specific symptoms. Interestingly, HIV-1 can lead to a depletion of CD4+ T cells within the gut associated
lymphoid tissue (GALT) with no concurrent development of gastrointestinal distress (Swanstrom and Coffin 2012). Viremia, as measured by viral RNA copies in the blood plasma, can range from <40 copies/ml to 100,000 copies/ml. AIDS develops when the CD4+ T cell count falls below 200 cells/μl blood plasmid (Lackner, Lederman et al. 2012). With early antiviral treatment, immune function improves, leading to a decreased risk of complications. It is estimated that people with HIV-1 who receive early treatment can approach survival rates similar to the uninfected population. In fact, with advent of highly active anti-retroviral therapy (HARRT), the causes of death in AIDS patients has shifted from opportunistic infections to cardiovascular disease, renal insufficiency and a variety of malignant disorders (Lackner, Lederman et al. 2012).

Patients infected with HIV-1 have a diminished T cell response as the initial target of infection is activated CD4+ T cells. CD4 is the primary receptor for HIV-1. Early in infection, the virus uses the cytokine CCR5 as a co-receptor. As the virus evolves and spreads, there is an emergence of viruses with altered cellular tropism. This causes the virus to spread to different cell types such as macrophages. Additionally, the virus glycoproteins can evolve to use a new co-receptor, CXCR4 (Wilen, Tilton et al. 2012). The emergence of so-called X4 and macrophage tropic viruses indicates a poor patient prognosis, as more cells can be infected and destroyed by the virus (Hunt, Harrigan et al. 2006; Swanstrom and Coffin 2012).

The HIV-1 genome consists of two copies of positive strand RNA packaged within the viral nucleocapsid complex (Lu, Heng et al. 2011). This is encapsidated by 1500 capsid protein (CA) monomers (Arhel 2010). The virus is packaged in an envelope
derived from the host cell plasma membrane (Figure 1). Upon infection of a target cell, the virus glycoprotein, gp120, engages the plasma membrane receptor CD4 (Freed 1998). Through the interaction of a co-receptor, fusion between the viral envelope and host cell plasma membrane occurs to allow for the release of viral core (consisting of viral CA and nucleoprotein complexed together) into the host cell cytoplasm. The virus undergoes a process of CA disassembly known as uncoating, either concurrent or immediately preceding viral reverse transcription. The reverse transcribed viral genome, which exists as a double stranded DNA product, translocates into the nucleus. The virus genome integrates into the host genome, a reaction that is catalyzed by the viral integrase protein (Figure 1). The initial steps of viral infection and replication have been extensively studied in an attempt to identify and develop antiviral targets.

**Tripartite motif containing proteins**

The tripartite motif (TRIM) family of proteins is a large, structurally diverse group of host proteins that are implicated in the prevention of viral infection (Perron, Stremlau et al. 2004; Stremlau, Owens et al. 2004; Uchil, Hinz et al. 2013; Versteeg, Rajsbaum et al. 2013). The TRIM is characterized N-terminally to C-terminally by a Really Interesting Gene (RING) domain, one or two Bbox domains, and a coiled–coil domain (CC) (Reddy, Etkin et al. 1992). The first large scale identification of TRIM family members was performed by Reymond et al. Using a bioinformatics approach, 37 TRIM family members, and 34 new TRIM splice variants in mammals were identified (Reymond, Meroni et al. 2001). This study demonstrated that TRIM proteins could
Figure 1 Steps in the retroviral life cycle. Schematic highlighting the process of viral entry and exit. The viral glycoprotein engages the primary receptor CD4 (co-receptors are not indicated). This leads to fusion of the viral membrane with the host cell plasma membrane to allow for the viral core to enter the cytoplasm. Partial uncoating of the viral nuclocapsid and reverse transcription occur in the cytoplasm. The reverse transcribed genome enters the nucleus and is incorporated into the host chromosome, a process catalyzed by the viral integrase. Viral transcription occurs via the cellular RNA polymerase II (RNAPII) and the viral RNA products are exported out of the nucleus. These products can serve as the new viral genome and be packaged into the viral particle or they can serve as the mRNA templates to produce viral proteins. The viral particle is assembled and released at the plasmid membrane.
assemble into unique cellular accumulations when exogenously expressed in HeLa or U2OS cells. Since this initial characterization, close to 100 mammalian TRIM proteins have been described (Han, D. et al. 2011). Many of these TRIM genes are controlled through interferon (IFN), both type I and II, indicating a conserved role for TRIM proteins in the host antiviral response (Carthagena, Bergamaschi et al. 2009). Additionally, many large scale, systematic screens of TRIM protein have shown this family to be involved in restriction of retroviruses, RNA viruses, and DNA viruses (Uchil, Quinlan et al. 2008; Uchil, Hinz et al. 2013; Versteeg, Rajsbaum et al. 2013).

The various domains of the TRIM confer unique functions on the protein, namely E3 ubiquitin ligase function from the RING domain, higher order multimerization from the B-box domain(s) and dimerization from the CC domain (Meroni and Diez-Roux 2005); (Reymond, Meroni et al. 2001). Diversity within the TRIM family comes from the C-terminal domain, with the majority of TRIM proteins containing a B30.2/SPRY domain, which allows for protein-protein interactions (Napolitano and Meroni 2012) (Figure 2). The RING domain of TRIM proteins has been implicated in protein ubiquitination on Lysine 48 (K48) or Lysine 63 (K63), which can lead to diverse outcomes within the cell such as proteasomal degradation, NFκB signaling, DNA repair and protein targeting to the lysosome (Deshaies and Joazeiro 2009; Ye and Rape 2009). Additionally, TRIM proteins can interact with E2 proteins important for SUMOylation and ISGylation (Zou and Zhang 2006; Chu and Yang 2011; Napolitano and Meroni 2012). Therefore, TRIM proteins may represent a cache of proteins involved in numerous cellular processes.
Figure 2. TRIM proteins are characterized by the C-terminal domain. There are over 100 human TRIM genes. TRIM proteins are identified by the conserved N-terminal RING-BBox-coiled coil (RBCC) domain. TRIM proteins are divided into 13 subfamilies based on the C-terminal domains.
**TRIM5α: Species specific retroviral restriction factor**

The first TRIM protein that was found to prevent viral infection was a variant of TRIM5, TRIM5α. Specifically, Stremlau et al. found that TRIM5α from rhesus macaques (rhTRIM5α) can potently prevent HIV-1 infection (Stremlau, Owens et al. 2004). Even before the identification of rhTRIM5α, it was known that rhesus macaques could not be productively infected by HIV-1; that this block in infection occurred after viral entry into target cells; and that this inhibition was dependent on the viral capsid (CA) (Bieniasz 2003). Indeed, Stremlau et al. demonstrated that rhTRIM5α acted early to prevent the formation of late reverse transcript (RT) products, which are necessary for productive infection (Stremlau, Owens et al. 2004). Interestingly, the human ortholog of TRIM5α (huTRIM5α) demonstrated a less potent block to HIV-1 infection, although it could potently inhibit N tropic murine leukemia virus (N-MLV) and equine infectious anemia virus (EIAV) (Keckesova, Ylinen et al. 2004; Perron, Stremlau et al. 2004; Stremlau, Owens et al. 2004; Yap, Nisole et al. 2004). B tropic MLV (B-MLV) is unaffected by either rhTRIM5α or huTRIM5α, unless residue 110 is changed to the amino acid in the N-MLV capsid. When this single amino acid change was made, B-MLV was now susceptible to huTRIM5α restriction (Perron, Stremlau et al. 2004). This led to the hypothesis that TRIM5α acts a species specific barrier to retroviral infection. Indeed, as more TRIM5α orthologs were identified from different primates, the evidence for this hypothesis grew as some primate TRIM5α genes could restrict simian immunodeficiency virus (SIV) isolated from a different primate species. For example, SIV isolated from macaques (SIV mac) was not restricted by rhTRIM5α. However, squirrel monkey
rhTRIM5α could restrict SIVmac (Nakayama and Shioda 2012). In all cases, the specific retroviral CA was targeted by the TRIM5α C-terminal domain, which in most TRIM5α orthologs contains a SPRY domain. The exception is found in Owl Monkeys and some macaques (Malim and Bieniasz 2012). Genetic events have led to the fusion of TRIM5 to cyclophilin A (CypA), in which CypA replaces the SPRY domain of the TRIM5 gene, generating a fusion protein known as TRIMCyp. The replacement of the SPRY domain with CypA still allows for TRIMCyp to recognize and bind the retroviral capsid, as CypA naturally binds the capsid. The TRIMCyp fusion proteins bind the CA of HIV-1 to various degrees among different species (Virgen, Kratovac et al. 2008; Price, Marzetta et al. 2009).

The SPRY domain of TRIM5α contains amino acid determinants that provide specificity for the retroviral capsid. Specifically, there are three variable regions within the SPRY domain of TRIM5α that have signatures of positive selection, indicating that residues within these regions can adapt to recognize different capsid of lentiviruses (Sawyer, Wu et al. 2005; Song, Gold et al. 2005; Stremlau, Perron et al. 2005; Ohkura, Yap et al. 2006). There are additional residues within the CC domain of TRIM5α that demonstrate similar positive selection (Johnson and Sawyer 2009). Collectively, these results indicate that rhTRIM5α acts as a retroviral restriction factor that recognizes specific retroviral capsids via evolutionarily selected residues within the C-terminal SPRY domain.
Possible mechanisms of retroviral restriction by rhTRIM5α

The mechanism of retroviral restriction by TRIM5α is not fully understood. While it was postulated that the E3 ubiquitin ligase activity found in the RING domain of TRIM5α could lead to the ubiquitination and proteasomal dependent degradation of the HIV-1 CA, no evidence to date has demonstrated this. Therefore, several models of TRIM5α mediated restriction have been proposed.

One of the earliest models proposed was described by Stremlau et al. In this model, TRIM5α can facilitate the rapid, premature degradation of the retroviral capsid (Stremlau, Perron et al. 2006). In these studies, TRIM5α led to the loss of CA protein that had not disassociated from the intact viral core, while not changing the total amount of CA protein. This led to the conclusion that TRIM5α can cause capsid disassembly while not degrading the CA protein itself (Stremlau, Perron et al. 2006). Similar results were observed for huTRIM5α and TRIMCyp (Diaz-Griffero, Kar et al. 2007).

A second model of TRIM5α restriction involves a two-step mechanism of restriction first proposed by Anderson et al. In this model, TRIM5α can bind the retroviral CA via the SPRY domain, which is sufficient to inhibit retroviral infection. The second step of this model proposed the disassembly of the virion via TRIM5α mediated proteasomal degradation (Sastri and Campbell 2011). Evidence for this model first came from studies performed by Wu et al. and Anderson et al. These studies identified a role for the proteasome in rhTRIM5α restriction by demonstrating the production of viral late RT products and competent pre-integration complexes (PICs) when rhTRIM5α restrictive cells are treated with the proteasomal inhibitor, MG132 (Wu, Anderson et al. 2006);
(Anderson, Campbell et al. 2006). In spite of this apparent relief of restriction when MG132 was present, 2 LTR circles, a measure of viral import into the nucleus, and infection (as measured by viral DNA integration into the host chromosome) remained restricted (Wu, Anderson et al. 2006). Similarly, the ability of TRIMCyp to restrict HIV-1 infectivity remained restricted with proteasomal inhibition (Perez-Caballero, Hatzioannou et al. 2005). Additionally, Campbell et al. described the sequestration of HIV-1 virions within rhTRIM5α cytoplasmic assemblies upon treatment with proteasomal inhibitors, although this sequestration was not necessary for viral restriction. Therefore, retroviral restriction mediated by rhTRIM5α involves a proteasomal-independent intermediate consisting of HIV-1 CA surrounded by rhTRIM5α (Campbell, Perez et al. 2008). However, the use of proteasomal inhibitors did not lead to the accumulation of rhTRIM5α protein, indicating that the ubiquitin-proteasome system is not the degradative pathway responsible for rhTRIM5α protein turnover.

A third model of TRIM5α restriction also describes the loss of CA in in TRIM5α expressing cells. This model proposes that the removal of the CA protein from the nucleoprotein complexes prevents proper infection from occurring (Chatterji, Bobardt et al. 2006). Specifically, HIV-1 infected cells expressing TRIM5α were fractionated into cytosolic and vesicular fractions, and specific CA protein degradation was measured. CA protein was degraded independent of the degradation of other nucleoprotein components. Furthermore, the authors demonstrated that CA degradation was proteasome independent, as degradation was unaffected with proteasomal inhibitors.
Cellular degradation pathways

Cellular protein turnover is mediated by two pathways in eukaryotic cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway. Together, these pathways control the degradation of proteins, leading to the recycling of the amino acid pool for new protein synthesis and the replenishing of energy stores. Additionally, both pathways have been implicated in a variety of human diseases such as Parkinson’s disease, viral and bacterial infections and control of the immune response.

Ubiquitin-proteasome system

The 26S proteasome is a multiprotein barrel complex that degrades individual, ubiquitinated proteins (Saeki and Tanaka 2012). Substrate proteins are often short-lived, indicating that the 26S proteasome is involved in attenuation of cellular signaling (Schreiber and Peter 2013). Proteins are degraded into small peptides which can be digested into single amino acids by the large protease, tripeptidyl peptidase (Chuang, Rockel et al. 2010). The 26S proteasome is comprised of the 20S proteolytic core and the 19S regulatory particle (Murata, Yashiroda et al. 2009). Degradation of proteins by the 26S proteasome requires ATP binding to subunits within the regulatory particle (Smith, Chang et al. 2007; Gillette, Kumar et al. 2008; Lander, Estrin et al. 2012). The 20S core contains the proteolytic residues within a central channel, and access is regulated by the 19S regulatory particle (Groll, Ditzel et al. 1997; Smith, Chang et al. 2007; Gillette, Kumar et al. 2008; Lander, Estrin et al. 2012). The 19S particle can either be at both ends of the channel or only at a single end (da Fonseca and Morris 2008). The 19S particle
also serves as the substrate recognition site and promotes deubiquitination, unfolding and translocation of the substrate proteins into the 20S core (Smith, Chang et al. 2007; Gillette, Kumar et al. 2008; Lander, Estrin et al. 2012). Within the base of the 19S particle lies the subunits that bind ATP to open the entry gate for substrate translocation (Tian, Park et al. 2011).

Proteins are targeted to the proteasome via a 76-amino acid protein called ubiquitin (Weissman 2001). The process of ubiquitination involves the covalent linkage of ubiquitin to a substrate protein via step-wise enzymatic reaction between the C-terminal glycine of ubiquitin to a lysine within the substrate protein (Kerscher, Felberbaum et al. 2006). When the site of conjugation occurs at a lysine within in the substrate protein, this is called monoubiquitination. Additionally, ubiquitin molecules can be linked together through one of the seven lysine molecules throughout the protein, yielding branched ubiquitin chains. Additionally, linear polyubiquitin chains are formed via the N-terminal α-amino group in ubiquitin (Schreiber and Peter 2013). The ubiquitin modification can occur in multiple sites throughout the ubiquitin molecule, with K48 and K63 linked ubiquitin chains being the most prevalent. It was thought that K48-linked polyubiquitin chains are responsible for targeting proteins to the proteasome for degradation (Chau, Tobias et al. 1989; Thrower, Hoffman et al. 2000). However, more and more evidence shows that all types of ubiquitin modifications (polyubiquitin chains at different lysines, monoubiquitination and multi-monoubiquitination) can lead to protein targeting to the proteasome (Kirkpatrick, Hathaway et al. 2006; Jin, Williamson et al. 2008). Ubiquitination is a multi-enzyme, stepwise process. The first step involves
the E1 ubiquitin-activating enzyme. Using ATP, a single ubiquitin molecule is activated at the C-terminal glycine to form a thiol-ester linkage with the cysteine residue of the E1 ubiquitin activating domain. This ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme via another thiol-ester linkage. In the final step, the ubiquitin is conjugated to a lysine residue within the target protein with the help of the E3 ubiquitin ligase enzyme (Yeh, Gong et al. 2000). The target protein could be an entirely different protein or the E3 ubiquitin ligase itself (Yamauchi, Wada et al. 2008). There are estimated to be hundreds of E3 ubiquitin ligases, and their congnate deubiquitinases (Nijman, Luna-Vargas et al. 2005). Substrate specificity is dictated by the E3 ubiquitin ligase (Nagy and Dikic 2010). In this way, the role for E3 ubiquitin ligases in proteasomal degradation has been heavily investigated.

**Autophagic-lysosomal degradation**

Autophagy is derived from the Greek words “auto” (self) and “phagy” (eating) and encompasses three separate pathways that all lead to degradation of proteins by lysosomes (Yorimitsu and Klionsky 2005). Degradation in the lysosome is mediated by proteases, lipases, nucleotidases and glycases, implicating the lysosome as the major organelle responsible for macromolecular recycling (Kroemer and Jaattela 2005; Park and Cuervo 2013). Autophagy is thought to be the main pathway activated to degrade target proteins during cellular stress; however numerous studies have shown that proteins targeted to the autophagy-lysosomal pathway under normal cellular conditions as well (Komatsu, Waguri et al. 2005; Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006;
Masse, Kaushik et al. 2006; Nakai, Yamaguchi et al. 2007; Liu, Wang et al. 2009). Three separate pathways encompass the autophagy-lysosomal pathway, delineated by the respective route of cargo delivery. These pathways are macroautophagy, microautophagy and chaperone mediated autophagy (CMA).

**Macroautophagy**

Macroautophagy is the bulk degradation of cytosolic proteins enclosed within a membrane that leads to the formation of the autophagosome. Macroautophagy can selectively and non-selectively degrade target proteins. There are more than 30 autophagy related genes (Atgs) responsible for macroautophagy (Klionsky, Codogno et al. 2010). Autophagosome formation occurs within the phagophore assembly site (PAS) (Schreiber and Peter 2013). Initiation of macroautophagy involves the inactivation of mTOR, the mammalian target of rapamycin, in response to specific stimuli such as reduced growth factors or cellular stress. Upon downregulation of mTOR, Ulk1/2 is dephosphorylated and its kinase activity is initiated. Dephosphorylation of Ulk1/2 leads to increased assembly of Ulk1/2-Agt13 complexes which are crucial for the initiation of macroautophagy (Weidberg, Shvets et al. 2011). Membrane lipids derived from the plasma membrane, the ER, the mitochondria and the Golgi apparatus are recruited to the PAS (Razi, Chan et al. 2009; Hailey, Rambold et al. 2010; Ravikumar, Moreau et al. 2010; Suzuki and Ohsumi 2010). Activated Ulk1/2 associates with the initiating phagophore membrane (Weidberg, Shvets et al. 2011). A second kinase complex is recruited to the site of autophagosomal formation. This complex is comprised of Beclin-
1, Vps34 and Vps15 as the core proteins (Furuya, Yu et al. 2005; Yan, Flinn et al. 2009). This complex phosphorylates the lipid molecules derived from the above cellular structures, specifically phosphoinositides (PIs), generating phosphatidylionsitol-3-phosphate (PI3P). PI3P recruits other effectors to the phagophore membrane, which in turn recruit other Atg proteins to the phagophore membrane (Reggiori, Komatsu et al. 2012).

Elongation of the phagophore membrane depends on the recruitment of two conjugation systems, the Atg5-Atg12-Atg16 complex and LC3 conjugation to phosphatidylethanolamine (LC3-II). These two conjugation systems resemble the conjugation of ubiquitin to target proteins via the stepwise conjugation of Atg proteins. The Atg5/12/16 system depends on the covalent and irreversible linkage of Atg12 to Atg5, mediated by Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme). Then, Atg16 interacts with Atg5, leading to the formation of a complex crucial for the early stages of autophagy. The conjugation of LC3 to PE is mediated by 1) cleavage by Atg4; 2) conjugation of cleaved LC3 to Atg7; 3) transfer to Atg3 (E2-like); and 4) covalent bond formed between LC3 and PE. This complex serves as a marker of the autophagosome until the degradation within the lysosome (Weidberg, Shvets et al. 2011). Both the Atg5/12/16 and LC3-PE conjugation systems are important for the isolation membrane elongation and autophagosomal closure. Additionally, LC3 has been implicated in cargo recruitment. The autophagosome then matures and fuses with late endosomes and lysosomes, which is mediated by SNARE and lipid-modifying proteins (Vergne and Deretic 2010; Dall'Armi, Devereaux et al. 2013; Park and Cuervo 2013). In addition to
bulk sequestration of cytoplasmic contents by the phagophore, there is increasing evidence of selective macroautophagy for certain cargos, such as mitochondria, pathogens, lipid droplets and other proteins. Selective macroautophagy is facilitated by the recognition of specific cargo by molecules such as p62, NBR1, or NDP52 and the binding of specific autophagy proteins, such as LC3. For example, the cellular scaffold proteins can recognize and bind ubiquitinated target proteins via the UBA domain. Then, through the interaction with LC3 via the LC3 interacting region (LIR), p62 can deliver ubiquitinated proteins to the autophagosome.

**Microautophagy**

Microautophagy is the engulfment of cytosolic contents via invagination on the lysosomal membrane and was first described by de Duve and Wattiaux (De Duve and Wattiaux 1966). Most of the research covering microautophagy has used yeast as the model organism (Mijaljica, Prescott et al. 2011). Similar to macroautophagy, microautophagy in yeast can be selective and non-selective (Kunz, Schwarz et al. 2004). Nonselective microautophagy is the random sequestration of cytosolic components. Selective microautophagy leads to the degradation of specific organelles (Farre, Krick et al. 2009). In higher eukaryotes, research suggests that microautophagy is present and active in addition to the well-established lysosomal degradation pathways such as macroautophagy and CMA. For example, Marzella et al. demonstrated that lysosomes can show microautophagy activity *in vitro* (Marzella, Ahlberg et al. 1980). Additionally, a lysosomal wrapping mechanism with features similar to microautophagy has been
described (Sakai and Ogawa 1982; Sakai, Araki et al. 1989). Finally, Moritmore et al. demonstrated a correlation between changes in the cellular environment and microautophagy in mouse hepatocytes during starvation (Mortimore, Hutson et al. 1983). More recently, Sahu et al. found that microautophagic mechanisms can facilitate cytosolic protein delivery to late endosomes. This led to the identification of a microautophagy-like, selective process distinct from CMA mediated by the cellular chaperone Hsc70 (Sahu, Kaushik et al. 2011).

**Chaperone mediated autophagy**

The term chaperone mediated autophagy (CMA) is somewhat of a misnomer as the pathway does not involve the *de novo* formation of the autophagosome. Instead, CMA occurs completely within the lysosome alone. CMA is a selective form of protein degradation mediated by the recognition of a pentapeptide motif by cellular chaperones. Backer et al. identified this sequence using the cleaved forms of RNase A, RNase S-peptide (residues 1-20) and RNase S-protein (residues 21-124). This cleavage event dramatically changed the half-life of the two fragments. Similar to full length RNase A, RNase S-peptide was degraded faster when cells were serum starved (Backer, Bourret et al. 1983). Furthermore, when the 20 amino acids of RNase S-peptide were conjugated to other proteins, their degradation increased upon serum removal (Backer and Dice 1986). In the case of RNase A, this pentapeptide motif consists of the amino acid sequence KFERQ (Dice, Chiang et al. 1986). Since the discovery of this motif, many other validated targets of CMA have been shown to contain a biochemically related sequence.
(Majeski and Dice 2004; Kaushik, Bandyopadhyay et al. 2011). In general, this motif consists of an glutamine (Q) preceded or followed by four amino acids consisting of a basic (lysine, K; arginine, R), an acidic (aspartic acid, D; glutamic acid, E), a bulky hydrophobic (phenylalanine, F; isoleucine, I; leucine, L; valine, V) and a repeated basic or hydrophobic amino acid (Dice, Terlecky et al. 1990). Additionally, some CMA targets such as α2-microglobulin and glyceraldehyde 3-phosphate dehydrogenase can utilize the related asparagine (N) in place of the Q. Therefore this sequence is very relaxed, relying more on the charge of the amino acids in the sequence, rather than the exact sequence itself (Bejarano and Cuervo 2010).

The CMA targeting motif is first recognized by the constitutive form of the heat shock protein Hsp70, Hsc70 (Chiang, Terlecky et al. 1989). Hsc70 is also involved in the folding of cytosolic proteins by recognition of exposed hydrophobic regions (Bejarano and Cuervo 2010). Hsc70 interacts with heat shock protein-90 (Hsp90), heat shock protein-40 (Hsp40), Bcl2-associated athanogene 1 protein (Bag-1), Hsp90-Hsp70 organizing protein (Hop) and Hsp70 interacting protein (Hip) proteins to facilitate translocation of the substrate protein into the lysosome (Agarraberes and Dice 2001). The recognition of the substrate protein by Hsc70 is governed by ATP/ADP binding (Chiang, Terlecky et al. 1989), with the ADP-bound form having the highest affinity for protein substrates (Agarraberes and Dice 2001). The various co-chaperones described above may play a role in protein unfolding, which is crucial for substrate translocation (Chiang, Terlecky et al. 1989).
Protein substrate recognition and translocation across the lysosomal membrane is mediated by a protein receptor (Cuervo and Dice 1996). This receptor is a specific isoform of the lysosomal associated membrane protein 2, LAMP2a. There are two LAMP proteins found in higher eukaryotes, LAMP1 and LAMP2. These membrane proteins are estimated to contribute approximately 50% of all proteins in the lysosomal membrane (Eskelinen 2006). LAMP1 and LAMP2 are type I transmembrane proteins with a single transmembrane domain, a large luminal domain and a C-terminal domain. These proteins are heavily glycosylated, with an apparent molecular weight of 120 kDa (Carlsson, Roth et al. 1988; Mane, Marzella et al. 1989). LAMP2 undergoes alternative splicing to generate three different isoforms, LAMP2a, b, and c (Gough, Hatem et al. 1995). Regulation of LAMP2a expression is governed by the lysosomal compartment and not transcriptional upregulation of LAMP2 (Cuervo and Dice 2000). This control includes the regulated cleavage of LAMP2a by two lysosomal membrane proteases (Cathepsin A and a membrane associated metalloprotease) or the distribution of LAMP2a between the lysosomal membrane and matrix. The latter controls the amount of the cytosolic tail on the lysosomal surface (Cuervo and Dice 2000; Cuervo, Mann et al. 2003). In CMA, LAMP2a interacts with protein substrates via four residues within the cytosolic tail of LAMP2a. These four residues mediate electrostatic interactions between substrate and receptor and are specific for LAMP2a as they are not found in the other LAMP2 isoforms (Cuervo and Dice 2000). When CMA is activated, LAMP2a is mobilized out of discrete membrane microdomains and away from Cathepsin A (Kaushik, Massey et al. 2006). Bandyopadhyay et al. found that upon substrate binding, LAMP2a monomers assemble to
form a 700 kDa multimeric complex on the lysosomal membrane (Bandyopadhyay, Kaushik et al. 2008). This complex can form a channel within the lysosomal membrane to allow the substrate protein to translocate across the membrane. This association is very transient, adding to the selectivity of CMA in addition to providing a mechanism to prevent lysosomal leakage. After substrate translocation, LAMP2a is quickly disassembled through the action of cytosolic Hsc70, glial fibrillary acidic protein (GFAP), and elongation factor 1α (EF1α) (Bandyopadhyay, Kaushik et al. 2008; Bandyopadhyay, Sridhar et al. 2010).

The translocation of the substrate protein across the lysosomal membrane is not a passive process. Rather, the lysosomal luminal form of Hsc70 (lys-Hsc70) engages the substrate to allow for its translocation across the lysosomal membrane (Agarraberes, Terlecky et al. 1997). Lys-hsc70 is the protein variant that confers CMA activity on a subset of lysosomes (Cuervo, Dice et al. 1997). Given that Hsc70 binds ATP/ADP, it is possible that substrate translocation is an active process, facilitated by protein unfolding by lys-Hsc70 (Agarraberes and Dice 2001). Conversely, Hsc70 could simply prevent the movement of the substrate back into the cytoplasm (Bejarano and Cuervo 2010).
Cross-talk between the UPS and autophagy-lysosomal pathway

Although the UPS and autophagy-lysosomal pathways degrade different proteins by different mechanisms, these systems do not act independently of each other. In fact, numerous studies have shown that a defect in one pathway can cause the upregulation of another, although examples of the converse (upregulation of one pathway causes the downregulation of another) have not been found. Additionally, proteins involved in either pathway can be degraded by the other. Finally, certain proteins have been shown to be degraded by both pathways, independent of the stress condition of the cell. Massey et al. demonstrated that in cells that were selectively deficient for LAMP2a, and therefore defective for CMA, there was an upregulation of macroautophagy (Massey, Kaushik et al. 2006). Additionally, Kaushik et al. found that by inhibiting macroautophagy with either pharmacological inhibitors or cells deficient for Atg5, there was an upregulation of CMA under normal nutritional conditions as well as stress conditions (Kaushik, Massey et al. 2008). Additionally, pharmacological inhibition of the proteasome can lead to the upregulation of macroautophagy in a variety of cellular systems (reviewed in (Park and Cuervo 2013). Interestingly, the catalytic core of the proteasome can be degraded by macroautophagy under nutritional stress (Cuervo, Palmer et al. 1995). Finally, the selective autophagy cargo-recognition protein p62 can be degraded by both the proteasome and macroautophagy (Myeku and Figueiredo-Pereira 2011).
Figure 3. Degradation of cellular proteins by the proteasome and autophagy-lysosomal systems. The proteasome degrades individual, often ubiquitinated, proteins (indicated by the light green spheres, left). Macroautophagy initiates on the isolation membrane as described in the text. Microautophagy involves the invagination of the lysosomal membrane to deliver cytosolic contents such as proteins and organelles. CMA depends on the recognition of a specific motif by cellular chaperones that facilitate the translocation of proteins across the lysosomal membrane.
Degradation of TRIM proteins

Many TRIM proteins contain E3 ubiquitin ligase function, which lies within the RING domain at the N-terminus of the protein. This function has led to the hypothesis that TRIM proteins are degraded by the proteasome. However, only a handful of these TRIM proteins have been shown to be definitively degraded by the proteasome. Alternatively, there are examples that shown TRIM proteins are sensitive to inhibitors of lysosomal degradation. The multiple pathways of degradation utilized by TRIM proteins highlights the little that is known about these proteins and how the degradation of TRIM proteins may play a role in their cellular functions. In particular, TRIM79α, TRIM50 and TRIM5α have all been shown to be sensitive to both degradation pathways.

TRIM79α: Flavivirus restriction factor

TRIM79α is a murine TRIM protein identified by Taylor et al. as a interacting partner of the flavivirus NS5 protein (Taylor, Lubick et al. 2011). The molecular organization of TRIM79α is similar to TRIM5α, with the requisite RING, BBox and CC domains followed by a C-terminal SPRY domain. Also similar to TRIM5α, the expression of TRIM79α can be induced by interferon. Interestingly, TRIM79α shows similar tissue distribution as murine TRIM30α, a mouse TRIM5α homolog (Shi, Deng et al. 2008; Tareen and Emerman 2011). TRIM79α has a protein half-life of about 90 minutes, similar to the half-life described for TRIM5α (Wu, Anderson et al. 2006). The interaction between TRIM79α and the flavivirus NS5 protein leads to the degradation of NS5, which ultimately prevents flavivirus infection (Taylor, Lubick et al. 2011).
Furthermore, the degradation of NS5 was species specific, in that TRIM79α prevented tick-borne encephalitis virus (TBEV) but not West Nile virus (WNV). The degradation of NS5 was not proteasomal dependent, as treatment with the lysosomal inhibitor \( \text{NH}_4\text{Cl} \) prevented NS5 degradation, while MG132 had no effect. In the absence of restriction sensitive virus, TRIM79α was sensitive to proteasomal inhibition, indicating that TRIM79α utilizes different degradation pathways to facilitate flavivirus restriction. It is hypothesized that as NS5 can form large protein complexes with other viral proteins, the TRIM79α-mediated degradation by the lysosomal pathway in the presence of the virus occurs more easily than proteasomal degradation of single proteins.

**TRIM50: Implicated in Williams Beuren syndrome**

TRIM50 is one of 28 genes on chromosome 7 that is deleted Williams-Beuren patients. This microdeletion of about 1.55-1.84 Mb occurs when partially homologous duplicons that flank the 28-gene region misalign and subsequently lead to deletion of this region during meiosis (Pober 2010). This rearrangement includes the genes encoding TRIM73 and TRIM74. Although the molecular mechanism is poorly defined, Williams-Beuren patients suffer from a multisystemic disease, impacting the cardiovascular, endocrine and nervous systems to name a few. (Micale, Fusco et al. 2008). Together, these data implicate TRIM proteins in multistystem development. Additionally, TRIM50 can localize to the tubulovesicular and canalicular membranes in gastric parietal cells, and can direct the formation of gastric vesicles in a PI3K dependent manner (Nishi, Aoyama et al. 2012). Thus, there is no evidence for this TRIM protein to act a restriction
factor; however, the data suggests that this protein is involved in multiple cellular systems.

Although the characterization of TRIM50 is limited, it has similar domain architecture to TRIM5α. Specifically, TRIM50 contains a RING, Bbox, CC and SPRY domain (Micale, Fusco et al. 2008). TRIM50 interacts with specific E2 enzymes to facilitate its autoubiquitination, which is dependent on the RING domain. TRIM50 ubiquitination increases upon treatment with MG132. Interestingly, TRIM50 steady-state protein levels increases in the presence of NH₄Cl, indicating that TRIM50 is degraded by autophagy-lysosomal pathway. Also of note, when autophagy is inhibited with NH₄Cl, TRIM50 localization to ubiquitin positive cellular accumulations decreases. Finally, TRIM50 can interact with p62, the selective autophagy protein that can shuttle proteins to both the proteasome and the autophagy-lysosomal system (Pankiv, Clausen et al. 2007; Geetha, Seibenhener et al. 2008; Fusco, Micale et al. 2012). Thus, TRIM50 represents another TRIM protein whose function is altered by inhibition of either the proteasome or the autophagy-lysosomal pathway.

TRIM5α: Association with proteins important for both degradative pathways

As discussed previously, TRIM5α-mediated restriction of retroviral infection includes proteasomal dependent and independent steps (Anderson, Campbell et al. 2006; Chatterji, Bobardt et al. 2006; Stremlau, Perron et al. 2006; Wu, Anderson et al. 2006). Interestingly, only one study to date has demonstrated that the degradation of TRIM5α is mediated by the proteasome. Rold and Aiken demonstrated that TRIM5α protein turnover
was sensitive to MG132 treatment only when large amounts of restriction-sensitive virus is present (Rold and Aiken 2008). The authors found that infection with HIV-1 led to a destabilization of rhTRIM5α but not of huTRIM5α. TRIMCyp was also destabilized in the presence of HIV-1. Interestingly, the stability of TRIMCyp increased in the presence of HIV-1 and cyclosporine A (CsA). CsA can prevent CA binding by cyclophillin A. Therefore, when restriction by TRIMCyp is relieved, TRIMCyp regains stability. It is postulated that TRIMCyp degradation depends on the recognition of the viral CA protein. Additionally, huTRIM5α, which can potently restrict N-MLV but not B-MLV and HIV-1, was destabilized upon N-MLV infection. Thus, various retroviruses can destabilize TRIM proteins that recognize species specific CA proteins, and this destabilization can be rescued with MG132.

The involvement of the proteasome in restriction and the degradation of rhTRIM5α in the presence of specific retroviruses suggests that perhaps a molecular switch exists that controls the degradative fate of TRIM5α, altering its endogenous degradation to proteasomal degradation in the presence of virus. Recently, O’Connor et al. described the direct interaction of the cellular scaffolding protein p62 with rhTRIM5α and huTRIM5α. Furthermore, p62 localized to TRIM5α cytoplasmic assemblies and knock down of p62 using specific siRNA led to a decrease in TRIM5α protein levels. Knock down of p62 in TRIM5α expressing cells also decreased retroviral restriction, which can likely be attributed to the decrease in TRIM5α protein (O’Connor, Pertel et al. 2010). p62 is a multi-domain, multifunctional protein whose expression is induced by interferon (Kim and Ozato 2009). p62 can shuttle proteins to the proteasome or to the
autophagy-lysosomal system (Pankiv, Clausen et al. 2007; Geetha, Seibenhener et al. 2008). p62 itself appears to be degraded by both pathways (Myeku and Figueiredo-Pereira 2011). Additionally, p62 can act as a cargo receptor for selective autophagy by binding ubiquitinated proteins via its UBA domain (Schreiber and Peter 2013). Therefore, it is possible that p62 acts as a molecular switch, altering the sensitivity of TRIM5α to different degradation pathways.

p62 is one of many TRIM5α cofactors that could play a role in the degradation of TRIM5α. Additionally, heat shock proteins have been shown to interact and co-localize with TRIM5α. Specifically, Hsp70, Hsp90 and Hsc70 can interact with TRIM5α (Diaz-Griffero, Li et al. 2006; Hwang, Holl et al. 2010). The association of Hsc70 with TRIM5α is interesting as Hsc70 is the cellular chaperone responsible for substrate recognition in both microautophagy and CMA (Chiang, Terlecky et al. 1989; Sahu, Kaushik et al. 2011). Additionally, Hsp70 and Hsp90 are co-chaperones that form a large complex upon substrate recognition during CMA (Majeski and Dice 2004). Interestingly, Hwang et al. propose that the association of Hsp70 assists in TRIM5α protein folding, while the role for Hsc70 in TRIM5α has not yet been identified (Hwang, Holl et al. 2010).

Finally, proteins important for ubiquitination and proteasomal degradation have been associated with TRIM5α (Yamauchi, Wada et al. 2008; Lukic, Hausmann et al. 2011; Pertel, Hausmann et al. 2011). TRIM5α has been shown to autoubiquitinate as well as ubiquitinate the related TRIM21 protein, which was dependent on the catalytic activity of the RING domain. Additionally, proteasomal inhibition did not stabilize TRIM5α
ubiquitination, demonstrating that TRIM5α is not degraded by the proteasome (Yamauchi, Wada et al. 2008). Both mono- and polyubiquitination of TRIM5α have been detected (Diaz-Griffero, Li et al. 2006; Yamauchi, Wada et al. 2008). Interestingly, although TRIM5α degradation is not dependent on the proteasome, Lukic et al. detected proteosomal subunits in TRIM5α cytoplasmic assemblies in the presence and absence of restriction-sensitive virus, as well as in the absence of proteasomal inhibitors (Lukic, Hausmann et al. 2011). Additionally, Pertel et al. demonstrated that TRIM5α can associate with the E2 complex, UBC13-UEV1A, that leads to the K63-linked ubiquitination of signaling molecules important for AP-1 and NFκB activation of the innate immune response (Pertel, Hausmann et al. 2011). Collectively, these data demonstrate that both proteasomal and autophagic proteins can interact with TRIM5α, and these interactions cannot definitely identify the degradative fate of TRIM5α.

**Summary and conclusions**

Previous studies have demonstrated that TRIM5 proteins are not degraded by the UPS, one of the degradation systems utilized by eukaryotic cells. We propose that TRIM5 proteins are degraded by the autophagy-lysosomal pathway. We hypothesize that TRIM5 proteins can localize to lysosomal marker proteins upon lysosomal inhibition, which prevents the cellular turnover of TRIM5 proteins. Furthermore, we hypothesize that TRIM5 proteins are degraded by CMA, which occurs solely within the lysosome. The route of degradation utilized by TRIM5 proteins in the absence of virus could have wide reaching implications. In particular, it could represent an unidentified aspect of
TRIM5-mediated retroviral restriction. Additionally, it could also shed light on how this diverse group of proteins is regulated by the host cell.
CHAPTER TWO

MATERIALS AND EXPERIMENTAL METHODS

Cell lines, viruses and pharmaceuticals

HeLa and 293T cell lines were obtained from the American Type Culture Collection. TE671 cells were a gift from Dr. Thomas Hope. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml ciprofloxacin. Cells were maintained in the presence of 5% CO$_2$ at 37°C.

To produce amphitropic MLV Env (A-MLV) pseudotyped HIV-1 GFP reporter viruses, 293T cells were transfected with 6 ug A-MLV plasmid and 4 ug of the proviral construct R7Δ EnvGFP in which the Nef gene was replaced with GFP. To produce A-MLV murine leukemia viruses, 293T cells were transfected with equal amounts of pCigB or pCigN packaging plasmids, YFP reporter vector and A-MLV Env. Transfections were performed with polyethylenimine (PEI). To assess virus infectivity, equivalent numbers of HeLa or CRFK cells were plated to assess HIV-1 or MLV reporter viruses, respectively. 14 hours post infection, the virus was removed and normal media was added. Infectivity was assessed by measuring GFP or YFP fluorescence 48-72 hours later by using a fluorescence-activated cell sorter (FACS) Canto II (Becton Dickinson) or Accuri c6 (BD...
Biosciences) flow cytometer. Bafilomycin A1 and MG132 (Cayman Chemical Company, Ann Arbor, Michigan, USA) were used at final concentrations of 100 nM and 1 µg/ml, respectively. Cycloheximide was used at a final concentration of 20 µg/ml.

**Recombinant DNA constructs and generation of stable cell lines**

Cells expressing yellow fluorescent protein-tagged WT rhTRIM5α (YFP-rhTRIM5α) and hemagglutinin (HA)-tagged WT rhTRIM5α have been described previously (Campbell, Dodding et al. 2007; Sastri, O’Connor et al. 2010). To generate mutations in the putative CMA sequences of rhTRIM5α, triple alanine mutants were introduced using SOEing PCR (Sastri, O’Connor et al. 2010). Subsequently, mutant rhTRIM5α PCR products were cloned into a pLNCX2 derived MLV retroviral plasmid encoding an N-terminal YFP protein tag in frame with rhTRIM5α using BamHI and XhoI. To generate HeLa cell lines expressing the mutant YFP-rhTRIM5α, 293T cells were transfected using the YFP-rhTRIM5α constructs, B-MLV packaging plasmid pCigB, and the envelope plasmid VSV-g. 48 hours after transfection, supernatant containing the retroviral particles was collected and filtered through a 0.45 µm syringe driven filter and applied to the HeLa cells. 48 hours later, DMEM media containing G418 replaced the original media to select for cells that were positively transduced and expressing YFP-rhTRIM5α. YFP-rhTRIM5α expression was confirmed by immunofluorescence and western blot.
Steady state protein analysis

Cell lines stably expressing the indicated TRIM5α tagged protein were treated with BafA1 and MG132 and cells were harvested at the indicated time points. Whole-cell lysates were prepared by treating $2 \times 10^5$ cells with lysis buffer (100 mM Tris, pH 8.0, 1% NP-40, 150 mM NaCl) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). After lysis of cells Laemmli 2x SDS sample buffer was added and samples were boiled for 5 min. Equal amounts of protein, based on cell number at the time of harvest, were loaded into a 10% polyacrylamide gel for SDS-PAGE. After separation of proteins via SDS-PAGE, proteins were transferred to nitrocellulose membranes and detected by incubation with the following antibodies: anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), anti-HA (clone 3F10) conjugated to horseradish peroxidase (HRP) (Roche Applied Science, Indianapolis, IN, USA), and anti-GFP (Clontech Laboratories, Inc, Mountain View, CA, USA). Secondary antibodies conjugated to HRP (Thermo Fisher Scientific, Waltham, MA, USA) were used where necessary, and antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescence was detected using a UVP EC3 imaging system (UVP LLCUpland, CA, USA).

Protein turnover assay

Cell lines stably expressing the indicated TRIM5α tagged protein were treated with cycloheximide alone or in the presence of BafA1 or MG132. Cells were harvested at
6 hours following cycloheximide addition. Equivalent amounts of protein from individual samples were subjected to SDS-PAGE and TRIM5α protein was detected by Western blot.

**Flow cytometry**

Equivalent numbers of cells stably expressing YFP-rhTRIM5α in a 12-well plate were treated with cycloheximide, BafA1, or MG132 for 6 h or 18 h, after which the cells were fixed in a final 10% formaldehyde-PBS solution. Protein levels were determined by mean fluorescence intensity (MFI) in the FITC channel for 10,000 events per sample using a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

**Detection of endogenous huTRIM5α**

TRIM5α antibodies were acquired from the NIH AIDS reagents and resources program. To measure endogenous TRIM5α upon proteasomal inhibition, TE671 cells were used due to their high level of endogenous huTRIM5α and their ability to resist N-MLV infection. Briefly, 1 x 10⁶ cells were evenly plated in a 6 well format. Cells were allowed to adhere to the plate and then DMEM supplemented with DMSO, BafA1 or MG132 was applied for 18 hours. Whole-cell lysates were prepared by treating cell pellets with lysis buffer (100 mM Tris, pH 8.0, 1% NP-40, 150 mM NaCl) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). After cell lysis, Laemmli 2x SDS sample buffer was added and samples were boiled for 5 min. Equal amounts of protein, based on cell number at the time of harvest, were loaded into a 10% polyacrylamide gel for SDS-PAGE. Proteins were transferred to nitrocellulose and
membranes were probed mouse anti-TRIM5α (IF8-4) for 1 hour in 0.6% BSA in 0.03% PBS-T. Membranes were washed and probed with goat anti-mouse IgG conjugated-HRP secondary antibody overnight at 4 degrees. huTRIM5α protein amounts were detected with Femto chemiluminescent substrate and quantified using ImageJ.

**Immunofluorescence microscopy**

Cells were allowed to adhere to fibronectin-treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], pH 6.8. Cells were permeabilized with 0.1% saponin, 10% normal donkey serum, 0.01% sodium azide in PBS. We used the following primary antibodies: rabbit anti-LC3b (Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-LAMP2 (BD Pharmigen, San Diego, CA, USA). Primary antibodies were secondarily labeled with fluorophore-conjugated donkey anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Images were collected with a DeltaVision microscope (Applied Precision, Issaquah, WA, USA) equipped with a digital camera (CoolSNAP HQ; Photometrics, Tucson, AZ, USA), using a 1.4-numerical aperture (NA) 100x objective lens, and were deconvolved with SoftWoRx software (Applied Precision, Issaquah, WA, USA).

**Image Analysis**

20 Z-stack images were acquired using identical acquisition parameters. Surfaces for cytoplasmic bodies in all samples analyzed were defined by using a fluorescence threshold (250 relative fluorescence units) for YFP-rhTRIM5α, and all YFP-rhTRIM5α
bodies over an area of 0.2 μm² were used in the analysis. Deconvolved images were analyzed for LC3b and LAMP2 maximum fluorescence intensity in cytoplasmic bodies using the Surface Finder function of the Imaris software package (Bitplane, Zurich, Switzerland) and the data were plotted in Prism (Graphpad Software Inc., La Jolla, CA, USA) for statistical analysis.

**siRNA transfections**

*Atg5 siRNA treatment:* To knockdown the expression of human Atg5 gene, we used two different small interfering RNAs: siRNA#1 (Cell Signaling, Danvers, MA, USA) consisting of 5’-GCCUGUAUGACUGCUUA-3’ and 5’-GCCUGUAUGACUCUUUA-3’ and siRNA#2 (Invitrogen, Carlsbad, CA, USA). The siRNA were transfected using RNAMax (Life Technologies, Grand Island, NY, USA) according to manufacturer’s instructions. Approximately 48 hrs following transfection with siRNA, the cells were treated with MG132 (10 μM) or BafA1 (20 nM) overnight and then scraped and collected in Laemmli buffer. Lysates were separated on an 8% or 10% polyacrylamide gel for SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and probed with α-Atg5 (Cell Signaling, Danvers, MA, USA), α-GFP, or α-β-actin antibodies.

*LAMP2 and Hsc70 siRNA treatment:* Subconfluent YFP-rhTRIM5α HeLa cells were plated equally in a 24 well plate. Control (sc-37007) and human LAMP2 and Hsc70-specific siRNAs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were
transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) twice within a 24 hour period. Cells were collected at 24, 48 and 72 hours post transfection and subjected to Western blot analysis. Equal amounts of protein was determined by cell number at the time of collection. Nitrocellulose membranes were probed with α-LAMP2, α-Hsc70 α-GFP, and α-β-actin and the appropriate secondary antibodies conjugated to HRP were used where necessary. Antibody complexes were detected using SuperSignal West Femto chemiluminescent substrate and chemiluminescence was detected using a ChemiDoc™ XRS+ (BioRad, Hercules, CA, USA).

*p62 siRNA treatment*: Subconfluent YFP-rhTRIM5α HeLa cells were plated equally in a 6 well plate. Control (sc-37007) and human p62-specific siRNAs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) twice within a 24 hour period. Cells were then evenly plated on fibronectin treated coverslips. Cells were allowed to adhere before the addition of DMEM containing BafA1 was added for 6 hours. Cells were then fixed and subject to immunofluorescence analysis as described above. Endogenous p62 was detected using a mouse anti-p62 antibody and endogenous LC3b was detected using a rabbit anti-LCb antibody. Image analysis was performed as described above.

**Quantitative Real-Time PCR for viral RT products**

Detection of viral RT products with pharmacological inhibition of degradation pathways: Untransduced HeLa cells or HeLa cells stably expressing HA-rhTRIM5α were seeded in 12-well plates at equal cell density per well and monolayers were treated
with BafA1 or MG132 concurrently with A-MLV Env-pseudotyped HIV-1-GFP infection for 18 hours at 37°C. Genomic DNA was harvested using a DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA was digested with 1 unit/µl DpnI (New England Biolabs, Ipswich, MA, USA) for 4 hours at 37°C to remove residual plasmid DNA. 50 ng of proviral DNA was quantified using primers specific for HIV-1 late RT products with SYBR green PCR reagent (Applied Biosystems, Carlsbad, California, USA) on a Realplex2 ep gradient Mastercycler (Eppendorf, Hauppauge, New York, USA). Each sample was normalized per 10 ng of total cellular DNA. Dilutions of proviral plasmid (10-fold) were used to generate standard curves for quantifying viral late RT products.

Detection of viral RT products and infectivity with LAMP2 siRNA treatment:
Untransduced HeLa cells or HeLa cells stably expressing hemagglutinin (HA)-rhTRIM5α were seeded in 6-well plates at equal cell density per well and monolayers were transfected with control or LAMP2 siRNA twice within 48 hours. 60 hours after the first transfection, cells were collected and re-plated in 24 well plates at equal cell density. After cells became adherent, undiluted A-MLV Env pseudotyped HIV-1 GFP, B-MLV YFP or N-MLV YFP were applied to the cells. BafA1 and MG132 were added where indicated. Cells were then spinoculated at 1200 x g for 2 hours at 13 degrees C. Immediately after spinoculation, viral inoculum was removed and fresh DMEM containing the indicated drugs was added. 12 hours post infection, cells were collected for viral late RT quantification. Genomic DNA was harvested and viral DNA was
isolated as described above. To measure viral infectivity, cells were kept in DMEM containing BafA1 or MG132 for 24 hours. The media was changed to normal DMEM at this point. At 48 hours post infection, cells were collected and fixed in 10% formaldehyde in PBS. Infectivity was measured by percent GFP or YFP fluorescence using a FACS Accuri c6 (BD Biosciences) flow cytometer. A minimum of 10,000 events were collected per sample.

**Statistical Analysis**

Data were statistically analyzed with GraphPad Prism 5 using one-way analysis of variance for comparison between no treatment, BafA1 or MG132 treated samples. Student’s t-test was used for the comparison of two independent groups (no treatment versus BafA1 treatment). For all tests, a P value of less than 0.05 was considered statistically significant. Chapter III
CHAPTER THREE

RESULTS

Identifying the cellular degradation pathway responsible for TRIM5α turnover

*Lysosomal inhibition alters rhTRIM5α localization*

To identify the degradative pathway responsible for the steady state degradation of rhTRIM5α, HeLa cells stably expressing YFP-rhTRIM5α were treated with inhibitors of the major degradative pathways, autophagy/lysosome or the UPS. To inhibit autophagy/lysosomal degradation, we used Bafilomycin A1 (BafA1), which inhibits lysosomal degradation by targeting the ATPase that leads to acidification of the lysosome (Bowman, Siebers et al. 1988). As the lysosome is the degradative compartment that fuses with autophagosomes, this will inhibit both macroautophagy and lysosomal degradation (microautophagy and CMA). To inhibit the proteasome, we used MG132, which prevents the entry of target proteins into the barrel proteasome (Lee and Goldberg 1998). Although proteasomal inhibition does not lead to an accumulation in rhTRIM5α protein, treating rhTRIM5α expressing cells with MG132 can lead to re-distribution of the protein into larger cytoplasmic accumulations (Anderson, Campbell et al. 2006; Wu, Anderson et al. 2006). Therefore we hypothesized that there would be a re-distribution of rhTRIM5α protein upon lysosomal inhibition of YFP-rhTRIM5α expressing cells were treated with BafA1 or MG132 for 6 hours. Cells were then fixed and stained with DAPI.
to visualize the nucleus. We found that upon treatment with BafA1, YFP-rhTRIM5α localized to smaller, more numerous puncta compared to control or MG132 treated cells (Figure 4 a-c). When the number of cytoplasmic bodies was quantified per cell using Imaris imaging software, we found that there was a statistically significant increase in the number of bodies per cell (Figure 4d). Additionally, we performed similar analysis with the lysosomal inhibitor NH₄Cl. We observed the same re-localization of YFP-rhTRIM5α protein with NH₄Cl treatment that was observed for BafA1 (data not shown). Given that BafA1 and NH₄Cl inhibit lysosomal degradation, we hypothesize that the accumulations of YFP-rhTRIM5α upon BafA1 treatment are not actually cytoplasmic bodies but rather represent YFP-rhTRIM5α that is not yet degraded within the lysosome. From these data, we conclude that the lysosomal degradation pathway is important for controlling the steady state protein levels and localization of rhTRIM5α.

Inhibition of the autophagy-lysosomal pathway prevents rhTRIMα degradation

Previously, proteasomal inhibition has been shown to relieve TRIM5α mediated restriction of late RT products without leading to an accumulation of TRIM5α protein (Wu, Anderson et al. 2006). Therefore, the mechanism of TRIM5α degradation remains unknown. We hypothesize that autophagy is responsible for the degradation of TRIM5α. To test this hypothesis, we utilized YFP-rhTRIM5α HeLa cell lines to measure rhTRIM5α protein in the presence of UPS or autophagy-lysosome inhibitors. Cells were
Figure 4 Subcellular localization of YFP-rhTRIM5α changes in the presence of BafA1 and MG132. HeLa cells stably expressing YFP-rhTRIM5α were seeded onto fibronectin treated coverslips for 18 hours and treated with BafA1 or MG132. (A-C). Representative images of cells left untreated, treated with MG132, or treated with BafA1. D). To quantify the number of rhTRIM5α cytoplasmic bodies in each treatment, 20 images were taken per treatment under identical acquisition parameters. Each image was analyzed using Imaris imaging software. The mean and standard error of the mean (SEM) are highlighted in red. ***, P<0.0001.
treated with BafA1 or MG132 for 18 hours. YFP-rhTRIM5α protein expression was assessed by western blot and flow cytometry. As shown in figure 5, compared to untreated cells, treatment with BafA1, but not MG132, led to an accumulation of rhTRIM5α, as measured by western blot. Densitometric analysis by ImageJ allowed us to quantify the rhTRIM5α protein levels with each treatment. As shown in figure 5b, rhTRIM5α protein increased ~2.5 fold with BafA1 treatment, while MG132 treatment did not significantly increase the protein level compared to untreated samples.

Total rhTRIM5α protein levels were also measured by flow cytometry. Cells were left untreated or treated with BafA1 or MG132 for 18 hours and then fixed with 10% formaldehyde in PBS. The mean fluorescence intensity (MFI) of YFP-rhTRIM5α was determined using a FACS Canto II flow cytometer. Untransduced HeLa cells were used to subtract background fluorescence. Similar to the results observed with the western blot, BafA1 treatment resulted in a ~2.5 fold increase in MFI of YFP-rhTRIM5α, whereas treatment with MG132 did not lead to an increase in TRIM5α protein (figure 5c). From these results, we conclude the lysosome is responsible for rhTRIM5α steady state protein degradation.

Inhibition of the autophagy-lysosomal pathway prevents rhTRIM5α protein turnover

To determine if rhTRIM5α protein turnover occurs within the lysosome, we measured YFP-rhTRIM5α protein levels in the presence of cycloheximide and degradation inhibitors. Cycloheximide prevents new protein translation and therefore, can be used to measure protein degradation. Briefly, we treated YFP-rhTRIM5α HeLa cells
Figure 5 rhTRIM5α protein is sensitive to the autophagy inhibitor BafA1. A) HeLa cells stably expressing YFP-rhTRIM5α were treated with BafA1, MG132 or left untreated for 18 hours. Equivalent amounts of cell lysates were analyzed via Western blot using antibodies for GFP and actin as a loading control. B) YFP-rhTRIM5α protein levels were quantified using ImageJ software. C) YFP-rhTRIM5α protein expression was determined by flow cytometry in YFP-rhTRIM5α positive and negative cells. Mean fluorescence intensity (MFI) was determined after background fluorescence was subtracted. Data are representative of at least three experiments.
with BafA1 or MG132 in the presence of cycloheximide for 6 hours. rhTRIM5α protein was analyzed by western blot and flow cytometry using the same conditions as the steady state analysis. As shown in Figure 6a and b, we found that treatment with cycloheximide for 6 hours led to a significant reduction of rhTRIM5α protein levels, as expected based on previous estimates of rhTRIM5α half-life (Wu, Anderson et al. 2006). In the presence of BafA1 treatment, the levels of rhTRIM5α remained nearly constant, indicating that rhTRIM5α was not degraded when the lysosome was inhibited. As expected, treatment with MG132 did not alter rhTRIM5α protein levels. Additionally, we quantified total cellular protein levels using flow cytometry. As shown in Figure 6c, BafA1 treatment inhibited rhTRIM5α degradation, while MG132 had no effect. These data led us to conclude that lysosomal degradation is the main pathway responsible for rhTRIM5α turnover.

*Endogenous huTRIM5α degradation occurs within the lysosome*

Recently, antibodies were developed by the Hope and Sundquist labs to detect endogenous human and rhesus TRIM5α. These antibodies were made available through the NIH AIDS reagents and resources. Using the TRIM5α antibody, IF8-4, we assessed endogenous huTRIM5α in TE671 cells after treatment with BafA1 or MG132 for 18 hours. As shown in figure 7, huTRIM5α was detected using this antibody, and in the presence of MG132, there was no significant increase in huTRIM5α protein levels compared to control. However, in the presence of BafA1 there was a significant increase in TRIM5α protein. When the relative ratio of huTRIM5α protein levels was normalized
Figure 6 Altered rhTRIM5α protein turnover in the presence of BafA1 or MG132.
A) HeLa cells stably expressing YFP-rhTRIM5α were plated in triplicate, left untreated or treated with cycloheximide alone (CHX), CHX and BafA1 (CHX+BafA1), or CHX and MG132 (CHX+MG132) for 6 hours. Equivalent amounts of cell lysates were analyzed via Western blot using antibodies for GFP and actin as a loading control. B) YFP-rhTRIM5α protein levels were quantified using ImageJ software. C) YFP-rhTRIM5α protein expression was determined by flow cytometry in YFP-rhTRIM5α positive and negative cells. Mean fluorescence intensity (MFI) was determined after background fluorescence was subtracted. Data are representative of at least three experiments. **, P<0.01, compared to CHX alone
using the endogenous β-actin levels as a loading control, there was almost a ~2 fold increase in protein levels (Lane 2). As a control, we also probed the membranes for LC3, as a measure of autophagy-lysosomal inhibition. As expected, the ratio of LC3bII/LC3bI was almost 2:1 when BafA1 was present, which indicates that autophagy was inhibited. In the presence of MG132, this ratio was 1:1, which indicates that autophagy was activated by the extended proteasomal inhibition (Mizushima and Yoshimori 2007). This would agree with the decrease we observe for huTRIM5α protein levels in the presence of MG132 (lane 3). From these data, we conclude that endogenous huTRIM5α is degraded by lysosomal degradation similar to HeLa cell lines stably expressing rhTRIM5α. Also, these data indicate that both rhesus and human TRIM5α are sensitive to lysosomal degradation.
Figure 7 Altered endogenous huTRIM5α protein degradation in the presence of BafA1. TE671 cells were treated with DMSO, BafA1 or MG132 for 18 hours. Equivalent amounts of cell lysates were analyzed via Western blot using antibodies for TRIM5α, LC3 and β-actin. The relative ratio of TRIM5/actin was calculated based on the densitometry for each sample compared to the DMSO control. The ratio of LC3II/LC3I is the absolute ratio based on densitometric analysis using ImageJ. Data are representative of at least three experiments.
TRIM5α is degradation via chaperone mediated autophagy

*TRIM5α localizes with the macroautophagy marker LC3*

Treatment with BafA1 inhibits all lysosomal degradation. This includes macroautophagy, microautophagy and chaperone mediated autophagy. To further characterize the lysosomal pathway that is responsible for TRIM5α degradation, we examined the co-localization of rhTRIM5α with the macroautophagy marker protein LC3b. HeLa cells stably expressing YFP-rhTRIM5α were treated with BafA1 for 6 hours. Cells were then fixed and permeabilized with Saponin to preserve internal membrane structures and endogenous LC3 was detected using a rabbit anti-LC3 antibody. Cells were also stained with DAPI to visualize the nucleus. Control and BafA1 treated cells were imaged under identical imaging parameters and 20 images were taken per treatment. The co-localization between YFP-rhTRIM5α and LC3b was measured using Imaris imaging software. Representative images and quantification are shown in Figure 8. Cells were stained with secondary only to control for background staining. Cells that were left untreated had moderate co-localization between YFP-rhTRIM5α and endogenous LC3b. When cells were treated with BafA1, the localization between these two proteins increased significantly. As we hypothesize that upon BafA1 treatment, rhTRIM5α localizes to smaller more numerous puncta that are lysosomes that cannot degrade their contents, it is possible that the increased localization between rhTRIM5α and LC3b indicates autolysosomes that are inhibited from degrading their contents.
**Figure 8** rhTRIM5α co-localizes with the autophagy marker LC3b. HeLa cells stably expressing YFP-rhTRIM5α were seeded onto fibronectin treated coverslips. Cells were left untreated or treated with BafA1 for 6 hours. Cells were fixed, permeabilized and immunostained with rabbit anti-LC3b and DAPI. (A and B). Representative images of cells left untreated (A) or treated with BafA1 (B). C). To quantify the number of rhTRIM5α cytoplasmic bodies that were positive for LC3b staining in each treatment, 20 images were taken per treatment under identical acquisition parameters. Each image was analyzed using Imaris imaging software. ***, P<0.0001
p62 does not alter BafA1 sensitivity or LC3 localization of TRIM5α

Recently, O’Connor et al. identified and characterized the TRIM5α co-factor, p62 (O’Connor, Pertel et al. 2010). p62 is a multifunctional protein that has been implicated in protein trafficking to degradative pathways, including proteasomal and autophagosomal degradation (Pankiv, Clausen et al. 2007; Geetha, Seibenhener et al. 2008). Additionally, it was shown that p62 was important for rhTRIM5α protein stability; when p62 was knocked down using siRNA, TRIM5α protein levels decreased. Therefore, we hypothesized that p62 was important for rhTRIM5α degradation. Specifically, we hypothesized that knockdown of p62 would decrease LC3b localization with rhTRIM5α. To test this hypothesis, HeLa cells stably expressing YFP-rhTRIM5α were treated with p62-specific siRNA for two days and then co-localization with LC3b was assessed in the same manner as described above. As shown in figure 9, p62 knockdown decreased overall TRIM5α protein expression, as expected. However, the degree to which LC3b localized to TRIM5α was not decreased compared to control treated cells. Additionally, when control or p62 siRNA treated cells were treated with BafA1, the increase of YFP-rhTRIM5α puncta, as well as the degree of co-localization with LC3b, remained the same. Therefore, we conclude that p62 is not important for the localization of rhTRIM5α to LC3b. Additionally, as p62 siRNA cells are still sensitive to BafA1, we hypothesize that p62 is not important for TRIM5α lysosomal degradation.

rhTRIM5α localizes with the lysosomal membrane protein LAMP2
Figure 9 rhTRIM5α co-localizes with the autophagy marker LC3b independent of p62. HeLa cells stably expressing YFP-rhTRIM5α were transfected with p62 specific siRNA. Cells were then seeded onto fibronectin treated coverslips. Cells were fixed, permeabilized and immunostained with rabbit anti-LC3b, mouse anti-p62 and DAPI. (A and B). Representative images of cells left untreated (A) or treated with BafA1 (B). C). To quantify the number of rhTRIM5α cytoplasmic bodies that were positive for LC3b staining in each treatment, 20 images were taken per treatment under identical acquisition parameters. Each image was analyzed using Imaris imaging software. ***, P<0.0001
It has been postulated that BafA1 can prevent the fusion of autophagosomes with lysosomes under certain conditions (Klionsky, Elazar et al. 2008). Therefore, in order to determine if the localization of YFP-rhTRIM5α after treatment with BafA1 was to lysosomes or autophagosomes that didn’t fuse with lysosomes, we assessed the co-localization of YFP-rhTRIM5α with LAMP2. LAMP2 is a lysosomal marker protein. Therefore, if YFP-rhTRIM5α was actually trapped in autophagosomes upon BafA1 treatment, we would expect that, with BafA1 treatment, there would be no change in localization with LAMP2. However, if YFP-rhTRIM5α was trapped in lysosomes or autolysosomes upon BafA1 treatment, we would expect that co-localization with LAMP2 would increase with BafA1 treatment. To measure co-localization with LAMP2, cells were seeded on glass coverslips and treated with BafA1 for 6 hours. To detect endogenous LAMP2, cells fixed, permeabilized with Saponin and stained with a mouse anti-LAMP2 antibody. As shown in figure 10, YFP-rhTRIM5α co-localized with LAMP2 in the absence of drug; however, in the presence of BafA1, there was increased localization between YFP-rhTRIM5α and LAMP2. Given that YFP-rhTRIM5α can also localize with LC3b, we hypothesize that YFP-rhTRIM5α is localized with lysosome or autolysosomes upon treatment with BafA1, and not autophagosomes that did not fuse with lysosomes.

*rhTRIM5α degradation is not changed with Atg5 knockdown*

The localization of rhTRIM5α with markers of both autophagosomal and lysosomal degradation led us to examine if rhTRIM5α is degraded by macroautophagy. As BafA1 will not differentiate between these two pathways, we used siRNA to inhibit
Figure 10 rhTRIM5α co-localizes with the autophagy marker LAMP2. HeLa cells stably expressing YFP-rhTRIM5α were seeded onto fibronectin treated coverslips. Cells were left untreated or treated with BafA1 for 6 hours. Cells were fixed, permeabilized and immunostained with mouse anti-LAMP2 and DAPI. (A and B). Representative images of cells left untreated (A) or treated with BafA1 (B). C). To quantify the number of rhTRIM5α cytoplasmic bodies that were positive for LC3b staining in each treatment, 20 images were taken per treatment under identical acquisition parameters. Each image was analyzed using Imaris imaging software. ***, P<0.0001
macroautophagy (Kaushik, Massey et al. 2008). Specifically, we treated cells with Atg5 siRNA to prevent for the formation of the isolation membrane in the initial stages of macroautophagy (Weidberg, Shvets et al. 2011). HeLa cells stably expressing YFP-rhTRIM5α were treated with control or Atg5 siRNA and then collected and lysed for western blot analysis. As shown in figure 11, knockdown of Atg5 did not result in the increase of TRIM5α protein compared to control siRNA treated cells (lanes 1 and 2). To determine if YFP-rhTRIM5α was degraded by the lysosome when macroautophagy was inhibited, siRNA treated cells were treated with BafA1 or MG132. Lanes 3 and 4 of figure 11 show that upon Atg5 knockdown, rhTRIM5α was still sensitive to BafA1. rhTRIM5α protein remained insensitive to MG132 when macroautophagy was inhibited. These results indicate lysosomal degradation, and not macroautophagy, is responsible for the degradation of rhTRIM5α.

Knock down of the CMA proteins LAMP2 and Hsc70 decreases rhTRIM5α degradation

The significance of protein degradation that occurs solely within the lysosome is increasing. Pathways that are classified as lysosomal degradation include chaperone mediated autophagy (CMA) and microautophagy. Previously, it was shown that rhTRIM5α can interact with Hsc70, a protein important for CMA (Hwang, Holl et al. 2010). These data, along with the data that show that rhTRIM5α is degraded within lysosomes and not autophagosomes, led us to hypothesize that rhTRIM5α is degraded by CMA. To test this hypothesis, we
Figure 11 rhTRIM5α protein is sensitive to BafA1 in the presence of Atg5 knockdown. HeLa cells stably expressing YFP-rhTRIM5α were treated with control or Atg5 siRNA. Cells were then treated with BafA1 or MG132 for 18 hours. Protein expression was measured by Western blot. Equivalent amounts of cell lysates were probed with antibodies to Atg5, YFP and actin. B) YFP-rhTRIM5α protein levels were quantified using ImageJ software. YFP-rhTRIM5α protein levels were determined as a percentage of control siRNA treated cells.
utilized LAMP2 siRNA to inhibit CMA. During CMA, LAMP2a protein multimerizes to form a channel from the cytosol to the interior of the lysosome; this allows for the substrate protein to enter the lysosome from the cytosol (Bandyopadhyay, Kaushik et al. 2008). Therefore, using LAMP2 siRNA will prevent the formation of the channel required for CMA and thus prevent rhTRIM5α degradation if CMA is the pathway responsible. Cells were treated with control or LAMP2 siRNA following a two day protocol and then cellular protein levels were assessed by western blot analysis. As shown in figure 12, knockdown of LAMP2 led to a ~2.5 fold increase in TRIM5α protein levels. Additionally, we used siRNA to the chaperone Hsc70, which recognizes and guides the CMA substrate protein to the lysosome to be degraded (Chiang, Terlecky et al. 1989). Therefore, we would expect that if rhTRIM5α was degraded by CMA, then knocking down Hsc70 would prevent the localization of rhTRIM5α to the lysosome and lead to an accumulation of YFP-rhTRIM5α. As shown in figure 13, similar to LAMP2 knockdown, Hsc70 siRNA led to an accumulation of YFP-rhTRIM5α. Therefore, we conclude that rhTRIM5α is degraded by the lysosomal pathway CMA.

*rhTRIM5α degradation by CMA is dependent on a motif in the coiled-coil domain*

CMA depends on the recognition of a pentapeptide motif by Hsc70 and other components of the chaperone complex to guide the substrate to the lysosome (Terlecky, Chiang et al. 1992). This pentapeptide motif is hypothesized to be in ~30% of cytosolic proteins, meaning the possible substrates for CMA are great in number (Chiang and Dice 1988). Additionally, the pentapeptide motif is
Figure 12 Reduced LAMP2 leads to increased rhTRIM5α protein. A) HeLa cells stably expressing YFP-rhTRIM5α were treated with control or LAMP2 siRNA for 48 hours. Protein expression was measured by Western blot. Equivalent amount of cell lysates were probed with antibodies to LAMP2, YFP and actin. B) YFP-rhTRIM5α protein levels were quantified using ImageJ software. YFP-rhTRIM5α protein levels were determined as a percentage of control siRNA treated cells. Black bars indicate the amount of LAMP2 while gray bars indicate the amount of YFP-rhTRIM5α. The numbers on each bar represent the fold change in protein levels compared to control siRNA treated cells.
Figure 13 Reduced Hsc70 and LAMP2 leads to increased rhTRIM5α protein. 
A) HeLa cells stably expressing YFP-rhTRIM5α were treated with control, LAMP2 or Hsc70 siRNA for 48 hours. Protein expression was measured by Western blot. Equivalent amount of cell lysates were probed with antibodies to LAMP2, Hsc70, YFP and actin. B) YFP-rhTRIM5α protein levels were quantified using ImageJ software. YFP-rhTRIM5α protein levels were determined as a percentage of control siRNA treated cells. Blue bars indicate the amount of LAMP2; the amount of Hsc70 is shown in red; YFP-rhTRIM5α is in green and actin is in purple. The numbers on each bar represent the fold change in protein levels compared to control siRNA treated cells.
characterized not by specific residues per se but rather by the location of certain charged residues within a motif. Therefore, we examined the coding sequence of rhTRIM5α for possible CMA motifs. We identified three possible motifs as shown in figure 14a. To examine these motifs in TRIM5α, we used SOEing PCR to introduce triple alanine residues within the middle of the motif and cloned these mutant TRIM5α cDNAs into retroviral vectors to generate YFP-expressing fusion TRIM5α proteins. To test if these mutations prevented CMA degradation, we assessed the protein sensitivity to BafA1 induced accumulation. Therefore, HeLa cells stably expressing the YFP-rhTRIM5α CMA mutants were treated with BafA1 for 6 hours. Cells were fixed and stained with DAPI to visualize the nucleus. Images were acquired under identical imaging parameters. Mutating the putative CMA sequences at residues 77-79 (L1 domain) and 258-260 (L2 domain) to contain triple alanine residues did not change the sensitivity of TRIM5α to BafA1 (Figure 14 c and e). Therefore, we conclude that these residues are not responsible for the degradation by CMA of TRIM5α. However, the residues from 190-192 (CC domain) did not demonstrate the BafA1 sensitivity that wild-type and the other mutant showed. In fact, there was no difference in this mutant rhTRIM5α localization when BafA1 was used to inhibit lysosomal degradation compared to the control, untreated cells (Figure 14 d). Therefore, we conclude that rhTRIM5α is degraded by CMA and that the CC domain contains the specific pentapeptide motif necessary for CMA recognition and degradation.
**Figure 14 Identification and characterization of putative CMA sequences in rhTRIM5α.**

A) The coding sequence of rhTRIM5α is shown, with known domains indicated. The underlined sequences highlight the putative CMA sequences based on their biochemical composition. * indicate sequences that are biochemically related to known CMA substrates (Adolase B and aspartate aminotransferase, respectively)

B-E) HeLa cells stably expressing YFP-rhTRIM5α WT (B), L1 CMA mutant (C), CC CMA mutant (D) and L2 CMA mutant (e), treated with DMSO (left panels) or BafA (right panels) for 6 hours.
**rhTRIM5α protein degradation is important for retroviral restriction**

*BafA1 does not change retroviral restriction of late viral RT products*

Given that CMA is responsible for the degradation of rhTRIM5α, we sought to determine if the degradation of rhTRIM5α is important for retroviral restriction. Namely, we sought to determine if the inhibition of rhTRIM5α degradation could change the restriction profile of this protein. rhTRIM5α can potently inhibit the accumulation of late reverse transcriptase products, which is an early step in viral replication that occurs after or concurrent with viral uncoating (Stremlau, Owens et al. 2004). Additionally, rhTRIM5α can inhibit the formation of 2LTR circles which act as a marker for viral genome entry into the nucleus (Wu, Anderson et al. 2006). The inhibition of these steps prevents the virus from integrating into the host genome and infection is inhibited.

Additionally, while the proteasome has no effect on the degradation of rhTRIM5α, it is known that treatment of TRIM5α expressing cells with proteasome inhibitors can relieve the restriction of late RT products, but retroviral infectivity remains restricted (Wu, Anderson et al. 2006). We hypothesize that inhibition of rhTRIM5α lysosomal degradation is important for the mechanism of retroviral restriction. To measure rhTRIM5α restriction of HIV-1 when lysosomal degradation is inhibited, cells were treated with BafA1 for 18 hours with concurrent amphotropic MLV envelope pseudotyped R7 GFP reporter virus. This envelope mediates the entry of viruses independent of viral endocytosis and therefore, infection will not decrease when cells are treated with BafA1. Cells were lysed and genomic and viral DNA was extracted. Viral
late RT products were quantified using real time PCR using viral specific primers. As shown in figure 15, treatment with BafA1 has no effect on the restriction of late RT products by rhTRIM5α. As expected, treatment of cells with MG132 rescued the production of viral late RT products, as described previously (Wu).

*LAMP2 knock down increases retroviral restriction of B-MLV by rhTRIM5α*

Although BafA1 did not change the restriction profile of rhTRIM5α, we hypothesized that the degradation of rhTRIM5α is important for retroviral restriction. Given that we hypothesize that rhTRIM5α is degraded by CMA, it is possible that BafA1 inhibits rhTRIM5α that is already sequestered in lysosomes. Therefore we sought to measure viral late RT products and infectivity when CMA specifically was inhibited with LAMP2 siRNA. If LAMP2α is responsible for the translocation of rhTRIM5α into the lysosomes, then when LAMP2 was knocked down, there should be an accumulation of rhTRIM5α in the cytoplasm, thus increasing the population of rhTRIM5α available to restrict retroviral infection. HeLa cells stably expressing HA-rhTRIM5α were knocked down for LAMP2 following a two day siRNA protocol. Cells were then infected with A-MLV R7 ΔEnv GFP for 12 hours concurrent with BafA1 treatment. At this point in the viral life cycle, viral late RT products are at a maximum and therefore easily detectable. Additionally, cells were infected with A-MLV pseudotyped B and N-MLV in the presence of BafA1 and MG132. The drug containing media was replaced with normal DMEM at 24 hours post infection, and cells were collected at 48 hours post infection to assess viral infectivity by GFP or YFP fluorescence. Figure 16a demonstrates that LAMP2 knock
Figure 15 rhTRIM5α mediated restriction of viral late RT products is not affected by lysosomal inhibition. Untransduced HeLa cells (A) or HeLa cells stably expressing HA-rhTRIM5α (B) were plated in equivalent numbers and infected with amphitropic MLV Env pseudo-pseudotyped HIV-1 reporter virus for 18 hours with BafA1 or MG132. Cells were harvested and viral DNA products were analyzed by quantitative PCR. Values were normalized to 10 ng of total DNA in identical samples. Error bars represent the SEM from triplicate samples. Values above the columns represent fold enhancement relative to the level for the untreated sample of that cell type.
down at 48 hours post infection was robust, with a near complete depletion of LAMP2 in both untransduced HeLa and HA-rhTRIM5α expressing cells compared to control siRNA treated cells. The results of the late RT analysis are shown in Figure 16b. Inhibiting lysosomal degradation either with BafA1 alone or LAMP2 siRNA treatment slightly diminished the production of late RT products in HeLa cells. This could be due in part to cellular toxicity of the combination of siRNA and drug treatment. In HA-rhTRIM5α expressing cells, the late RT products remain restricted, indicating that inhibiting TRIM5α degradation does not relieve restriction. This is in contrast to treatment with the proteasomal inhibitor MG132, which can relieve the restriction of viral late RT products to the levels of HeLa cells. Interesting, in HA-rhTRIM5α expressing cells that were both knocked down for LAMP2 and treated with BafA1 there was a statistically significant decrease compared to DMSO treated cells. Although BafA1 led to slight a diminution of late RT products in the presence of BafA1 alone, these results suggests that there is a rhTRIM5α dependent decrease in viral late RT products when all lysosomal degradation is inhibited.

We also measured the effect of LAMP2 knock down on viral infectivity, specifically HIV-1, B-MLV and N-MLV. N-MLV was completely restricted in HeLa cells as expected, even though infectious virus was produced as evidenced by the titration of this virus on CRFK cells (Figure 15d). Similar to the results obtained for the late RT assay, BafA1 caused a slight decrease in viral infectivity, independent of TRIM5α expression (Figure 15c). As we used virus pseudotyped with A-MLV envelope, the decrease may be due to cellular toxicity, as BafA1 should not have affected viral entry.
We found that rhTRIM5α cells remained restricted for HIV-1 infection (left panel). However, we detected a rhTRIM5α-dependent decrease in B-MLV infectivity when LAMP2 was knocked down (right panel). This decrease was potentiated in the presence of BafA1. The complete inhibition of B-MLV infection in the presence may due to cellular toxicity from the extended MG132 treatment, as there was increased cell death prior to analysis. These data suggest that rhTRIM5α can restrict B-MLV infection when lysosomal degradation is inhibited, indicating a role for the lysosomal degradation in the restriction profile of rhTRIM5α.
Figure 16 rhTRIM5α mediated restriction of B-MLV is enhanced by LAMP2 siRNA. A) Untransduced HeLa cells or HeLa cells stably expressing HA-rhTRIM5α were treated with control or LAMP2 specific siRNA for 2 days. Cell lysates were analyzed for LAMP2 protein levels 48 hours later. B) siRNA treated cells were plated in equivalent numbers and infected with amphotropic MLV Env pseudotyped HIV-1 reporter virus for 12 hours with BafA1. Cells were harvested and viral DNA products were analyzed by quantitative PCR. Quantity of viral late RT products is expressed as a ratio to actin. Error bars represent the SEM from triplicate samples. * indicates a p>0.05 from a student’s T test. C) siRNA treated cells were plated in equivalent numbers and infected with A-MLV Env pseudotyped HIV-1 and B-MLV reporter viruses for 24 hours in the presence of BafA1 or MG132. Cells were collected 48 hours post infection and infectivity was assessed by flow cytometry. D) Titration of A-MLV Env pseudotyped B- and N-MLV on CRFK cells.
CHAPTER FOUR

DISCUSSION

Degradation of rhTRIM5α and huTRIM5α by the autophagy-lysosomal pathway

Many studies have found that retroviral restriction by TRIM5α includes a step that is sensitive to proteasomal inhibition. It is well established that this step allows the production of viral late RT products but 2LTR circles and infectivity remains inhibited (Wu, Anderson et al. 2006). Additionally, proteasomal inhibition reveals that during rhTRIM5α-mediated restriction, a functional preintegration complex forms and these complexes are competent for nuclear import (Anderson, Campbell et al. 2006). Also, proteasomal inhibition results in the accumulation of HIV-1 virions in TRIM5α containing cytoplasmic assemblies (Campbell, Perez et al. 2008). In spite of these data, proteasomal inhibition does not inhibit TRIM5α degradation, unless restriction sensitive virus is present (Rold and Aiken 2008). Therefore, the degradative pathway responsible for TRIM5α turnover and the role for this degradation in TRIM5α-mediated restriction was unclear.

In order to understand which cellular pathway is responsible for TRIM5α degradation, we used pharmacological inhibitors of the two main degradative pathways, the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway. Upon treatment of YFP-rhTRIM5α expressing HeLa cells with BafA1, an inhibitor of
lysosomal degradation, or MG132, which inhibits the UPS, we found that both treatments led to the re-distribution of YFP-rhTRIM5α. Inhibition of the UPS by MG132 caused YFP-rhTRIM5α to localize to fewer, larger cytoplasmic accumulations, as previously described (Wu, Anderson et al. 2006; Campbell, Perez et al. 2008). Inhibition of the autophagy lysosomal pathway by BafA1 led to the formation of smaller, more numerous YFP-rhTRIM5α accumulations (Figure 3). As BafA1 prevents degradation within the lysosome, it is possible that the localization of YFP-rhTRIM5α to these smaller accumulations represents YFP-rhTRIM5α trapped within lysosomes that cannot be degraded within this department. In support of this fact, we found that BafA1 increases the localization of YFP-rhTRIM5α to LC3 and LAMP2, proteins that serve as markers for the autophagosome and the lysosome, respectively (Figures 7 and 9). Additionally, we found that BafA1 treatment inhibits both the steady-state degradation and cellular turnover of rhTRIM5α (Figures 4 and 5). Collectively, these data indicate that YFP-rhTRIM5α is degraded by the autophagy-lysosomal pathway.

Since the discovery of rhTRIM5α as the cellular protein responsible for the species specific inhibition of HIV-1 and other retroviral infections, most studies of TRIM5 proteins have relied on cell lines expressing epitope tagged versions of this protein. This is due in part to the lack of reliable antibodies for the detection of endogenous TRIM5 proteins. The difficulty in generating specific antibodies can be attributed to the structural similarities between TRIM proteins (see Figure 2). However, recent collaboration between the Sundquist and Hope labs have generated monoclonal antibodies that can reliably and specifically detect endogenous human and rhesus
TRIM5α. Therefore, we used these antibodies to determine if endogenous human TRIM5α is also sensitive to inhibition of lysosomal degradation by BafA1. We found that in TE671, a human cell line that can inhibit N-MLV infection via huTRIM5α (Perron, Stremlau et al. 2004), there was a specific increase in huTRIM5α protein levels (Figure 6). Interestingly, treatment with MG132 resulted in a slight diminution of huTRIM5α protein in this assay. This decrease could possibly indicate that extended proteasomal inhibition in TE671 cells can increase lysosomal flux more rapidly than HeLa cells, as we saw no decrease in protein with MG132 in HeLa cells. These data were supported by the relatively similar levels of LC3 I and LC3II, which indicates increased degradation through the autophagy-lysosomal pathway (Mizushima and Yoshimori 2007).

Previously, it was reported that TRIM5 proteins are only sensitive to proteasomal degradation when restriction sensitive virus is present (Rold and Aiken 2008). This change in degradation was found to be conserved amongst rhesus, human and owl monkey TRIM5 proteins. Interestingly, TRIM5α proteins only become sensitive to proteasomal inhibition when destabilized by the retroviral CA protein. It is possible that with the stress of infection, TRIM5α proteins are degraded by the cell in order to ensure cellular survival, as has been the case for other proteins. Therefore, the proteasomal degradation of TRIM5α solely in the presence of restriction sensitive virus does not indicate the natural route of TRIM5α protein degradation.

TRIM proteins can act as E3 ubiquitin-ligases, an activity conferred by the N-terminal RING domain (Napolitano and Meroni 2012). Additionally, TRIM5α was found to ubiquitinate itself as well as other cellular proteins (Diaz-Griffero, Li et al. 2006;
Specifically, TRIM5α led to the K63-linked polyubiquitination of TAK1, a cellular protein necessary for AP-1 and NFκB innate immune signaling. Traditionally, K48-linked ubiquitin is thought to signal proteasomal degradation, while K63-linked ubiquitination can signal other fates for the substrate protein, such as protein localization or signaling. A recent publication revealed that both K48 and K63 linked ubiquitination can facilitate lysosomal degradation (Zhang, Xu et al. 2013), expanding the cellular role for ubiquitination. The present study does not address the role for TRIM5α ubiquitination and lysosomal degradation, although the hypothesis that ubiquitination of TRIM5α leads to lysosomal degradation deserves further study. Ultimately, the present study suggests that the ubiquitination of TRIM5α does not signal its degradation by the proteasome.

Degradation of rhTRIM5α by chaperone mediated autophagy

Our data demonstrate that the inhibition of lysosomal degradation with BafA1 prevents the degradation of TRIM5α. However, there are multiple cellular pathways that lead to lysosomal degradation. Interestingly, TRIM5α has been shown to directly act with cellular proteins involved in two of these pathways, Hsc70 and p62 (Hwang, Holl et al. 2010; O'Connor, Pertel et al. 2010). Hsc70 is chaperone that guides substrate proteins to the lysosome in chaperone mediated autophagy (CMA) while p62 is a cargo receptor protein involved in selective macroautophagy (Terlecky, Chiang et al. 1992; Schreiber and Peter 2013). We found that upon p62 knock down with specific siRNA, there was no change in BafA1 sensitivity of rhTRIM5α. Additionally, there was no change in the
degree of localization of LC3 to YFP-rhTRIM5α assemblies (Figure 8). These data indicate that p62 is not responsible for the selective macroautophagic degradation of rhTRIM5α. We also found that upon inhibition of macroautophagy with Atg5 specific siRNA, rhTRIM5α protein degradation remained inhibited with BafA1 treatment (Figure 10). Collectively, these data indicate that macroautophagy is not the pathway that degrades rhTRIM5α in the lysosome. However, we found that when we specifically inhibited CMA dependent lysosomal degradation by knocking down LAMP2, the protein receptor for CMA, and Hsc70, there was an accumulation of rhTRIM5α protein, in the absence of BafA1 treatment (Figures 11 and 12).

All validated substrates of CMA contain a pentapeptide motif that is recognized by Hsc70 to facilitate their degradation within the lysosome (Majeski and Dice 2004). However, this sequence is relies on the organization of certain charge residues, and not on the specific amino acids to direct substrates to the lysosome. The sequence consists of a glutamine (Q) preceded or followed by four amino acids consisting of a basic (lysine, K; arginine, R), an acidic (aspartic acid, D; glutamic acid, E), a bulky hydrophobic (phenylalanine, F; isoleucine, I; leucine, L; valine, V) and a repeated basic or hydrophobic amino acid (Dice, Terlecky et al. 1990). Additionally, some CMA targets can utilize the related asparagines (N) in place of the Q. Additionally, the CMA motif can be found at any position within the protein. However, the protein must be exposed for Hsc70 to bind the protein through unfolding or loss of interacting proteins that mask the motif (Kaushik and Cuervo 2012). In this way, single proteins of a multimer may be degraded by CMA. Finally, post translational modification can impart the necessary
charge to a given amino acid in a manner that now makes the protein targeted for CMA (Thompson, Aiken et al. 2009; Lv, Li et al. 2011). Using these criteria we identified three putative sequences within rhTRIM5α. Two of these motifs (QLREI, 189-192 and KRIEN, residues 257-261) are biochemically similar to the validated CMA targets aldolase B and aspartate aminotransferase, respectively (Majeski and Dice 2004). The putative sequence at residues 76-80 does not share biochemical similarities to known sequences; however it does meet the requirement as a CMA target sequence.

Additionally, all three motifs are well conserved between rhesus, human and gorilla TRIM5α, with the most diversity found in the residues that span 257-261 (data not shown). We mutated the core three residues of each of these motifs to alanines and expressed the mutant TRIM5α protein as a YFP fusion protein. When stably expressed in HeLa cells, the mutations of residues 77-79 and 258-260 remained sensitive to BafA1 (Figure 13). However, the mutation of residues 190-192 was not sensitive to BafA1. Interestingly, the two mutants that demonstrated BafA1 sensitivity formed cytoplasmic assemblies that resemble wild-type rhTRIM5α, while the mutation at 190-192 was diffuse and remained diffuse with BafA1 treatment. The coiled-coil domain, which is where these residues lie, is important for TRIM protein dimerization (Reddy, Etkin et al. 1992). Additionally, the coiled coil of rhTRIM5α has been shown to be under positive selective pressure to recognize the retroviral capsid (Johnson and Sawyer 2009). Specifically, residues 186, 214 and 229 of the coiled coil domain can modulate retrovirus specificity (Maillard, Ecco et al. 2010). However, no study has identified residues 189-192 to be important to retroviral restriction. Future studies to investigate the role of the
putative CMA motif in the coiled-coil domain of rhTRIM5α in retroviral restriction will allow us to further understand the mechanism of restriction.

**rhTRIM5α degradation alters the retroviral restriction profile**

As proteasomal involvement in TRIM5α mediated restriction has been well documented, we sought to understand if the lysosomal degradation of rhTRIM5α contributed to retroviral restriction. We assessed the possible role of lysosomal degradation two ways. First, we treated HA-rhTRIM5α expressing HeLa cells concurrently with BafA1 during A-MLV Env psuedotyped HIV-1 reporter virus. We then analyzed the viral late RT products for changes in restriction in the presence of BafA1. As shown in figure 14, BafA1 did not change the restriction profile in this analysis, while MG132 relieved the restriction of late RT products to the levels of the HeLa control. To assess the role for lysosomal degradation in another, we measured viral late RT products and infectivity in the presence of LAMP2 knock down. We hypothesize that BafA1 treatment sequesters rhTRIM5α to lysosomes, thereby preventing TRIM5α availability to restrict the incoming virus particle. Therefore, we inhibited TRIM5α localization to the lysosome using LAMP2, as the transcript variant LAMP2a is important for CMA-dependent localization to the lysosome. Under these conditions, we found a small, but statistically significant difference in the amount of viral late RT products between LAMP2 siRNA treated cells with or without BafA1 in rhTRIM5α expressing cells. We hypothesize that as LAMP2 siRNA can increase the amount of rhTRIM5α protein, this slight diminution in viral RT products represent the ability of TRIM5α to recognize the
viral capsid more efficiently because the protein is stabilized. The particular cell line used in this experiment expresses a large amount of rhTRIM5α. Therefore, we would expect in a cell line that expressed a more biologically relevant amount of TRIM5α, this difference would increase.

When we measured B-MLV infectivity in the presence of LAMP2 knock down, we saw a similar trend to the viral late RT assay. That is, LAMP2 knock down in rhTRIM5α expressing cells prevented B-MLV restriction more efficiently the control siRNA treated cells. B-MLV infection is normally uninhibited by TRIM5α expression. However, similar to the results of the late RT data, it is possible that increased rhTRIM5α available to recognize the retroviral capsid allows for more restriction to occur. Therefore, we conclude that the degradation of rhTRIM5α through the lysosome via CMA is important for restriction of HIV-1 and B-MLV. It is possible that the mechanism of restriction in this case arises from the increased amount of restriction factor present upon LAMP2 knock down.

**Conclusion**

This study presents evidence that the retroviral restriction factor rhTRIM5α is degraded via the lysosomal pathway chaperone mediated autophagy. We have demonstrated that inhibition of chaperone mediated autophagy prevents the degradation of rhTRIM5α, possibly through the recognition of a pentapeptide motif in the coiled-coil region of rhTRIM5α. Additionally, we found that when CMA is inhibited specifically using siRNA, there is an increase in rhTRIM5α mediated restriction of B-MLV, which is
normally unrestricted by TRIM5α. Therefore, the cellular degradation of rhTRIM5α may participate in the mechanism of retroviral restriction.
Figure 17 Model of CMA-mediated degradation of TRIM5α

A. TRIM5α protein degradation in the absence of restriction sensitive virus. The basal degradation of TRIM5α is insensitive to proteasomal inhibition. Treatment of TRIM5α expressing cells with lysosomal inhibitors such as BafA1 prevents CMA and leads to phenotypic changes in TRIM5α protein localization and turnover. B. TRIM5α protein degradation in the presence of restriction sensitive virus. During retroviral infection, TRIM5α binds determinants in the retroviral capsid that lead to the destabilization of TRIM5α and the capsid protein itself. Under these conditions, TRIM5α protein localization and turnover is sensitive to proteasomal inhibitors, indicating a change in the degradation pathway utilized by the host cell.
REFERENCES


VITA

Rachel Sarah Nelson was born November 27th, 1984 in Akron, Ohio to Dana Offerman and Larry Nelson. She attended the University of Wisconsin-Madison, majoring in Molecular biology and Latin American, Iberian and Caribbean Studies. Rachel completed her senior thesis in the laboratory of Nadine Connor, PhD, studying the effects of aging on estrogen receptor alpha and beta in tongue muscles to better understand the molecular mechanism of swallowing. She received her Bachelor of Science degree in August 2007.

From November 2007 to July 2009, Rachel worked as an Associate Research Specialist at the Influenza Research Institute in Madison, WI, headed by Yoshihiro Kawaoka, DVM, PhD. Under the guidance of senior scientist, Shinji Watanabe, PhD, she studied the molecular pathogenesis of Influenza and Ebola viruses.

Rachel entered the Department of Microbiology and Immunology at Loyola University Chicago in August of 2009. She joined the laboratory of Edward Campbell, Ph.D. in June 2010, where she has been investigating the cellular degradation of TRIM5α, as well as the characterization of the TRIM family of proteins. During her time at Loyola, Rachel presented her research at the American Society for Virology in the summer of 2012.

After completing her Master of Science degree, Rachel is pursuing a job in the pharmaceutical industry or public health. She plans on attending graduate school to get her Master of Public Health in the future.