



2013

## Identifying and Characterizing the Degradative Pathway of the Retroviral Restriction Factor RhTRIM5 $\alpha$

Rachel Nelson  
*Loyola University Chicago*

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LOYOLA UNIVERSITY CHICAGO

IDENTIFYING AND CHARACTERIZING THE DEGRADATIVE PATHWAY OF  
THE RETROVIRAL RESTRICTION FACTOR RHTRIM5 $\alpha$

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

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## 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.

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## 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<sup>+</sup> T cells. The cellular host protein TRIM5 $\alpha$  found in rhesus macaques (rhTRIM5 $\alpha$ ) 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 rhTRIM5 $\alpha$  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 TRIM5 $\alpha$ . Interestingly, treatment of rhTRIM5 $\alpha$  expressing cells with proteasomal inhibitors does not alter rhTRIM5 $\alpha$  protein turnover. The role of a second cellular degradation pathway, the autophagy-lysosomal pathway, in TRIM5 $\alpha$  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, TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  is degraded via chaperone mediated autophagy. Using inhibitors of lysosomal degradation, we show that rhTRIM5 $\alpha$  protein turnover decreases and rhTRIM5 $\alpha$  subcellular localization is altered. Additionally, we show that human TRIM5 $\alpha$  protein degradation is inhibited when

lysosomal degradation is inhibited. These data indicate that lysosomal degradation is responsible for TRIM5 $\alpha$  protein turnover. We found that treatment with lysosomal inhibitors increased the co-localization between TRIM5 $\alpha$  and proteins important for autophagy-lysosomal degradation. We show that when macroautophagy is inhibited, TRIM5 $\alpha$  protein degradation remains sensitive to lysosomal inhibition. We found that genetic inhibition of CMA prevented rhTRIM5 $\alpha$  turnover, indicating that CMA may be the pathway responsible for rhTRIM5 $\alpha$  degradation. Furthermore, we identify a CMA targeting motif within the coiled-coil domain of rhTRIM5 $\alpha$  that decreases rhTRIM5 $\alpha$  sensitivity to lysosomal inhibitors, indicating that rhTRIM5 $\alpha$  is degraded by CMA .

Our data suggests that rhTRIM5 $\alpha$  degraded by the lysosome, specifically chaperone mediated autophagy. Therefore, we hypothesized that the lysosomal degradation of TRIM5 $\alpha$  is important for viral restriction. When rhTRIM5 $\alpha$  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 TRIM5 $\alpha$ . Therefore, we conclude that TRIM5 $\alpha$  is degraded by chaperone mediated autophagy and that inhibition of chaperone mediated autophagy can enhance TRIM5 $\alpha$  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 20<sup>th</sup> and 21<sup>st</sup> 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/ $\mu$ l blood plasma (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).

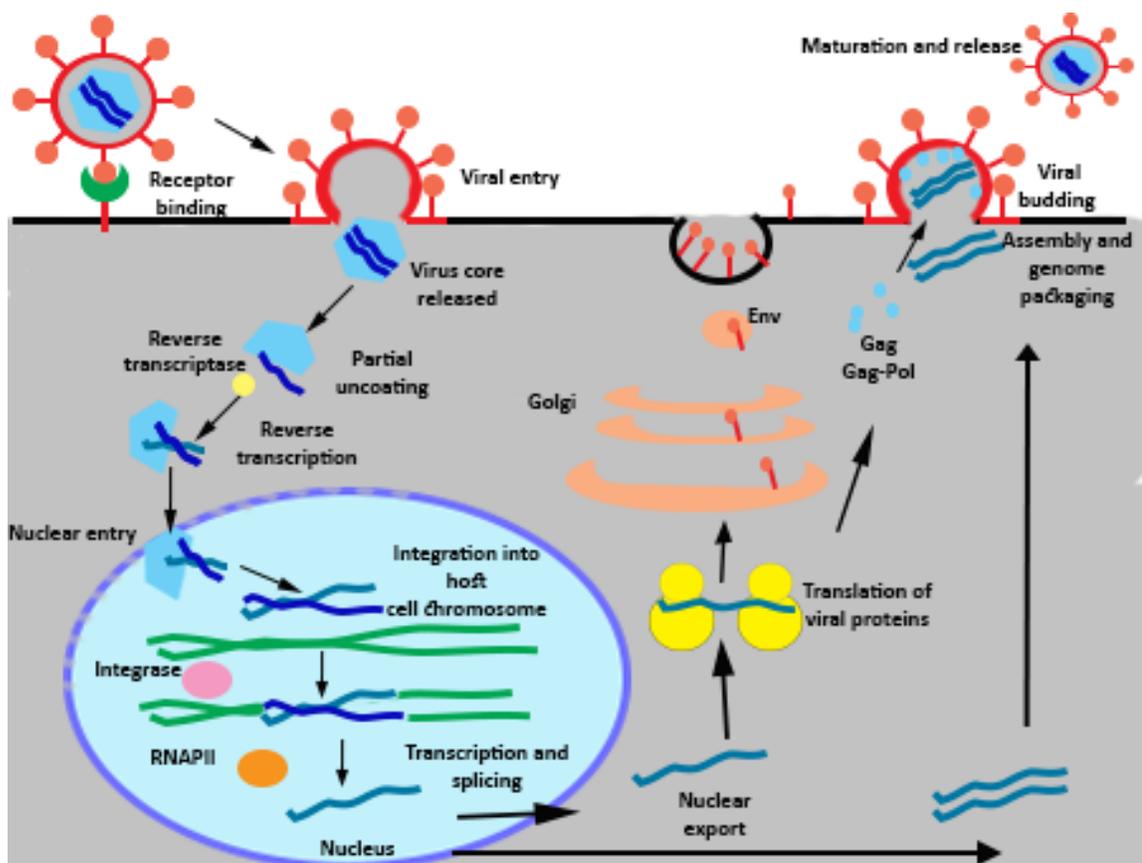
Patients infected with HIV-1 have a diminished T cell response as the initial target of infection is activated CD4+ T cells. CD4 is the primary receptor for HIV-1. Early in infection, the virus uses the cytokine CCR5 as a co-receptor. As the virus evolves and spreads, there is an emergence of viruses 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 (Wilens, 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

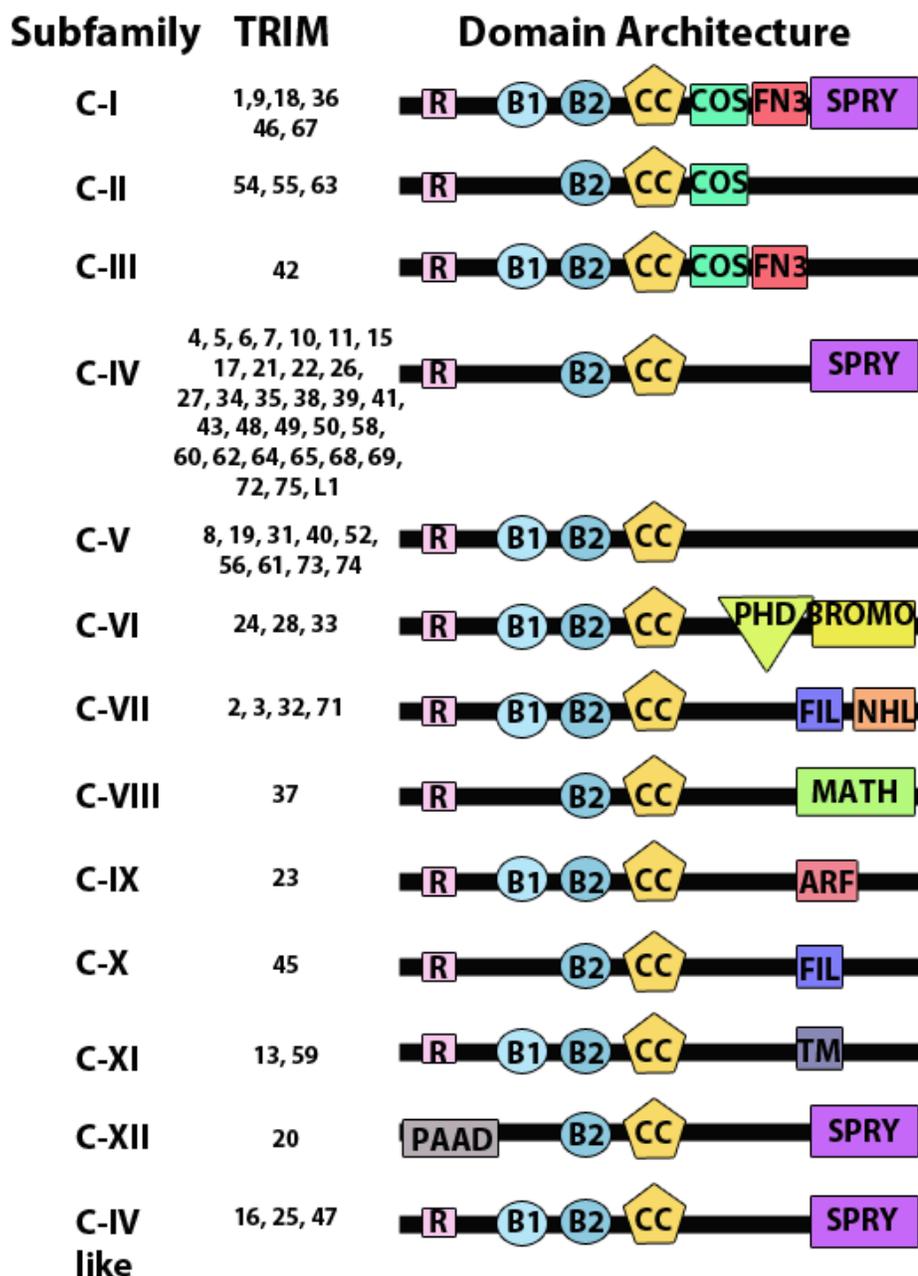


**Figure 1 Steps in the retroviral life cycle.** Schematic highlighting the process of viral entry and exit. The viral glycoprotein engages the primary receptor CD4 (co-receptors are not indicated). This leads to fusion of the viral membrane with the host cell plasma membrane to allow for the viral core to enter the cytoplasm. Partial uncoating of the viral nucleocapsid 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 (Carthagen, 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, NF $\kappa$ B 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.

### **TRIM5 $\alpha$ : Species specific retroviral restriction factor**

The first TRIM protein that was found to prevent viral infection was a variant of TRIM5, TRIM5 $\alpha$ . Specifically, Stremlau et al. found that TRIM5 $\alpha$  from rhesus macaques (rhTRIM5 $\alpha$ ) can potentially prevent HIV-1 infection (Stremlau, Owens et al. 2004). Even before the identification of rhTRIM5 $\alpha$ , 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 rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  (huTRIM5 $\alpha$ ) demonstrated a less potent block to HIV-1 infection, although it could potentially 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 rhTRIM5 $\alpha$  or huTRIM5 $\alpha$ , 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 huTRIM5 $\alpha$  restriction (Perron, Stremlau et al. 2004). This led to the hypothesis that TRIM5 $\alpha$  acts as a species specific barrier to retroviral infection. Indeed, as more TRIM5 $\alpha$  orthologs were identified from different primates, the evidence for this hypothesis grew as some primate TRIM5 $\alpha$  genes could restrict simian immunodeficiency virus (SIV) isolated from a different primate species. For example, SIV isolated from macaques (SIV<sub>mac</sub>) was not restricted by rhTRIM5 $\alpha$ . However, squirrel monkey

rhTRIM5 $\alpha$  could restrict SIV<sub>mac</sub> (Nakayama and Shioda 2012). In all cases, the specific retroviral CA was targeted by the TRIM5 $\alpha$  C-terminal domain, which in most TRIM5 $\alpha$  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 TRIM5 $\alpha$  contains amino acid determinants that provide specificity for the retroviral capsid. Specifically, there are three variable regions within the SPRY domain of TRIM5 $\alpha$  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 TRIM5 $\alpha$  that demonstrate similar positive selection (Johnson and Sawyer 2009). Collectively, these results indicate that rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$**

The mechanism of retroviral restriction by TRIM5 $\alpha$  is not fully understood. While it was postulated that the E3 ubiquitin ligase activity found in the RING domain of TRIM5 $\alpha$  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 TRIM5 $\alpha$  mediated restriction have been proposed.

One of the earliest models proposed was described by Stremlau et al. In this model, TRIM5 $\alpha$  can facilitate the rapid, premature degradation of the retroviral capsid (Stremlau, Perron et al. 2006). In these studies, TRIM5 $\alpha$  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 TRIM5 $\alpha$  can cause capsid disassembly while not degrading the CA protein itself (Stremlau, Perron et al. 2006). Similar results were observed for huTRIM5 $\alpha$  and TRIMCyp (Diaz-Griffero, Kar et al. 2007).

A second model of TRIM5 $\alpha$  restriction involves a two-step mechanism of restriction first proposed by Anderson et al. In this model, TRIM5 $\alpha$  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 TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  restriction by demonstrating the production of viral late RT products and competent pre-integration complexes (PICs) when rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  cytoplasmic assemblies upon treatment with proteasomal inhibitors, although this sequestration was not necessary for viral restriction. Therefore, retroviral restriction mediated by rhTRIM5 $\alpha$  involves a proteasomal-independent intermediate consisting of HIV-1 CA surrounded by rhTRIM5 $\alpha$  (Campbell, Perez et al. 2008). However, the use of proteasomal inhibitors did not lead to the accumulation of rhTRIM5 $\alpha$  protein, indicating that the ubiquitin-proteasome system is not the degradative pathway responsible for rhTRIM5 $\alpha$  protein turnover.

A third model of TRIM5 $\alpha$  restriction also describes the loss of CA in in TRIM5 $\alpha$  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 TRIM5 $\alpha$  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 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  $\alpha$ -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 cognate 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;

Massey, 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 phosphatidylinositol-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, Mortimore 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 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 such as  $\alpha$ 2-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 1 $\alpha$  (EF1 $\alpha$ ) (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).



### **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, TRIM79 $\alpha$ , TRIM50 and TRIM5 $\alpha$  have all been shown to be sensitive to both degradation pathways.

### **TRIM79 $\alpha$ : Flavivirus restriction factor**

TRIM79 $\alpha$  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 TRIM79 $\alpha$  is similar to TRIM5 $\alpha$ , with the requisite RING, BBox and CC domains followed by a C-terminal SPRY domain. Also similar to TRIM5 $\alpha$ , the expression of TRIM79 $\alpha$  can be induced by interferon. Interestingly, TRIM79 $\alpha$  shows similar tissue distribution as murine TRIM30 $\alpha$ , a mouse TRIM5 $\alpha$  homolog (Shi, Deng et al. 2008; Tareen and Emerman 2011). TRIM79 $\alpha$  has a protein half-life of about 90 minutes, similar to the half-life described for TRIM5 $\alpha$  (Wu, Anderson et al. 2006). The interaction between TRIM79 $\alpha$  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 TRIM79 $\alpha$  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 NH<sub>4</sub>Cl prevented NS5 degradation, while MG132 had no effect. In the absence of restriction sensitive virus, TRIM79 $\alpha$  was sensitive to proteasomal inhibition, indicating that TRIM79 $\alpha$  utilizes different degradation pathways to facilitate flavivirus restriction. It is hypothesized that as NS5 can form large protein complexes with other viral proteins, the TRIM79 $\alpha$ -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 in 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 multisystem 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 as 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 TRIM5 $\alpha$ . 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 NH<sub>4</sub>Cl, indicating that TRIM50 is degraded by autophagy-lysosomal pathway. Also of note, when autophagy is inhibited with NH<sub>4</sub>Cl, 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.

### **TRIM5 $\alpha$ : Association with proteins important for both degradative pathways**

As discussed previously, TRIM5 $\alpha$ -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 TRIM5 $\alpha$  is mediated by the proteasome. Rold and Aiken demonstrated that TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  but not of huTRIM5 $\alpha$ . 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 cyclophilin 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, huTRIM5 $\alpha$ , 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 rhTRIM5 $\alpha$  in the presence of specific retroviruses suggests that perhaps a molecular switch exists that controls the degradative fate of TRIM5 $\alpha$ , 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 rhTRIM5 $\alpha$  and huTRIM5 $\alpha$ . Furthermore, p62 localized to TRIM5 $\alpha$  cytoplasmic assemblies and knock down of p62 using specific siRNA led to a decrease in TRIM5 $\alpha$  protein levels. Knock down of p62 in TRIM5 $\alpha$  expressing cells also decreased retroviral restriction, which can likely be attributed to the decrease in TRIM5 $\alpha$  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 TRIM5 $\alpha$  to different degradation pathways.

p62 is one of many TRIM5 $\alpha$  cofactors that could play a role in the degradation of TRIM5 $\alpha$ . Additionally, heat shock proteins have been shown to interact and co-localize with TRIM5 $\alpha$ . Specifically, Hsp70, Hsp90 and Hsc70 can interact with TRIM5 $\alpha$  (Diaz-Griffero, Li et al. 2006; Hwang, Holl et al. 2010). The association of Hsc70 with TRIM5 $\alpha$  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 TRIM5 $\alpha$  protein folding, while the role for Hsc70 in TRIM5 $\alpha$  has not yet been identified (Hwang, Holl et al. 2010).

Finally, proteins important for ubiquitination and proteasomal degradation have been associated with TRIM5 $\alpha$  (Yamauchi, Wada et al. 2008; Lukic, Hausmann et al. 2011; Pertel, Hausmann et al. 2011). TRIM5 $\alpha$  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 TRIM5 $\alpha$

ubiquitination, demonstrating that TRIM5 $\alpha$  is not degraded by the proteasome (Yamauchi, Wada et al. 2008). Both mono- and polyubiquitination of TRIM5 $\alpha$  have been detected (Diaz-Griffero, Li et al. 2006; Yamauchi, Wada et al. 2008). Interestingly, although TRIM5 $\alpha$  degradation is not dependent on the proteasome, Lukic et al. detected proteasomal subunits in TRIM5 $\alpha$  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 TRIM5 $\alpha$  can associate with the E2 complex, UBC13-UEV1A, that leads to the K63-linked ubiquitination of signaling molecules important for AP-1 and NF $\kappa$ B activation of the innate immune response (Pertel, Hausmann et al. 2011). Collectively, these data demonstrate that both proteasomal and autophagic proteins can interact with TRIM5 $\alpha$ , and these interactions cannot definitely identify the degradative fate of TRIM5 $\alpha$ .

### **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<sub>2</sub> at 37°C. To produce amphitropic MLV Env (A-MLV) pseudotyped HIV-1 GFP reporter viruses, 293T cells were transfected with 6 µg A-MLV plasmid and 4 µg 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 rhTRIM5 $\alpha$  (YFP-rhTRIM5 $\alpha$ ) and hemagglutinin (HA)-tagged WT rhTRIM5 $\alpha$  have been described previously (Campbell, Dodding et al. 2007; Sastri, O'Connor et al. 2010). To generate mutations in the putative CMA sequences of rhTRIM5 $\alpha$ , triple alanine mutants were introduced using SOEing PCR (Sastri, O'Connor et al. 2010). Subsequently, mutant rhTRIM5 $\alpha$  PCR products were cloned into a pLNCX2 derived MLV retroviral plasmid encoding an N-terminal YFP protein tag in frame with rhTRIM5 $\alpha$  using BamHI and XhoI. To generate HeLa cell lines expressing the mutant YFP-rhTRIM5 $\alpha$ , 293T cells were transfected using the YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$ . YFP-rhTRIM5 $\alpha$  expression was confirmed by immunofluorescence and western blot.

### **Steady state protein analysis**

Cell lines stably expressing the indicated TRIM5 $\alpha$  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- $\beta$ -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 TRIM5 $\alpha$  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 TRIM5 $\alpha$  protein was detected by Western blot.

### **Flow cytometry**

Equivalent numbers of cells stably expressing YFP-rhTRIM5 $\alpha$  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 huTRIM5 $\alpha$**

TRIM5 $\alpha$  antibodies were acquired from the NIH AIDS reagents and resources program. To measure endogenous TRIM5 $\alpha$  upon proteasomal inhibition, TE671 cells were used due to their high level of endogenous huTRIM5 $\alpha$  and their ability to resist N-MLV infection. Briefly,  $1 \times 10^6$  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-TRIM5 $\alpha$  (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. huTRIM5 $\alpha$  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-rhTRIM5 $\alpha$ , and all YFP-rhTRIM5 $\alpha$

bodies over an area of  $0.2 \mu\text{m}^2$  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'-GCCUGUAUGUACUGCUUUA-3' and 5'-GCCUGUAUGUACUGCUUUA-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  $\mu\text{M}$ ) 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  $\alpha$ -Atg5 (Cell Signaling, Danvers, MA, USA),  $\alpha$ -GFP, or  $\alpha$ - $\beta$ -actin antibodies.

*LAMP2 and Hsc70 siRNA treatment:* Subconfluent YFP-rhTRIM5 $\alpha$  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  $\alpha$ -LAMP2 ,  $\alpha$ -Hsc70  $\alpha$ -GFP, and  $\alpha$ - $\beta$ -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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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/ $\mu$ l Dpn1 (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)-rhTRIM5 $\alpha$  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 TRIM5 $\alpha$ turnover**

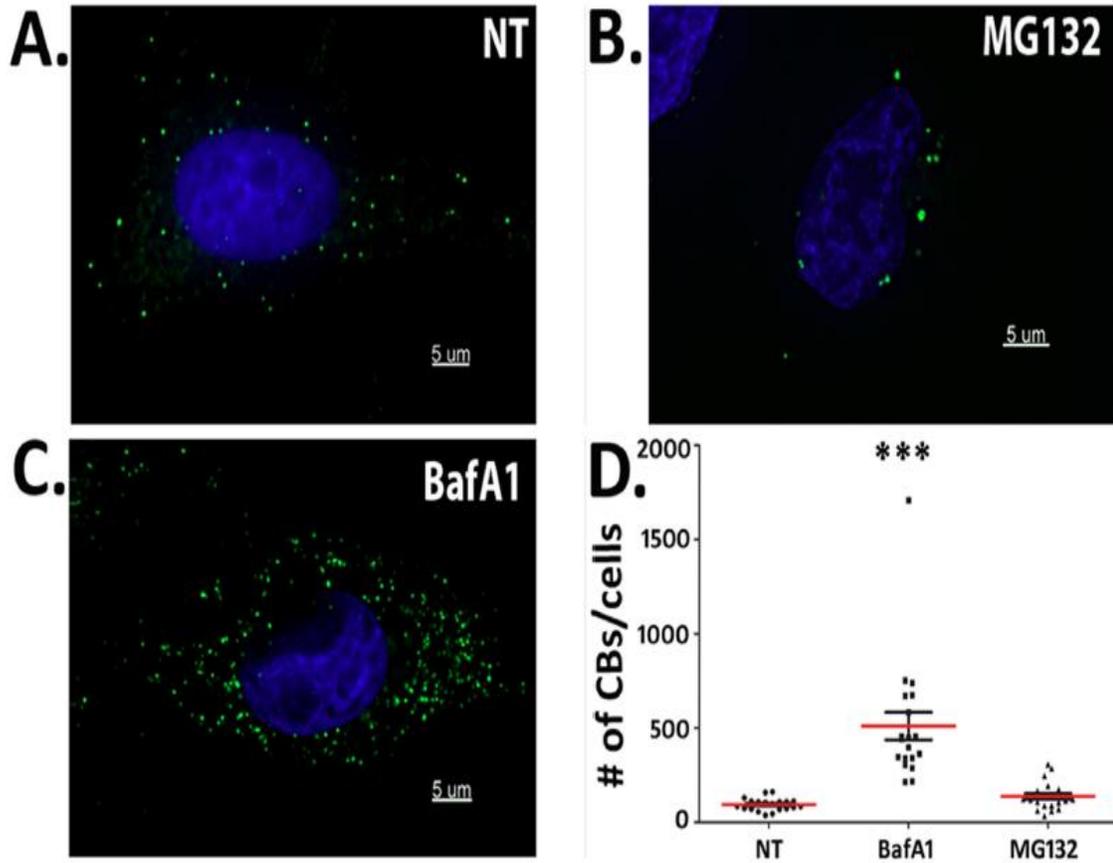
##### *Lysosomal inhibition alters rhTRIM5 $\alpha$ localization*

To identify the degradative pathway responsible for the steady state degradation of rhTRIM5 $\alpha$ , HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  protein, treating rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  protein upon lysosomal inhibition of YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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 NH<sub>4</sub>Cl. We observed the same re-localization of YFP-rhTRIM5 $\alpha$  protein with NH<sub>4</sub>Cl treatment that was observed for BafA1 (data not shown). Given that BafA1 and NH<sub>4</sub>Cl inhibit lysosomal degradation, we hypothesize that the accumulations of YFP-rhTRIM5 $\alpha$  upon BafA1 treatment are not actually cytoplasmic bodies but rather represent YFP-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$ .

#### *Inhibition of the autophagy-lysosomal pathway prevents rhTRIM $\alpha$ degradation*

Previously, proteasomal inhibition has been shown to relieve TRIM5 $\alpha$  mediated restriction of late RT products without leading to an accumulation of TRIM5 $\alpha$  protein (Wu, Anderson et al. 2006). Therefore, the mechanism of TRIM5 $\alpha$  degradation remains unknown. We hypothesize that autophagy is responsible for the degradation of TRIM5 $\alpha$ . To test this hypothesis, we utilized YFP-rhTRIM5 $\alpha$  HeLa cell lines to measure rhTRIM5 $\alpha$  protein in the presence of UPS or autophagy-lysosome inhibitors. Cells were



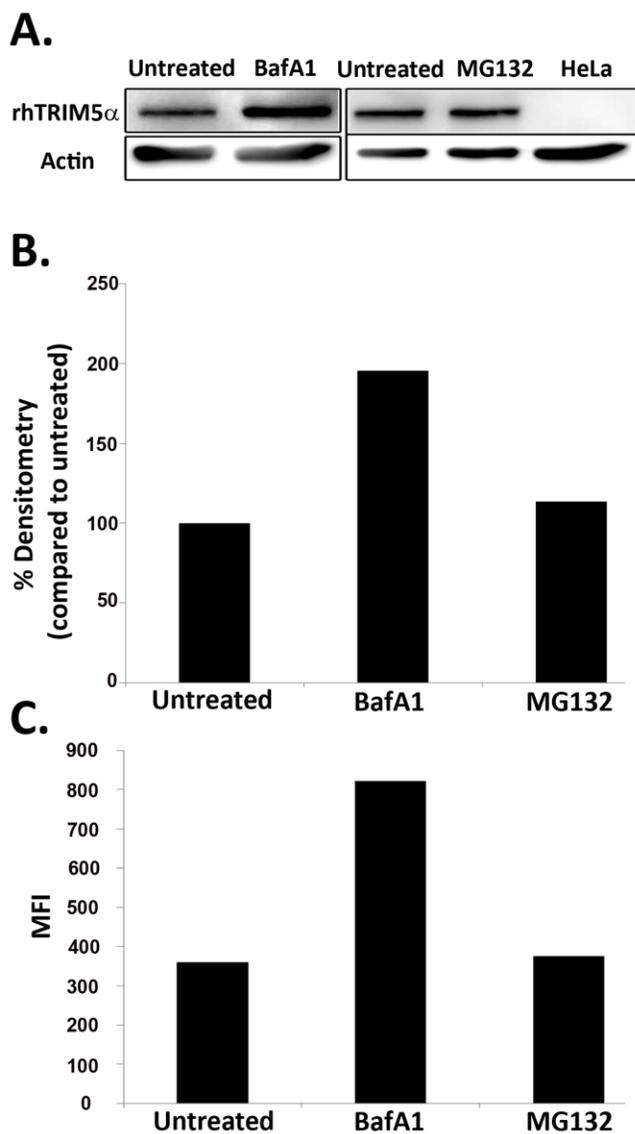
**Figure 4 Subcellular localization of YFP-rhTRIM5 $\alpha$  changes in the presence of BafA1 and MG132.** HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were seeded onto fibronectin treated coverslips for 18 hours and treated with BafA1 or MG132. (A-C). Representative images of cells left untreated, treated with MG132, or treated with BafA1. D). To quantify the number of rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$ , as measured by western blot. Densitometric analysis by ImageJ allowed us to quantify the rhTRIM5 $\alpha$  protein levels with each treatment. As shown in figure 5b, rhTRIM5 $\alpha$  protein increased ~2.5 fold with BafA1 treatment, while MG132 treatment did not significantly increase the protein level compared to untreated samples.

Total rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$ , whereas treatment with MG132 did not lead to an increase in TRIM5 $\alpha$  protein (figure 5c). From these results, we conclude the lysosome is responsible for rhTRIM5 $\alpha$  steady state protein degradation.

#### *Inhibition of the autophagy-lysosomal pathway prevents rhTRIM $\alpha$ protein turnover*

To determine if rhTRIM5 $\alpha$  protein turnover occurs within the lysosome, we measured YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  HeLa cells

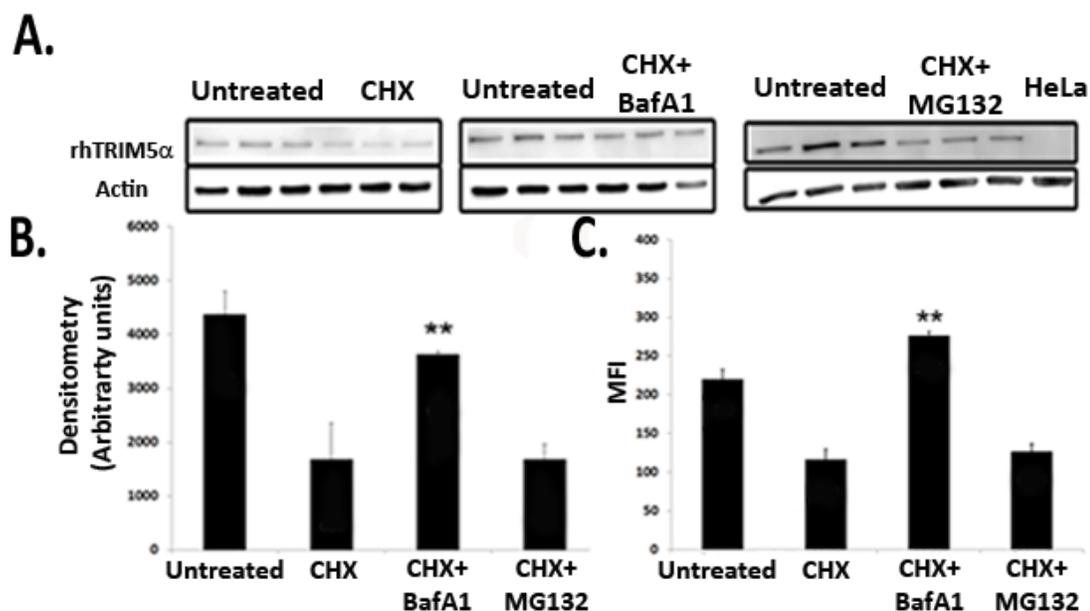


**Figure 5 rhTRIM5 $\alpha$  protein is sensitive to the autophagy inhibitor BafA1.** A) HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were treated with BafA1, MG132 or left untreated for 18 hours. Equivalent amounts of cell lysates were analyzed via Western blot using antibodies for GFP and actin as a loading control. B) YFP-rhTRIM5 $\alpha$  protein levels were quantified using ImageJ software. C) YFP-rhTRIM5 $\alpha$  protein expression was determined by flow cytometry in YFP-rhTRIM5 $\alpha$  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. rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  protein levels, as expected based on previous estimates of rhTRIM5 $\alpha$  half-life (Wu, Anderson et al. 2006). In the presence of BafA1 treatment, the levels of rhTRIM5 $\alpha$  remained nearly constant, indicating that rhTRIM5 $\alpha$  was not degraded when the lysosome was inhibited. As expected, treatment with MG132 did not alter rhTRIM5 $\alpha$  protein levels. Additionally, we quantified total cellular protein levels using flow cytometry. As shown in Figure 6c, BafA1 treatment inhibited rhTRIM5 $\alpha$  degradation, while MG132 had no effect. These data led us to conclude that lysosomal degradation is the main pathway responsible for rhTRIM5 $\alpha$  turnover.

*Endogenous huTRIM5 $\alpha$  degradation occurs within the lysosome*

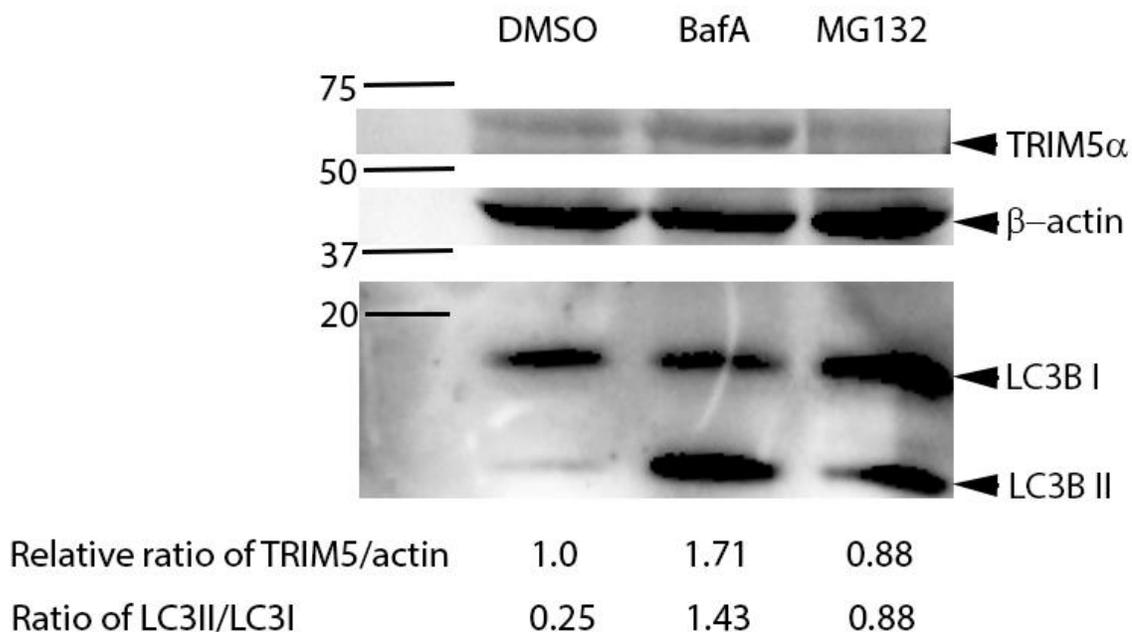
Recently, antibodies were developed by the Hope and Sundquist labs to detect endogenous human and rhesus TRIM5 $\alpha$ . These antibodies were made available through the NIH AIDS reagents and resources. Using the TRIM5 $\alpha$  antibody, IF8-4, we assessed endogenous huTRIM5 $\alpha$  in TE671 cells after treatment with BafA1 or MG132 for 18 hours. As shown in figure 7, huTRIM5 $\alpha$  was detected using this antibody, and in the presence of MG132, there was no significant increase in huTRIM5 $\alpha$  protein levels compared to control. However, in the presence of BafA1 there was a significant increase in TRIM5 $\alpha$  protein. When the relative ratio of huTRIM5 $\alpha$  protein levels was normalized



**Figure 6 Altered rhTRIM5 $\alpha$  protein turnover in the presence of BafA1 or MG132.**

A) HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  protein levels were quantified using ImageJ software. C) YFP-rhTRIM5 $\alpha$  protein expression was determined by flow cytometry in YFP-rhTRIM5 $\alpha$  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  $\beta$ -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 huTRIM5 $\alpha$  protein levels in the presence of MG132 (lane 3). From these data, we conclude that endogenous huTRIM5 $\alpha$  is degraded by lysosomal degradation similar to HeLa cell lines stably expressing rhTRIM5 $\alpha$ . Also, these data indicate that both rhesus and human TRIM5 $\alpha$  are sensitive to lysosomal degradation.

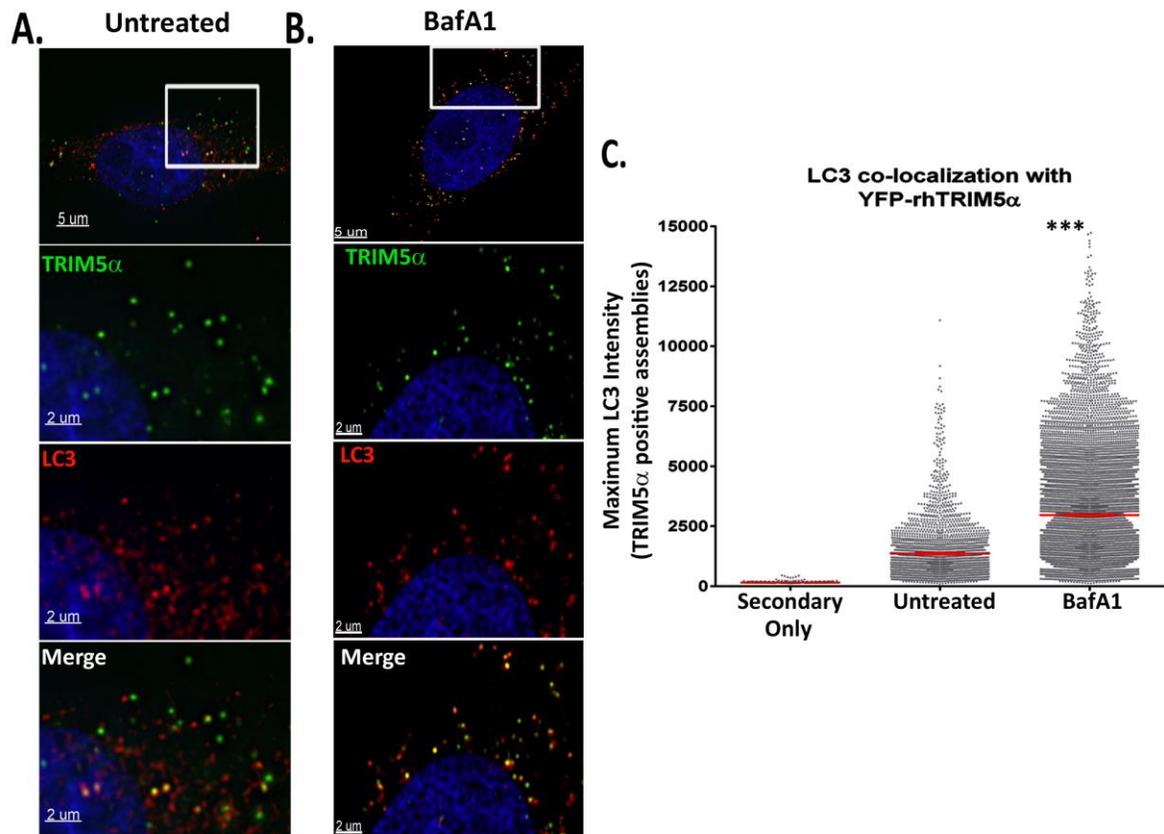


**Figure 7 Altered endogenous huTRIM5 $\alpha$  protein degradation in the presence of BafA1.** TE671 cells were treated with DMSO, BafA1 or MG132 for 18 hours. Equivalent amounts of cell lysates were analyzed via Western blot using antibodies for TRIM5 $\alpha$ , LC3 and  $\beta$ -actin. The relative 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.

## **TRIM5 $\alpha$ is degradation via chaperone mediated autophagy**

### *TRIM5 $\alpha$ 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 TRIM5 $\alpha$  degradation, we examined the co-localization of rhTRIM5 $\alpha$  with the macroautophagy marker protein LC3b. HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  and endogenous LC3b. When cells were treated with BafA1, the localization between these two proteins increased significantly. As we hypothesize that upon BafA1 treatment, rhTRIM5 $\alpha$  localizes to smaller more numerous puncta that are lysosomes that cannot degrade their contents, it is possible that the increased localization between rhTRIM5 $\alpha$  and LC3b indicates autolysosomes that are inhibited from degrading their contents.

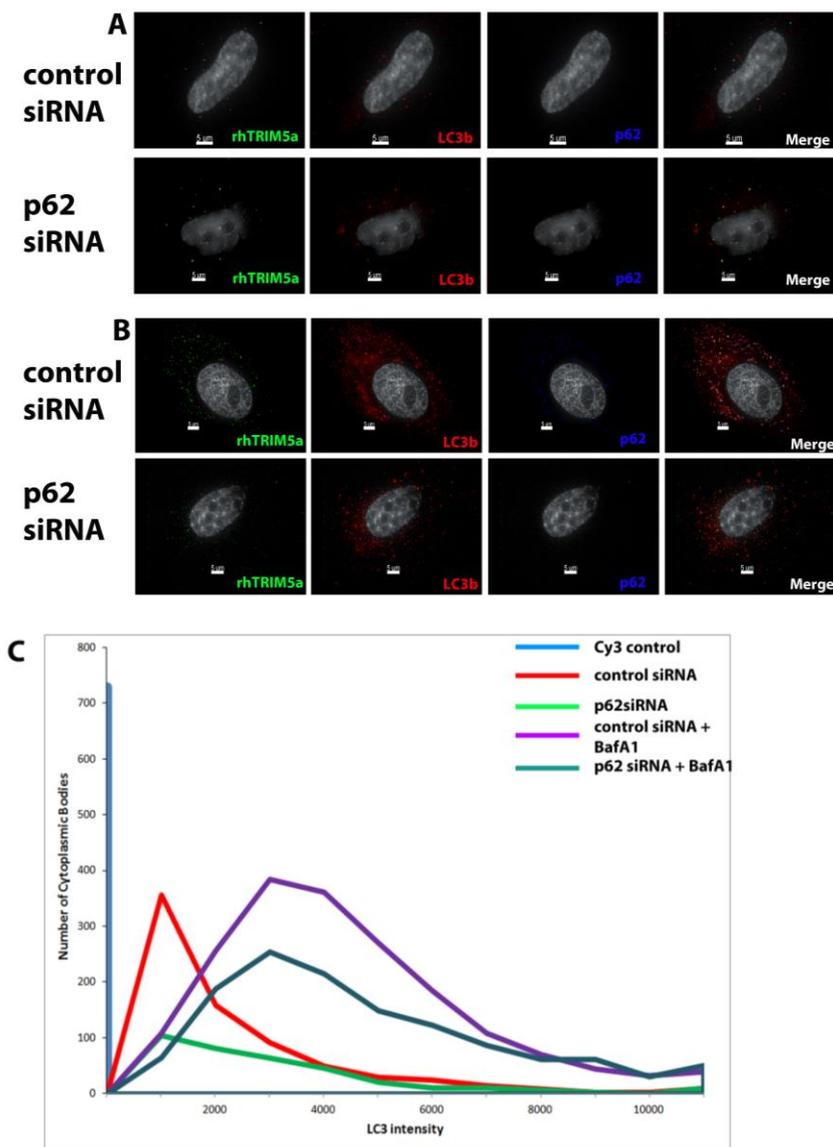


**Figure 8 rhTRIM5 $\alpha$  co-localizes with the autophagy marker LC3b.** HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were seeded onto fibronectin treated coverslips. Cells were left untreated or treated with BafA1 for 6 hours. Cells were fixed, permeabilized and immunostained with rabbit anti-LC3b and DAPI. (A and B). Representative images of cells left untreated (A) or treated with BafA1 (B). C). To quantify the number of rhTRIM5 $\alpha$  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 TRIM5 $\alpha$*

Recently, O'Connor et al. identified and characterized the TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  protein stability; when p62 was knocked down using siRNA, TRIM5 $\alpha$  protein levels decreased. Therefore, we hypothesized that p62 was important for rhTRIM $\alpha$  degradation. Specifically, we hypothesized that knockdown of p62 would decrease LC3b localization with rhTRIM5 $\alpha$ . To test this hypothesis, HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  protein expression, as expected. However, the degree to which LC3b localized to TRIM5 $\alpha$  was not decreased compared to control treated cells. Additionally, when control or p62 siRNA treated cells were treated with BafA1, the increase of YFP-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  to LC3b. Additionally, as p62 siRNA cells are still sensitive to BafA1, we hypothesize that p62 is not important for TRIM5 $\alpha$  lysosomal degradation.

*rhTRIM5 $\alpha$  localizes with the lysosomal membrane protein LAMP2*



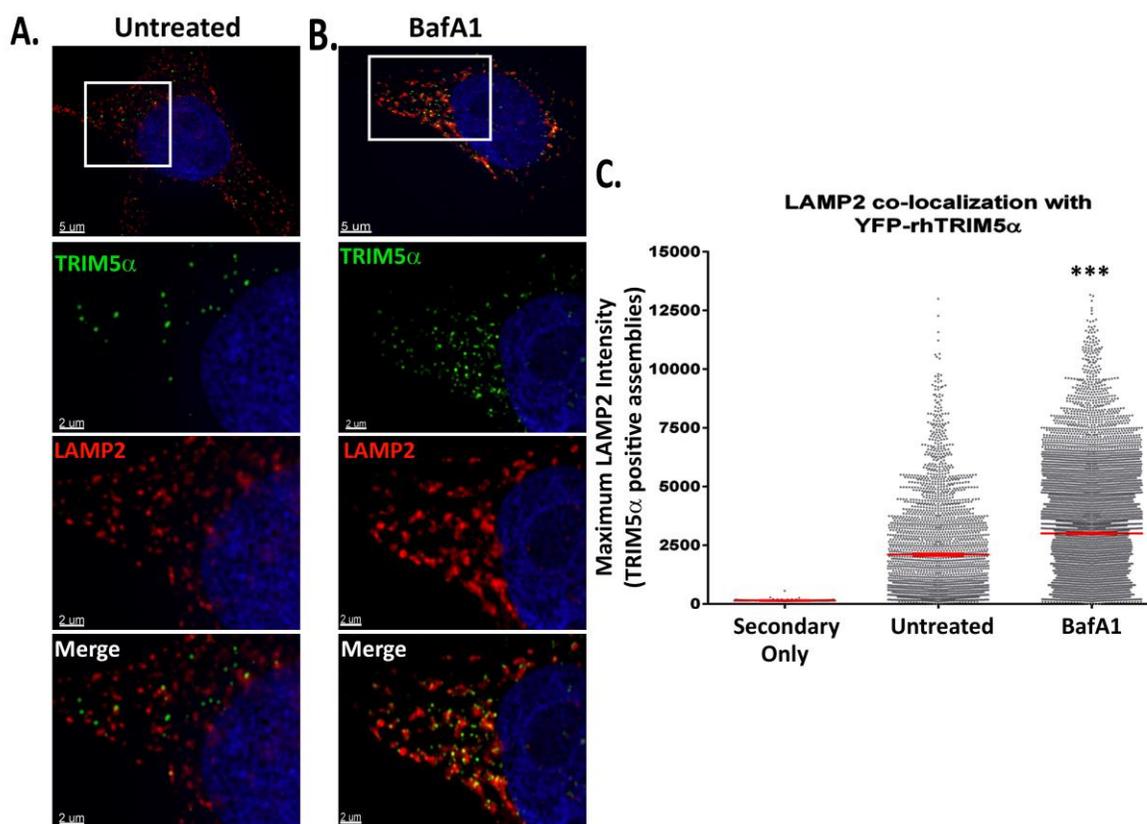
**Figure 9 rhTRIM5 $\alpha$  co-localizes with the autophagy marker LC3b independent of p62.**

HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  after treatment with BafA1 was to lysosomes or autophagosomes that didn't fuse with lysosomes, we assessed the co-localization of YFP-rhTRIM5 $\alpha$  with LAMP2. LAMP2 is a lysosomal marker protein. Therefore, if YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  co-localized with LAMP2 in the absence of drug; however, in the presence of BafA1, there was increased localization between YFP-rhTRIM5 $\alpha$  and LAMP2. Given that YFP-rhTRIM5 $\alpha$  can also localize with LC3b, we hypothesize that YFP-rhTRIM5 $\alpha$  is localized with lysosome or autolysosomes upon treatment with BafA1, and not autophagosomes that did not fuse with lysosomes.

*rhTRIM5 $\alpha$  degradation is not changed with Atg5 knockdown*

The localization of rhTRIM5 $\alpha$  with markers of both autophagosomal and lysosomal degradation led us to examine if rhTRIM5 $\alpha$  is degraded by macroautophagy. As BafA1 will not differentiate between these two pathways, we used siRNA to inhibit

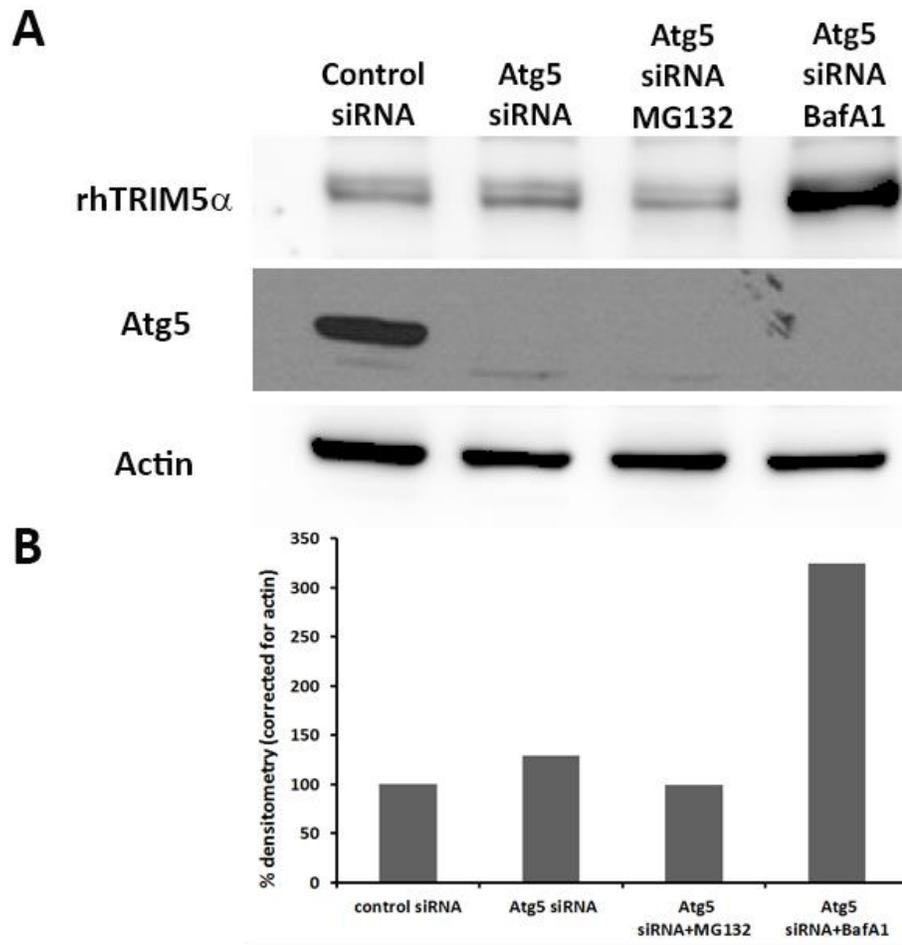


**Figure 10 rhTRIM5 $\alpha$  co-localizes with the autophagy marker LAMP2.** HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were seeded onto fibronectin treated coverslips. Cells were left untreated or treated with BafA1 for 6 hours. Cells were fixed, permeabilized and immunostained with mouse anti-LAMP2 and DAPI. (A and B). Representative images of cells left untreated (A) or treated with BafA1 (B). C). To quantify the number of rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  protein compared to control siRNA treated cells (lanes 1 and 2). To determine if YFP-rhTRIM5 $\alpha$  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, rhTRIM5 $\alpha$  was still sensitive to BafA1. rhTRIM5 $\alpha$  protein remained insensitive to MG132 when macroautophagy was inhibited. These results indicate lysosomal degradation, and not macroautophagy, is responsible for the degradation of rhTRIM5 $\alpha$ .

*Knock down of the CMA proteins LAMP2 and Hsc70 decreases rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  can interact with Hsc70, a protein important for CMA (Hwang, Holl et al. 2010). These data, along with the data that show that rhTRIM5 $\alpha$  is degraded within lysosomes and not autophagosomes, led us to hypothesize that rhTRIM5 $\alpha$  is degraded by CMA. To test this hypothesis, we

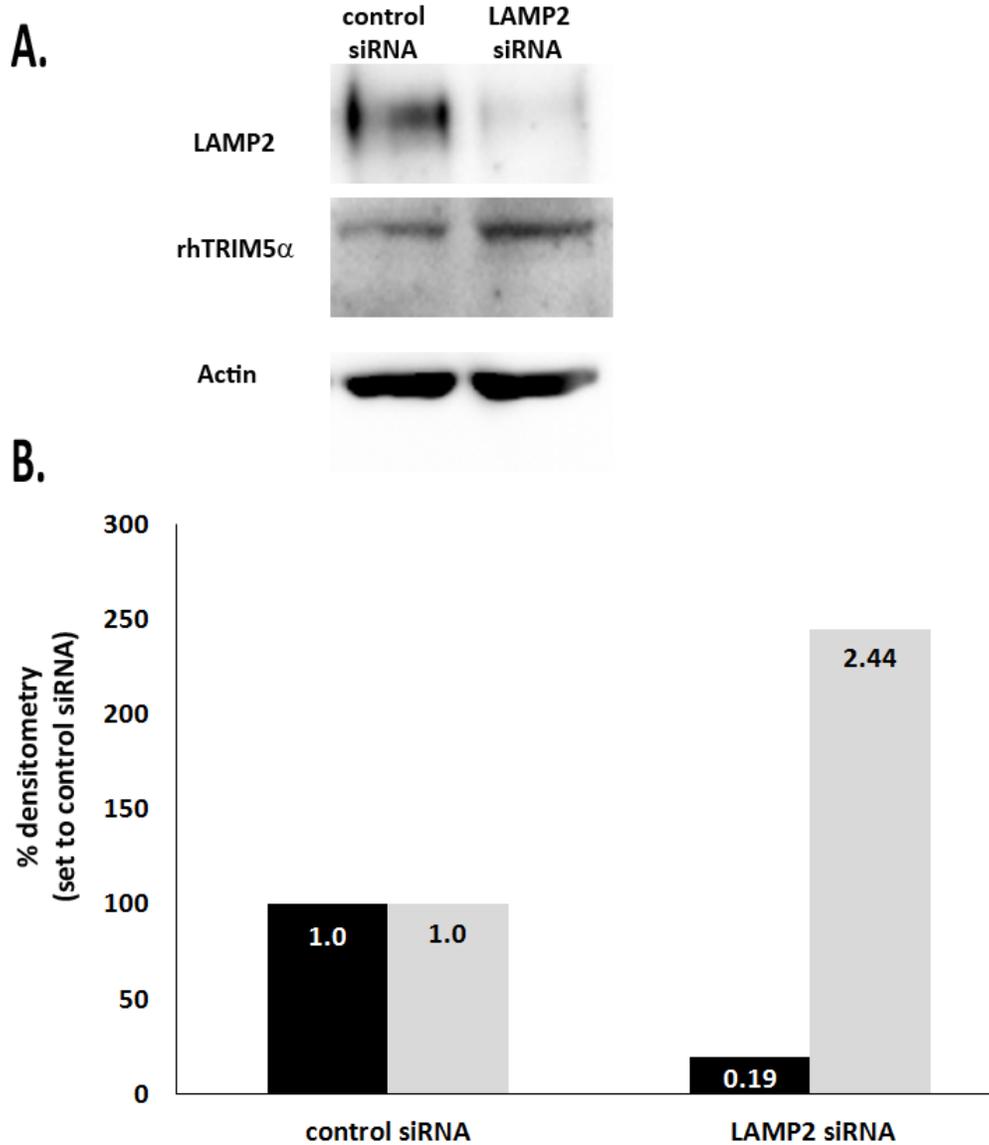


**Figure 11 rhTRIM5 $\alpha$  protein is sensitive to BafA1 in the presence of Atg5 knockdown.** HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were treated with control or Atg5 siRNA. Cells were then treated with BafA1 or MG132 for 18 hours. Protein expression was measured by Western blot. Equivalent amounts of cell lysates were probed with antibodies to Atg5, YFP and actin. B) YFP-rhTRIM5 $\alpha$  protein levels were quantified using ImageJ software. YFP-rhTRIM5 $\alpha$  protein levels were determined as a percentage of control siRNA treated cells.

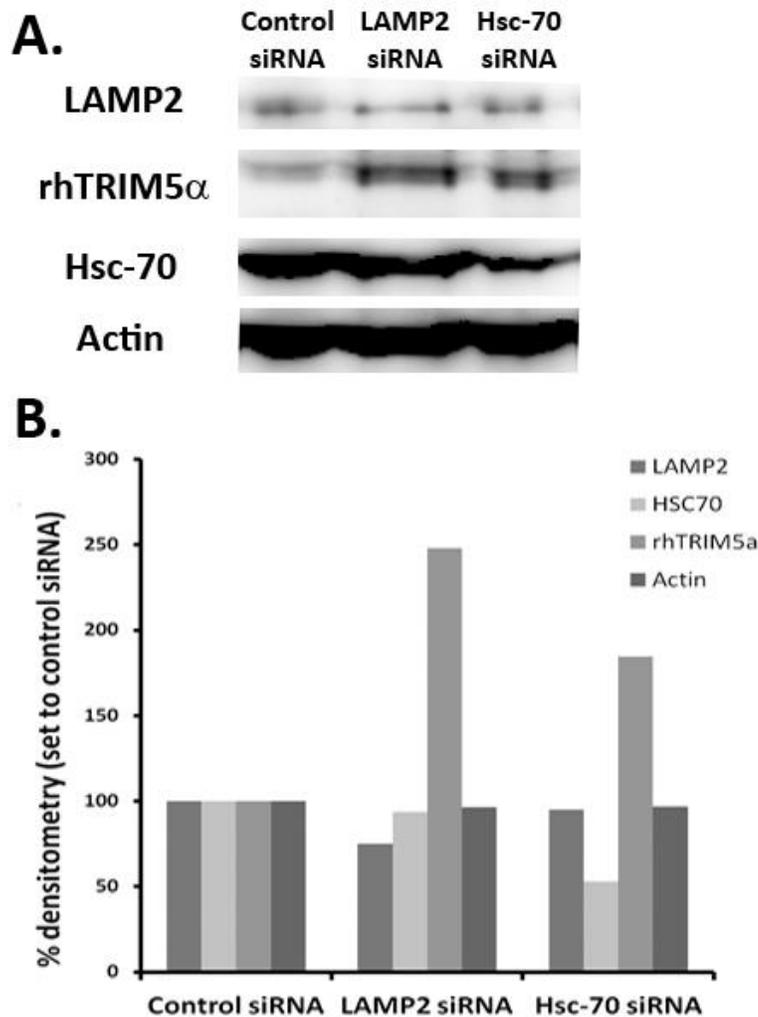
utilized LAMP2 siRNA to inhibit CMA. During CMA, LAMP2a protein multimerizes to form a channel from the cytosol to the interior of the lysosome; this allows for the substrate protein to enter the lysosome from the cytosol (Bandyopadhyay, Kaushik et al. 2008). Therefore, using LAMP2 siRNA will prevent the formation of the channel required for CMA and thus prevent rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  was degraded by CMA, then knocking down Hsc70 would prevent the localization of rhTRIM5 $\alpha$  to the lysosome and lead to an accumulation of YFP-rhTRIM5  $\alpha$ . As shown in figure 13, similar to LAMP2 knockdown, Hsc70 siRNA led to an accumulation of YFP-rhTRIM5 $\alpha$ . Therefore, we conclude that rhTRIM5 $\alpha$  is degraded by the lysosomal pathway CMA.

*rhTRIM5 $\alpha$  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



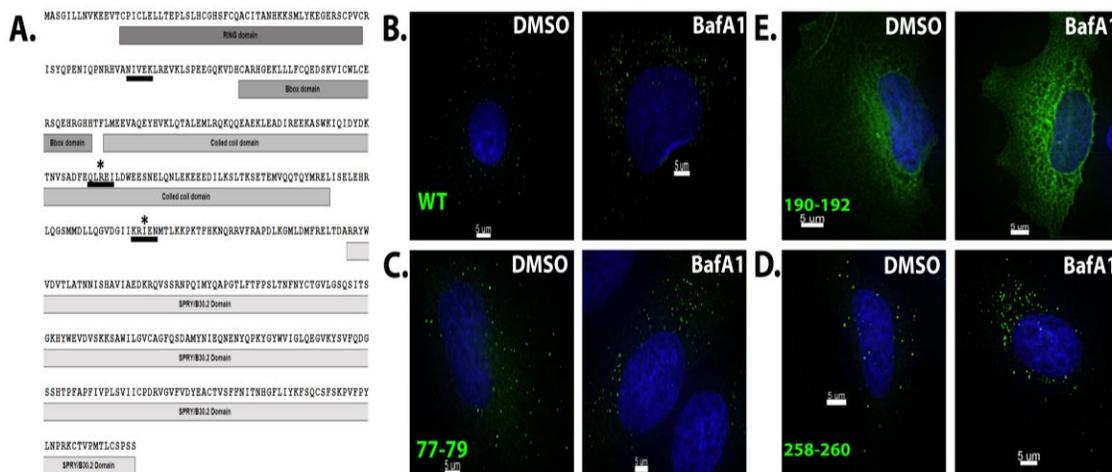
**Figure 12 Reduced LAMP2 leads to increased rhTRIM5 $\alpha$  protein.** A) HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  were treated with control or LAMP2 siRNA for 48 hours. Protein expression was measured by Western blot. Equivalent amount of cell lysates were probed with antibodies to LAMP2, YFP and actin. B) YFP-rhTRIM5 $\alpha$  protein levels were quantified using ImageJ software. YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$ . 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 rhTRIM5 $\alpha$  protein.**

A) HeLa cells stably expressing YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  protein levels were quantified using ImageJ software. YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  for possible CMA motifs. We identified three possible motifs as shown in figure 14a. To examine these motifs in TRIM5 $\alpha$ , we used SOEing PCR to introduce triple alanine residues within the middle of the motif and cloned these mutant TRIM5 $\alpha$  cDNAs into retroviral vectors to generate YFP-expressing fusion TRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  to BafA1 (Figure 14 c and e). Therefore, we conclude that these residues are not responsible for the degradation by CMA of TRIM5 $\alpha$ . 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 rhTRIM5 $\alpha$  localization when BafA1 was used to inhibit lysosomal degradation compared to the control, untreated cells (Figure 14 d). Therefore, we conclude that rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$ .** A) The coding sequence of rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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.

## **rhTRIM5 $\alpha$ protein degradation is important for retroviral restriction**

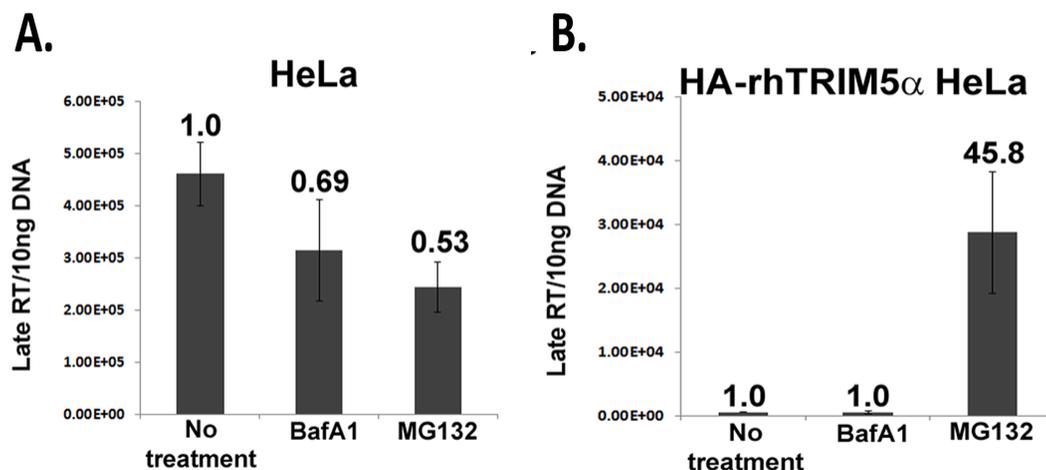
### *BafA1 does not change retroviral restriction of late viral RT products*

Given that CMA is responsible for the degradation of rhTRIM5 $\alpha$ , we sought to determine if the degradation of rhTRIM5 $\alpha$  is important for retroviral restriction. Namely, we sought to determine if the inhibition of rhTRIM5 $\alpha$  degradation could change the restriction profile of this protein. rhTRIM5 $\alpha$  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, rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$ , it is known that treatment of TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  lysosomal degradation is important for the mechanism of retroviral restriction. To measure rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$ . 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 rhTRIM5 $\alpha$*

Although BafA1 did not change the restriction profile of rhTRIM5 $\alpha$ , we hypothesized that the degradation of rhTRIM5 $\alpha$  is important for retroviral restriction. Given that we hypothesize that rhTRIM5 $\alpha$  is degraded by CMA, it is possible that BafA1 inhibits rhTRIM5 $\alpha$  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 LAMP2a is responsible for the translocation of rhTRIM5 $\alpha$  into the lysosomes, then when LAMP2 was knocked down, there should be an accumulation of rhTRIM5 $\alpha$  in the cytoplasm, thus increasing the population of rhTRIM5 $\alpha$  available to restrict retroviral infection. HeLa cells stably expressing HA-rhTRIM5 $\alpha$  were knocked down for LAMP2 following a two day siRNA protocol. Cells were then infected with A-MLV R7  $\Delta$ Env 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

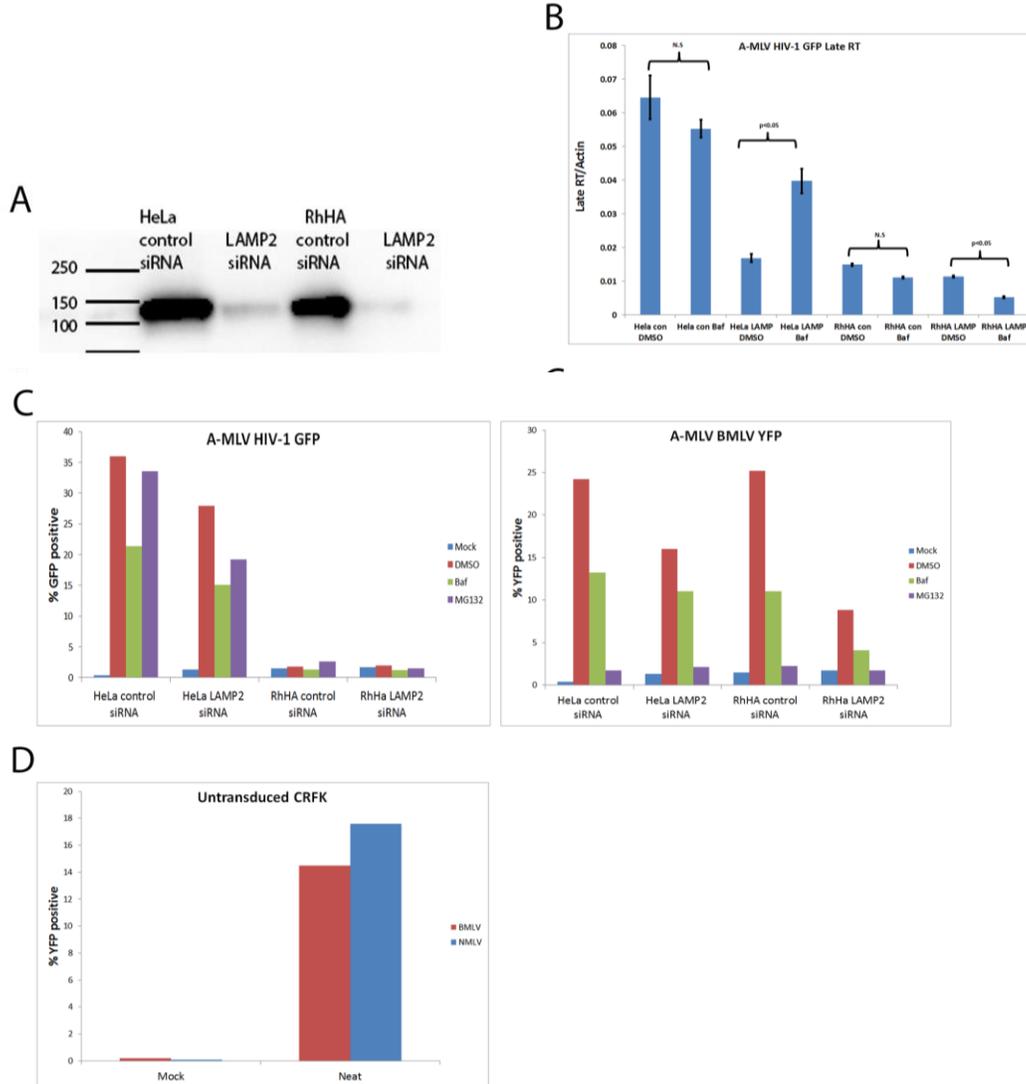


**Figure 15 rhTRIM5 $\alpha$  mediated restriction of viral late RT products is not affected by lysosomal inhibition.** Untransduced HeLa cells (A) or HeLa cells stably expressing HA-rhTRIM5 $\alpha$  (B) were plated in equivalent numbers and infected with amphotropic MLV Env pseudotyped HIV-1 reporter 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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  expressing cells, the late RT products remain restricted, indicating that inhibiting TRIM5 $\alpha$  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. Interestingly, in HA-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  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 TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  cells remained restricted for HIV-1 infection (left panel). However, we detected a rhTRIM5 $\alpha$ -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 rhTRIM5 $\alpha$  can restrict B-MLV infection when lysosomal degradation is inhibited, indicating a role for the lysosomal degradation in the restriction profile of rhTRIM5 $\alpha$ .



**Figure 16 rhTRIM5 $\alpha$  mediated restriction of B-MLV is enhanced by LAMP2 siRNA.** A) Untransduced HeLa cells or HeLa cells stably expressing HA-rhTRIM5 $\alpha$  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 amphitropic 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 rhTRIM5 $\alpha$ and huTRIM5 $\alpha$ by the autophagy-lysosomal pathway**

Many studies have found that retroviral restriction by TRIM5 $\alpha$  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 rhTRIM5 $\alpha$ -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 TRIM5 $\alpha$  containing cytoplasmic assemblies (Campbell, Perez et al. 2008). In spite of these data, proteasomal inhibition does not inhibit TRIM5 $\alpha$  degradation, unless restriction sensitive virus is present (Rold and Aiken 2008). Therefore, the degradative pathway responsible for TRIM5 $\alpha$  turnover and the role for this degradation in TRIM5 $\alpha$ -mediated restriction was unclear.

In order to understand which cellular pathway is responsible for TRIM5 $\alpha$  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-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$ . Inhibition of the UPS by MG132 caused YFP-rhTRIM5 $\alpha$  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-rhTRIM5 $\alpha$  accumulations (Figure 3). As BafA1 prevents degradation within the lysosome, it is possible that the localization of YFP-rhTRIM5 $\alpha$  to these smaller accumulations represents YFP-rhTRIM5 $\alpha$  trapped within lysosomes that cannot be degraded within this department. In support of this fact, we found that BafA1 increases the localization of YFP-rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  (Figures 4 and 5). Collectively, these data indicate that YFP-rhTRIM5 $\alpha$  is degraded by the autophagy-lysosomal pathway.

Since the discovery of rhTRIM5 $\alpha$  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

TRIM5 $\alpha$ . Therefore, we used these antibodies to determine if endogenous human TRIM5 $\alpha$  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 huTRIM5 $\alpha$  (Perron, Stremlau et al. 2004), there was a specific increase in huTRIM5 $\alpha$  protein levels (Figure 6). Interestingly, treatment with MG132 resulted in a slight diminution of huTRIM5 $\alpha$  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, TRIM5 $\alpha$  proteins only become sensitive to proteasomal inhibition when destabilized by the retroviral CA protein. It is possible that with the stress of infection, TRIM5 $\alpha$  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 TRIM5 $\alpha$  solely in the presence of restriction sensitive virus does not indicate the natural route of TRIM5 $\alpha$  protein degradation.

TRIM proteins can act as E3 ubiquitin-ligases, an activity conferred by the N-terminal RING domain (Napolitano and Meroni 2012). Additionally, TRIM5 $\alpha$  was found to ubiquitinate itself as well as other cellular proteins (Diaz-Griffero, Li et al. 2006;

Yamauchi, Wada et al. 2008; Pertel, Hausmann et al. 2011). Specifically, TRIM5 $\alpha$  led to the K63-linked polyubiquitination of TAK1, a cellular protein necessary for AP-1 and NFkB 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 TRIM5 $\alpha$  ubiquitination and lysosomal degradation, although the hypothesis that ubiquitination of TRIM5 $\alpha$  leads to lysosomal degradation deserves further study. Ultimately, the present study suggests that the ubiquitination of TRIM5 $\alpha$  does not signal its degradation by the proteasome.

### **Degradation of rhTRIM5 $\alpha$ by chaperone mediated autophagy**

Our data demonstrate that the inhibition of lysosomal degradation with BafA1 prevents the degradation of TRIM5 $\alpha$ . However, there are multiple cellular pathways that lead to lysosomal degradation. Interestingly, TRIM5 $\alpha$  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 rhTRIM5 $\alpha$ . Additionally, there was no change in the

degree of localization of LC3 to YFP-rhTRIM5 $\alpha$  assemblies (Figure 8). These data indicate that p62 is not responsible for the selective macroautophagic degradation of rhTRIM5 $\alpha$ . We also found that upon inhibition of macroautophagy with Atg5 specific siRNA, rhTRIM5 $\alpha$  protein degradation remained inhibited with BafA1 treatment (Figure 10). Collectively, these data indicate that macroautophagy is not the pathway that degrades rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  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 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 rhTRIM5 $\alpha$ . 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 TRIM5 $\alpha$ , 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 TRIM5 $\alpha$  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 rhTRIM5 $\alpha$ , 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 rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  in retroviral restriction will allow us to further understand the mechanism of restriction.

### **rhTRIM5 $\alpha$ degradation alters the retroviral restriction profile**

As proteasomal involvement in TRIM5 $\alpha$  mediated restriction has been well documented, we sought to understand if the lysosomal degradation of rhTRIM5 $\alpha$  contributed to retroviral restriction. We assessed the possible role of lysosomal degradation two ways. First, we treated HA-rhTRIM5 $\alpha$  expressing HeLa cells concurrently with BafA1 during A-MLV Env pseudotyped 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 rhTRIM5 $\alpha$  to lysosomes, thereby preventing TRIM5 $\alpha$  availability to restrict the incoming virus particle. Therefore, we inhibited TRIM5 $\alpha$  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 rhTRIM5 $\alpha$  expressing cells. We hypothesize that as LAMP2 siRNA can increase the amount of rhTRIM5 $\alpha$  protein, this slight diminution in viral RT products represent the ability of TRIM5 $\alpha$  to recognize the

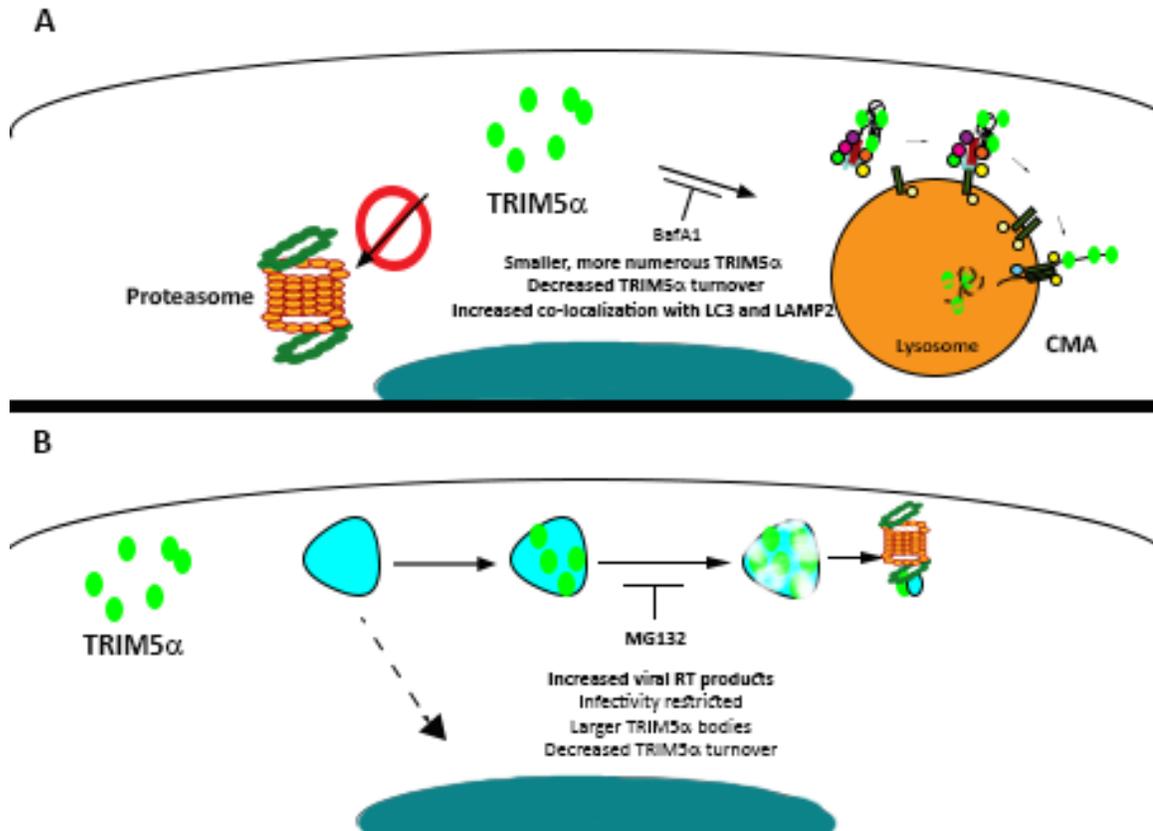
viral capsid more efficiently because the protein is stabilized. The particular cell line used in this experiment expresses a large amount of rhTRIM5 $\alpha$ . Therefore, we would expect in a cell line that expressed a more biologically relevant amount of TRIM5 $\alpha$ , this difference would increase.

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 rhTRIM5 $\alpha$  expressing cells prevented B-MLV restriction more efficiently than control siRNA treated cells. B-MLV infection is normally uninhibited by TRIM5 $\alpha$  expression. However, similar to the results of the late RT data, it is possible that increased rhTRIM5 $\alpha$  available to recognize the retroviral capsid allows for more restriction to occur. Therefore, we conclude that the degradation of rhTRIM5 $\alpha$  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 rhTRIM5 $\alpha$  is degraded via the lysosomal pathway chaperone mediated autophagy. We have demonstrated that inhibition of chaperone mediated autophagy prevents the degradation of rhTRIM5 $\alpha$ , possibly through the recognition of a pentapeptide motif in the coiled-coil region of rhTRIM5 $\alpha$ . Additionally, we found that when CMA is inhibited specifically using siRNA, there is an increase in rhTRIM5 $\alpha$  mediated restriction of B-MLV, which is

normally unrestricted by TRIM5 $\alpha$ . Therefore, the cellular degradation of rhTRIM5 $\alpha$  may participate in the mechanism of retroviral restriction.



**Figure 17 Model of CMA-mediated degradation of TRIM5 $\alpha$**  A. TRIM5 $\alpha$  protein degradation in the absence of restriction sensitive virus. The basal degradation of TRIM5 $\alpha$  is insensitive to proteasomal inhibition. Treatment of TRIM5 $\alpha$  expressing cells with lysosomal inhibitors such as BafA1 prevents CMA and leads to phenotypic changes in TRIM5 $\alpha$  protein localization and turnover. B. TRIM5 $\alpha$  protein degradation in the presence of restriction sensitive virus. During retroviral infection, TRIM5 $\alpha$  binds determinants in the retroviral capsid that lead to the destabilization of TRIM5 $\alpha$  and the capsid protein itself. Under these conditions, TRIM5 $\alpha$  protein localization and turnover is sensitive to proteasomal inhibitors, indicating a change in the degradation pathway utilized by the host cell.

## REFERENCES

- Agarraberes, F. A. and J. F. Dice (2001). "A molecular chaperone complex at the lysosomal membrane is required for protein translocation." *J Cell Sci* **114**: 2491-2499.
- Agarraberes, F. A., S. R. Terlecky, et al. (1997). "An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation." *J Cell Biol* **137**: 825-834.
- Anderson, J. L., E. M. Campbell, et al. (2006). "Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins." *J Virol* **80**: 9754-9760.
- Arhel, N. (2010). "Revisiting HIV-1 uncoating." *Retrovirology* **7**: 96.
- Backer, J. M., L. Bourret, et al. (1983). "Regulation of catabolism of microinjected ribonuclease A requires the amino-terminal 20 amino acids." *Proc Natl Acad Sci USA* **80**: 2166-2170.
- Backer, J. M. and J. F. Dice (1986). "Covalent linkage of ribonuclease S-peptide to microinjected proteins causes their intracellular degradation to be enhanced during serum withdrawal." *Proc Natl Acad Sci USA* **83**: 5830-5834.
- Bandyopadhyay, U., S. Kaushik, et al. (2008). "The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane." *Mol Cell Biol* **28**: 5747-5763.
- Bandyopadhyay, U., S. Sridhar, et al. (2010). "Identification of regulators of chaperone-mediated autophagy." *Mol Cell* **39**: 535-547.
- Bejarano, E. and A. M. Cuervo (2010). "Chaperone-mediated autophagy." *Proc Am Thorac Soc* **7**: 29-39.
- Bieniasz, P. D. (2003). "Restriction factors: a defense against retroviral infection." *Trends in Microbiology* **11**: 286-291.
- Bowman, E. J., A. Siebers, et al. (1988). "Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells." *Proceedings of the National Academy of Sciences* **85**: 7972-7976.

- Campbell, E. M., M. P. Dodding, et al. (2007). "TRIM5 alpha cytoplasmic bodies are highly dynamic structures." *Mol Biol Cell* **18**: 2102-2111.
- Campbell, E. M., O. Perez, et al. (2008). "Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha." *J Cell Biol* **180**: 549-561.
- Carlsson, S. R., J. Roth, et al. (1988). "Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylectosaminoglycan." *J Biol Chem* **263**: 18911-18919.
- Carthagen, L., A. Bergamaschi, et al. (2009). "Human TRIM gene expression in response to interferons." *PLoS ONE* **4**: e4894.
- Chatterji, U., M. D. Bobardt, et al. (2006). "Trim5alpha accelerates degradation of cytosolic capsid associated with productive HIV-1 entry." *J Biol Chem* **281**: 37025-37033.
- Chau, V., J. W. Tobias, et al. (1989). "A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein." *Science* **243**: 1576-1583.
- Chiang, H. L. and J. F. Dice (1988). "Peptide sequences that target proteins for enhanced degradation during serum withdrawal." *J Biol Chem* **263**: 6797-6805.
- Chiang, H. L., S. R. Terlecky, et al. (1989). "A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins." *Science* **246**: 382-385.
- Chu, Y. and X. Yang (2011). "SUMO E3 ligase activity of TRIM proteins." *Oncogene* **30**: 1108-1116.
- Chuang, C. K., B. Rockel, et al. (2010). "Hybrid molecular structure of the giant protease tripeptidyl peptidase II." *Nat Struct Mol Biol* **17**: 990-996.
- Cuervo, A. M. and J. F. Dice (1996). "A receptor for the selective uptake and degradation of proteins by lysosomes." *Science* **273**: 501-503.
- Cuervo, A. M. and J. F. Dice (2000). "Regulation of lamp2a levels in the lysosomal membrane." *Traffic* **1**: 570-583.
- Cuervo, A. M. and J. F. Dice (2000). "Unique properties of lamp2a compared to other lamp2 isoforms." *J Cell Sci* **113**: 4441-4450.

- Cuervo, A. M., J. F. Dice, et al. (1997). "A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins." *J Biol Chem* **272**: 5606-5615.
- Cuervo, A. M., L. Mann, et al. (2003). "Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor." *EMBO J* **22**: 47-59.
- Cuervo, A. M., A. Palmer, et al. (1995). "Degradation of proteasomes by lysosomes in rat liver." *Eur J Biochem* **227**: 792-800.
- da Fonseca, P. C. A. and E. P. Morris (2008). "Structure of the Human 26S Proteasome: Subunit Radial Displacements Open the Gate into the Proteolytic Core." *Journal of Biological Chemistry* **283**: 23305-23314.
- Dall'Armi, C., K. A. Devereaux, et al. (2013). "The role of lipids in the control of autophagy." *Curr Biol* **23**: R33-45.
- De Duve, C. and R. Wattiaux (1966). "Functions of lysosomes." *Annu Rev Physiol* **28**: 435-492.
- Deshaias, R. J. and C. A. P. Joazeiro (2009). "RING Domain E3 Ubiquitin Ligases." *Annual Review of Biochemistry* **78**: 399-434.
- Diaz-Griffero, F., A. Kar, et al. (2007). "Comparative requirements for the restriction of retrovirus infection by TRIM5alpha and TRIMCyp." *Virology* **369**: 400-410.
- Diaz-Griffero, F., X. Li, et al. (2006). "Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5." *Virology* **349**: 300-315.
- Dice, J. F., H. L. Chiang, et al. (1986). "Regulation of catabolism of microinjected ribonuclease A. Identification of residues 7-11 as the essential pentapeptide." *J Biol Chem* **261**: 6853-6859.
- Dice, J. F., S. R. Terlecky, et al. (1990). "A selective pathway for degradation of cytosolic proteins by lysosomes." *Semin Cell Biol* **1**: 449-455.
- Eskelinen, E. L. (2006). "Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy." *Mol Aspects Med* **27**: 495-502.
- Farre, J. C., R. Krick, et al. (2009). "Turnover of organelles by autophagy in yeast." *Curr Opin Cell Biol* **21**: 522-530.

- Freed, E. O. (1998). "HIV-1 gag proteins: diverse functions in the virus life cycle." *Virology* **251**: 1-15.
- Furuya, N., J. Yu, et al. (2005). "The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function." *Autophagy* **1**: 46-52.
- Fusco, C., L. Micale, et al. (2012). "The E3-Ubiquitin Ligase TRIM50 Interacts with HDAC6 and p62, and Promotes the Sequestration and Clearance of Ubiquitinated Proteins into the Aggresome." *PLoS One* **7**: e40440.
- Geetha, T., M. L. Seibenhener, et al. (2008). "p62 serves as a shuttling factor for TrkA interaction with the proteasome." *Biochem Biophys Res Commun* **374**: 33-37.
- Gillette, T. G., B. Kumar, et al. (2008). "Differential Roles of the COOH Termini of AAA Subunits of PA700 (19 S Regulator) in Asymmetric Assembly and Activation of the 26 S Proteasome." *Journal of Biological Chemistry* **283**: 31813-31822.
- Gottlieb, M. S., R. Schroff, et al. (1981). "Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency." *N Engl J Med* **305**: 1425-1431.
- Gough, N. R., C. L. Hatem, et al. (1995). "The family of LAMP-2 proteins arises by alternative splicing from a single gene: characterization of the avian LAMP-2 gene and identification of mammalian homologs of LAMP-2b and LAMP-2c." *DNA Cell Biol* **14**: 863-867.
- Groll, M., L. Ditzel, et al. (1997). "Structure of 20S proteasome from yeast at 2.4A resolution." *Nature* **386**: 463-471.
- Hailey, D. W., A. S. Rambold, et al. (2010). "Mitochondria supply membranes for autophagosome biogenesis during starvation." *Cell* **141**: 656-667.
- Han, K., L. D., et al. (2011). "Identification of a Genomic Reservoir for New TRIM Genes in Primate Genomes." *PLoS Genet* **7**: e1002388.
- Hara, T., K. Nakamura, et al. (2006). "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice." *Nature* **441**: 885-889.
- Hatakeyama, S. (2011). "TRIM proteins and cancer." *Nat Rev Cancer* **11**: 792-804.

- Hunt, P. W., P. R. Harrigan, et al. (2006). "Prevalence of CXCR4 tropism among antiretroviral-treated HIV-1-infected patients with detectable viremia." *J Infect Dis* **194**: 926-930.
- Hwang, C. Y., J. Holl, et al. (2010). "Hsp70 interacts with the retroviral restriction factor TRIM5alpha and assists the folding of TRIM5alpha." *J Biol Chem* **285**: 7827-7837.
- Jin, L., A. Williamson, et al. (2008). "Mechanism of Ubiquitin-Chain Formation by the Human Anaphase-Promoting Complex." *Cell* **133**: 653-665.
- Johnson, W. E. and S. L. Sawyer (2009). "Molecular evolution of the antiretroviral TRIM5 gene." *Immunogenetics* **61**: 163-176.
- Kaushik, S., U. Bandyopadhyay, et al. (2011). "Chaperone-mediated autophagy at a glance." *J Cell Sci* **124**: 495-499.
- Kaushik, S. and A. M. Cuervo (2012). "Chaperone-mediated autophagy: a unique way to enter the lysosome world." *Trends Cell Biol* **22**: 407-417.
- Kaushik, S., A. C. Massey, et al. (2006). "Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy." *EMBO J* **25**: 3921-3933.
- Kaushik, S., A. C. Massey, et al. (2008). "Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy." *Mol Biol Cell* **19**: 2179-2192.
- Keckesova, Z., L. M. Ylinen, et al. (2004). "The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities." *Proc Natl Acad Sci USA* **101**: 10780-10785.
- Kerscher, O., R. Felberbaum, et al. (2006). "Modification of Proteins by Ubiquitin and Ubiquitin-Like Proteins." *Annual Review of Cell and Developmental Biology* **22**: 159-180.
- Kim, J. Y. and K. Ozato (2009). "The sequestosome 1/p62 attenuates cytokine gene expression in activated macrophages by inhibiting IFN regulatory factor 8 and TNF receptor-associated factor 6/NF-kappaB activity." *J Immunol* **182**: 2131-2140.
- Kirkpatrick, D. S., N. A. Hathaway, et al. (2006). "Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology." *Nat Cell Biol* **8**: 700-710.

- Klionsky, D. J., P. Codogno, et al. (2010). "A comprehensive glossary of autophagy-related molecules and processes." *Autophagy* **6**: 438-448.
- Klionsky, D. J., Z. Elazar, et al. (2008). "Does bafilomycin A1 block the fusion of autophagosomes with lysosomes?" *Autophagy* **4**: 849-850.
- Komatsu, M., S. Waguri, et al. (2006). "Loss of autophagy in the central nervous system causes neurodegeneration in mice." *Nature* **441**: 880-884.
- Komatsu, M., S. Waguri, et al. (2005). "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice." *J Cell Biol* **169**: 425-434.
- Kroemer, G. and M. Jaattela (2005). "Lysosomes and autophagy in cell death control." *Nat Rev Cancer* **5**: 886-897.
- Kunz, J. B., H. Schwarz, et al. (2004). "Determination of four sequential stages during microautophagy in vitro." *J Biol Chem* **279**: 9987-9996.
- Lackner, A. A., M. M. Lederman, et al. (2012). "HIV pathogenesis: the host." *Cold Spring Harb Perspect Med* **2**: a007005.
- Lander, G. C., E. Estrin, et al. (2012). "Complete subunit architecture of the proteasome regulatory particle." *Nature* **482**: 186-191.
- Lee, D. H. and A. L. Goldberg (1998). "Proteasome inhibitors: valuable new tools for cell biologists." *Trends Cell Biol* **8**: 397-403.
- Liu, H., P. Wang, et al. (2009). "Degradation of regulator of calcineurin 1 (RCAN1) is mediated by both chaperone-mediated autophagy and ubiquitin proteasome pathways." *FASEB J* **23**: 3383-3392.
- Lu, K., X. Heng, et al. (2011). "Structural determinants and mechanism of HIV-1 genome packaging." *J Mol Biol* **410**: 609-633.
- Lukic, Z., S. Hausmann, et al. (2011). "TRIM5alpha associates with proteasomal subunits in cells while in complex with HIV-1 virions." *Retrovirology* **8**: 93.
- Lv, L., D. Li, et al. (2011). "Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth." *Mol Cell* **42**: 719-730.
- Maillard, P. V., G. Ecco, et al. (2010). "The specificity of TRIM5 alpha-mediated restriction is influenced by its coiled-coil domain." *J Virol* **84**: 5790-5801.

- Majeski, A. E. and J. F. Dice (2004). "Mechanisms of chaperone-mediated autophagy." *Int J Biochem Cell Biol* **36**: 2435-2444.
- Malim, M. H. and P. D. Bieniasz (2012). "HIV Restriction Factors and Mechanisms of Evasion." *Cold Spring Harb Perspect Med* **2**: a006940.
- Mane, S. M., L. Marzella, et al. (1989). "Purification and characterization of human lysosomal membrane glycoproteins." *Arch Biochem Biophys* **268**: 360-378.
- Marzella, L., J. Ahlberg, et al. (1980). "In vitro uptake of particles by lysosomes." *Exp Cell Res* **129**: 460-466.
- Massey, A. C., S. Kaushik, et al. (2006). "Consequences of the selective blockage of chaperone-mediated autophagy." *Proc Natl Acad Sci USA* **103**: 5805-5810.
- Masur, H., M. A. Michelis, et al. (1981). "An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestation of cellular immune dysfunction." *N Engl J Med* **305**: 1431-1438.
- Meroni, G. and G. Diez-Roux (2005). "TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases." *BioEssays* **27**: 1147-1157.
- Micale, L., C. Fusco, et al. (2008). "Williams-Beuren syndrome TRIM50 encodes an E3 ubiquitin ligase." *Eur J Hum Genet* **16**: 1038-1049.
- Mijaljica, D., M. Prescott, et al. (2011). "Microautophagy in mammalian cells: revisiting a 40-year-old conundrum." *Autophagy* **7**: 673-682.
- Mizushima, N. and T. Yoshimori (2007). "How to interpret LC3 immunoblotting." *Autophagy* **3**: 542-545.
- Mortimore, G. E., N. J. Hutson, et al. (1983). "Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeeding." *Proc Natl Acad Sci USA* **80**: 2179-2183.
- Murata, S., H. Yashiroda, et al. (2009). "Molecular mechanisms of proteasome assembly." *Nat Rev Mol Cell Biol* **10**: 104-115.
- Myeku, N. and M. E. Figueiredo-Pereira (2011). "Dynamics of the degradation of ubiquitinated proteins by proteasomes and autophagy: association with sequestosome 1/p62." *J Biol Chem* **286**: 22426-22440.

- Nagy, V. and I. Dikic (2010). "Ubiquitin ligase complexes: from substrate selectivity to conjugational specificity." *Biol Chem* **391**: 163-169.
- Nakai, A., O. Yamaguchi, et al. (2007). "The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress." *Nat Med* **13**: 619-624.
- Nakayama, E. E. and T. Shioda (2012). "TRIM5a and species tropism of HIV/SIV." *Frontiers in Microbiology* **3**: 13.
- Napolitano, L. M. and G. Meroni (2012). "TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation." *IUBMB Life* **64**: 64-71.
- Nedelsky, N. B., P. K. Todd, et al. (2008). "Autophagy and the ubiquitin-proteasome system: Collaborators in neuroprotection." *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1782**: 691-699.
- Nijman, S. M. B., M. P. A. Luna-Vargas, et al. (2005). "A Genomic and Functional Inventory of Deubiquitinating Enzymes." *Cell* **123**: 773-786.
- Nishi, M., F. Aoyama, et al. (2012). "TRIM50 Protein Regulates Vesicular Trafficking for Acid Secretion in Gastric Parietal Cells." *Journal of Biological Chemistry* **287**: 33523-33532.
- O'Connor, C., T. Pertel, et al. (2010). "p62/sequestosome-1 associates with and sustains the expression of retroviral restriction factor TRIM5alpha." *J Virol* **84**: 5997-6006.
- Ohkura, S., M. W. Yap, et al. (2006). "All three variable regions of the TRIM5alpha B30.2 domain can contribute to the specificity of retrovirus restriction." *J Virol* **80**: 8554-8565.
- Pankiv, S., T. H. Clausen, et al. (2007). "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy." *J Biol Chem* **282**: 24131-24145.
- Park, C. and A. M. Cuervo (2013). "Selective Autophagy: Talking with the UPS." *Cell Biochem Biophys* **67**: 3-13.
- Perez-Caballero, D., T. Hatzioannou, et al. (2005). "Restriction of Human Immunodeficiency Virus Type 1 by TRIM-CypA Occurs with Rapid Kinetics and Independently of Cytoplasmic Bodies, Ubiquitin, and Proteasome Activity." *J Virol* **79**: 15567-15572.

- Perron, M. J., M. Stremlau, et al. (2004). "TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells." *Proc Natl Acad Sci USA* **101**: 11827-11832.
- Pertel, T., S. Hausmann, et al. (2011). "TRIM5 is an innate immune sensor for the retrovirus capsid lattice." *Nature* **472**: 361-365.
- Piot, P. and T. C. Quinn (2013). "Response to the AIDS pandemic--a global health model." *N Engl J Med* **368**: 2210-2218.
- Pober, B. R. (2010). "Williams–Beuren Syndrome." *New England Journal of Medicine* **362**: 239-252.
- Price, A. J., F. Marzetta, et al. (2009). "Active site remodeling switches HIV specificity of antiretroviral TRIMCyp." *Nat Struct Mol Biol* **16**: 1036-1042.
- Ravikumar, B., K. Moreau, et al. (2010). "Plasma membrane helps autophagosomes grow." *Autophagy* **6**: 1184-1186.
- Razi, M., E. Y. Chan, et al. (2009). "Early endosomes and endosomal coatome are required for autophagy." *J Cell Biol* **185**: 305-321.
- Reddy, B. A., L. D. Etkin, et al. (1992). "A novel zinc finger coiled-coil domain in a family of nuclear proteins." *Trends Biochem Sci* **17**: 344-345.
- Reggiori, F., M. Komatsu, et al. (2012). "Selective types of autophagy." *Int J Cell Biol* **2012**: 156272.
- Reymond, A., G. Meroni, et al. (2001). "The tripartite motif family identifies cell compartments." *EMBO J* **20**: 2140-2151.
- Rold, C. J. and C. Aiken (2008). "Proteasomal degradation of TRIM5alpha during retrovirus restriction." *PLoS Pathog* **4**: e1000074.
- Saeki, Y. and K. Tanaka (2012). "Assembly and Function of the Proteasome." *Methods Mol Biol* **832**: 315-337.
- Sahu, R., S. Kaushik, et al. (2011). "Microautophagy of cytosolic proteins by late endosomes." *Dev Cell* **20**: 131-139.
- Sakai, M., N. Araki, et al. (1989). "Lysosomal movements during heterophagy and autophagy: with special reference to nematolysosome and wrapping lysosome." *J Electron Microscop Tech* **12**: 101-131.

- Sakai, M. and K. Ogawa (1982). "Energy-dependent lysosomal wrapping mechanism (LWM) during autophagolysosome formation." *Histochemistry* **76**: 479-488.
- Sastri, J. and E. M. Campbell (2011). "Recent insights into the mechanism and consequences of TRIM5alpha retroviral restriction." *AIDS Res Hum Retroviruses* **27**: 231-238.
- Sastri, J., C. O'Connor, et al. (2010). "Identification of residues within the L2 region of rhesus TRIM5alpha that are required for retroviral restriction and cytoplasmic body localization." *Virology* **405**: 259-266.
- Sawyer, S. L., L. I. Wu, et al. (2005). "Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain." *Proc Natl Acad Sci USA* **102**: 2832-2837.
- Schacker, T., A. C. Collier, et al. (1996). "Clinical and epidemiologic features of primary HIV infection." *Ann Intern Med* **125**: 257-264.
- Schreiber, A. and M. Peter (2013). "Substrate recognition in selective autophagy and the ubiquitin-proteasome system." *Biochimica et Biophysica Acta* **2013**: Mar 29 [Epub ahead of print].
- Shi, M., W. Deng, et al. (2008). "TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation." *Nat Immunol* **9**: 369-377.
- Smith, D. M., S.-C. Chang, et al. (2007). "Docking of the Proteasomal ATPases' Carboxyl Termini in the 20S Proteasome's  $\alpha$  Ring Opens the Gate for Substrate Entry." *Molecular Cell* **27**: 731-744.
- Song, B., B. Gold, et al. (2005). "The B30.2(SPRY) domain of the retroviral restriction factor TRIM5alpha exhibits lineage-specific length and sequence variation in primates." *J Virol* **79**: 6111-6121.
- Stoye, J. P. (2012). "Studies of endogenous retroviruses reveal a continuing evolutionary saga." *Nat Rev Microbiol* **10**: 395-406.
- Stremlau, M., C. M. Owens, et al. (2004). "The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys." *Nature* **427**: 848-853.

- Stremlau, M., M. Perron, et al. (2006). "Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor." *Proc Natl Acad Sci USA* **103**: 5514-5519.
- Stremlau, M., M. Perron, et al. (2005). "Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction." *J Virol* **79**: 3139-3145.
- Suzuki, K. and Y. Ohsumi (2010). "Current knowledge of the pre-autophagosomal structure (PAS)." *FEBS Lett* **584**: 1280-1286.
- Swanstrom, R. and J. Coffin (2012). "HIV-1 pathogenesis: the virus." *Cold Spring Harb Perspect Med* **2**: a007443.
- Tareen, S. U. and M. Emerman (2011). "Human Trim5alpha has additional activities that are uncoupled from retroviral capsid recognition." *Virology* **409**: 113-120.
- Taylor, R. T., Kirk J. Lubick, et al. (2011). "TRIM79 $\alpha$ , an Interferon-Stimulated Gene Product, Restricts Tick-Borne Encephalitis Virus Replication by Degrading the Viral RNA Polymerase." *Cell Host Microbe* **10**: 185-196.
- Terlecky, S. R., H. L. Chiang, et al. (1992). "Protein and peptide binding and stimulation of in vitro lysosomal proteolysis by the 73-kDa heat shock cognate protein." *J Biol Chem* **267**: 9202-9209.
- Thompson, L. M., C. T. Aiken, et al. (2009). "IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome." *J Cell Biol* **187**: 1083-1099.
- Thrower, J. S., L. Hoffman, et al. (2000). "Recognition of the polyubiquitin proteolytic signal." *EMBO J* **19**: 94-102.
- Tian, G., S. Park, et al. (2011). "An asymmetric interface between the regulatory and core particles of the proteasome." *Nat Struct Mol Biol* **18**: 1259-1267.
- Uchil, P. D., A. Hinz, et al. (2013). "TRIM Protein-Mediated Regulation of Inflammatory and Innate Immune Signaling and Its Association with Antiretroviral Activity." *J Virol* **87**: 257-272.
- Uchil, P. D., B. D. Quinlan, et al. (2008). "TRIM E3 ligases interfere with early and late stages of the retroviral life cycle." *PLoS Pathog* **4**: e16.
- Vergne, I. and V. Deretic (2010). "The role of PI3P phosphatases in the regulation of autophagy." *FEBS Lett* **584**: 1313-1318.

- Versteeg, Gijs A., R. Rajsbaum, et al. (2013). "The E3-Ligase TRIM Family of Proteins Regulates Signaling Pathways Triggered by Innate Immune Pattern-Recognition Receptors." *Immunity* **38**: 384-398.
- Virgen, C. A., Z. Kratovac, et al. (2008). "Independent genesis of chimeric TRIM5-cyclophilin proteins in two primate species." *Proc Natl Acad Sci USA* **105**: 3563-3568.
- Weidberg, H., E. Shvets, et al. (2011). "Biogenesis and cargo selectivity of autophagosomes." *Annu Rev Biochem* **80**: 125-156.
- Weissman, A. M. (2001). "Themes and variations on ubiquitylation." *Nat Rev Mol Cell Biol* **2**: 169-178.
- Wilens, C. B., J. C. Tilton, et al. (2012). "HIV: cell binding and entry." *Cold Spring Harb Perspect Med* **2**: a006866.
- Wu, X., J. L. Anderson, et al. (2006). "Proteasome inhibitors uncouple rhesus TRIM5 $\alpha$  restriction of HIV-1 reverse transcription and infection." *Proc Natl Acad Sci USA* **103**: 7465-7470.
- Yamauchi, K., K. Wada, et al. (2008). "Ubiquitination of E3 ubiquitin ligase TRIM5 $\alpha$  and its potential role." *FEBS J* **275**: 1540-1555.
- Yan, Y., R. J. Flinn, et al. (2009). "hVps15, but not Ca<sup>2+</sup>/CaM, is required for the activity and regulation of hVps34 in mammalian cells." *Biochem J* **417**: 747-755.
- Yap, M. W., S. Nisole, et al. (2004). "Trim5 $\alpha$  protein restricts both HIV-1 and murine leukemia virus." *Proc Natl Acad Sci USA* **101**: 10786-10791.
- Ye, Y. and M. Rape (2009). "Building ubiquitin chains: E2 enzymes at work." *Nat Rev Mol Cell Biol* **10**: 755-764.
- Yeh, E. T. H., L. Gong, et al. (2000). "Ubiquitin-like proteins: new wines in new bottles." *Gene* **248**: 1-14.
- Yorimitsu, T. and D. J. Klionsky (2005). "Autophagy: molecular machinery for self-eating." *Cell Death Differ* **12**: 1542-1552.
- Zhang, L., M. Xu, et al. (2013). "Both K63 and K48 ubiquitin linkages signal lysosomal degradation of the LDL receptor." *J Lipid Res* **54**: 1410-1420.

Zou, W. and D. E. Zhang (2006). "The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase." *J Biol Chem* **281**: 3989-3994.

## VITA

Rachel Sarah Nelson was born November 27<sup>th</sup>, 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 Carribean 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 TRIM5 $\alpha$ , 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.