FUNCTIONAL CHARACTERIZATION OF THE P97 ADAPTOR PROTEIN UBXD1

A Dissertation submitted to
The Temple University Graduate Board

in Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

by
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January, 2012

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ABSTRACT

Title: Functional characterization of the p97 adaptor protein UBXD1

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Temple University 2011

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p97 is a member of the AAA family of proteins (ATPase Associated with various cellular Activities). It is a highly conserved and abundant protein and functions in numerous ubiquitin-mediated processes including ERAD. Endoplasmic Reticulum Associated Degradation is the process by which misfolded/ubiquitinated proteins translocate out of the ER and migrate to the proteasome for degradation. p97 maintains substrate misfolding and mediates its exit from the ER and trafficking to the 26S proteasome. It also plays important roles in protein trafficking, the cell-cycle, apoptosis and homeotypic Golgi Apparatus and Endoplasmic Reticulum membrane fusion after mitosis. In addition, p97 plays a role in the aggresome-autophagy degradation pathway, which handles the ubiquitin-mediated destruction of aggregate-prone, misfolded, cytosolic proteins. p97 mutation is the causative alteration in the disorder, IBMPFD, which is marked by defects in autophagy. This broad diversity of function is mediated through p97’s interaction with a large group of adaptor proteins. Many of these adaptors harbor both p97 interaction motifs and ubiquitin association domains. However, more than half of known p97 adaptors do not. Their function is largely unknown. UBXD1 is one known
adaptor for p97 that does not have a ubiquitin association domain (UBA), and has been shown to have decreased interaction with IBMPFD mutant p97\textsuperscript{R155H} and p97\textsuperscript{A232E}. Recently, it has been suggested to perform a role in protein trafficking, specifically in monoubiquitinated caveolin-1 internalization and trafficking to the endosome. A novel high abundance UBXD1 interacting partner has been identified via solution-based mass spectrometric analyses. ERGIC-53, the namesake of the ER-Golgi Intermediate Compartment, has been shown to be involved in bi-directional trafficking between the ER and Golgi. The association between UBXD1 and ERGIC-53 is unique among UBX family members. Deletional analysis has shown that unlike p97, the ERGIC-53-UBXD1 interaction takes place in the extreme amino terminus of UBXD1, (within the first 10 amino acids) which is predicted by computer modeling to form a hydrophobic binding pocket. Further site-directed mutagenesis work has clearly shown four amino acids (3 highly hydrophobic) are crucial for maintaining this interaction. They have been modelled to form a conserved alpha-helix. βCOP, a primary member of the COPI coatamer complex which is involved in protectively coating ERGIC-53 positive vesicles, is also thought to be involved with the ERGIC-53-UBXD1-p97 pathway. βCOP has been identified as a UBXD1-independent interactor with p97. Modest UBXD1 over-expression using a ponasterone inducible system has shown that UBXD1 modulates ERGIC-53 localization. Additionally, a functional link between UBXD1, p97 and ERGIC-53 in autophagy has been discovered through the use of a highly efficient, miR30-based, inducible knockdown system. Upon individual knockdown of UBXD1, p97 and ERGIC-53, autophagic markers p62 and LC3-II accumulate at relatively high levels in normal culture conditions, strongly suggesting a role in mediating basal
autophagy. However, when placed under starvation conditions, autophagy progresses and p62 is degraded. It is speculated from these studies that a p97/UBXD1 complex plays a role in regulating the trafficking of ERGIC-53 positive vesicles and this activity plays an important role in autophagy.
Successful science is an endeavor that cannot be done alone. It only takes one hand to grasp a pipette, but countless other hands to use it successfully. It is a great pleasure to thank those hands. First, I would like to thank my advisor, Dr. Dale Haines for his dedication to his academic promise. Through the trials and tribulations we have endured together, he has provided me the guidance and tutelage necessary to show me what it takes to succeed. My committee members, Dr. Scott Shore, Dr. Xavier Graña and Dr. Nora Engel, have provided the constant feedback, ideas and scientific/emotional guidance necessary for me to produce research that I am proud of. In addition to my formal mentors, my peers and co-workers have provided a crucial pool of knowledge and support that has made this work possible. Past members of the lab, Dr. Natalia Scherbik, Dr. Sabyasachi Bhattacharya, Dr. Christopher Carbone, Meghan Jordan and Mr. Dane Kyle MS are dear friends that have truly contributed to making this endeavor a success. Dane, especially, has been a key force in the progression of this work towards its publication. Friends and coworkers too numerous to name have also provided a sanctuary to comisserate and motivate. Finally, I need to thank my family and friends who have consistently and eternally supplied their advice and support helping me through the worst of times and celebrating with me through the best of times. My parents and brother have always been my strongest allies. Nothing in life can compare to the love and support garnered from your family. To say my wife, Kathleen, and my son, Henry have supported me, would be greatly understating their impact. Above this work; without them I wouldn’t be possible.
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<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>AAA</td>
<td>ATPase associated with various activities</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-diphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
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<tr>
<td>BiP</td>
<td>Binding Immunoglobulin Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>Celsius</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DUB</td>
<td>De-ubiquitinating enzyme</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation</td>
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<tr>
<td>ERGIC-53</td>
<td>ER-Golgi Intermediate Compartment 53 kDa</td>
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<tr>
<td>FTLD</td>
<td>Frontotemporal lobar degeneration</td>
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<tr>
<td>FTLD-U</td>
<td>Frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>HA</td>
<td>Hemmagglutinin</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Buffered Salt Solution</td>
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<tr>
<td>His</td>
<td>Histidine</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IBMPFD</td>
<td>Inclusion body myopathy/Paget disease of the bone/frontotemporal dementia</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>IP/MS</td>
<td>Immunoprecipitation/Mass Spectrometry</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LRS</td>
<td>LC3 recognition sequence</td>
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<tr>
<td>LSD</td>
<td>Lysosome Storage Disease</td>
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<tr>
<td>MAP LC3</td>
<td>Microtubule-associated protein 1 light chain 3</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>miR</td>
<td>Micro-RNA</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>MVB</td>
<td>Multivesicular Inclusion Bodies</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>NTP</td>
<td>Nucleotide Tri-phosphate</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDI</td>
<td>Protein di-sulfide isomerase</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PUB</td>
<td>Peptide:N-glycanase/UBA or UBX-containing proteins</td>
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<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
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<td>Ser/Thr</td>
<td>Serine/Threonine</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td>SNARE</td>
<td>Soluble NSF Attachment Protein Receptor</td>
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<td>SV40</td>
<td>Simian Virus 40</td>
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<td>Ub</td>
<td>Ubiquitin</td>
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<td>UBA</td>
<td>Ubiquitin Association</td>
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<tr>
<td>UBD</td>
<td>Ubiquitin Binding Domain</td>
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<tr>
<td>UBX</td>
<td>Ubiquitin Regulatory X</td>
</tr>
<tr>
<td>UDP</td>
<td>Uracil-diphosphate</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>UPS</td>
<td>Ubiquitin-Proteasome System</td>
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<tr>
<td>VIM</td>
<td>VCP interaction motif</td>
</tr>
<tr>
<td>VTC</td>
<td>Vesicular tubular cluster</td>
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<tr>
<td>ZN$^{2+}$</td>
<td>Zinc ion</td>
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CHAPTER 1

INTRODUCTION

1.1 Ubiquitin Signalling: General Overview

Ubiquitination, or ubiquitylation, is a reversible, covalent, post-translational protein modification in which a small 76 amino acid, 8 kDa ubiquitin (Ub) moiety is covalently conjugated onto a lysine residue of a target protein. This is accomplished through the joint action of three families of enzymes. The E1 ubiquitin-activating enzymes bind individual ubiquitin molecules in an ATP-dependent manner via a high-energy thioester linkage between the ubiquitin carboxy terminus and a cysteine side chain on the E1 enzyme, readying it for transfer onto the necessary E2 ubiquitin-conjugating enzyme. E2 enzymes then work in concert with E3 ubiquitin-ligase enzymes that are responsible for recognizing specific target protein’s degradation signals (degrons). E2-E3 complexes bind to target substrates and the ubiquitin protein is transferred onto the appropriate lysine residue of the target protein. There are approximately 8 E1 enzymes, 30 predicted E2 enzymes and hundreds of identified E3 enzymes. This allows for multiple combinations of the E1, E2 and E3 enzymes, however the E3 enzyme is responsible for substrate specificity (Alberts et al. 2002) (Schulman, Harper 2009). The addition of one ubiquitin moiety is called -ubiquitination. Some proteins undergo mono-ubiquitination at
multiple lysines. This is termed multi-ubiquitination. Mono-ubiquitination has been shown to serve as a signal mediating receptor endocytosis, protein trafficking, DNA repair and histone regulation. Following the target protein mono-ubiquitination, additional ubiquitin moieties are often added in a step-wise manner, forming long chains of variable length (termed polyubiquitination) (Hoeller, Dikic 2010) (Levkowitz et al. 1998) (Li et al. 2003) (Stewart et al. 2009) (Ramaekers, Wouters 2011). Directed by E2 enzymes, each additional ubiquitin is conjugated via its carboxy-terminus onto a specific lysine in the ubiquitin molecule that preceeds it. Ubiquitin contains 7 lysines (K6, K11, K27, K29, K33, K48 and K63), making it capable of forming long chains of a variety of linkages (Peng et al. 2003). This process is reversible; deubiquitinating enzymes (DUBs) can trim back polyubiquitin chains or cleave and remove them outright (Shi, Grossman 2010) (Reyes-Turcu, Ventii & Wilkinson 2009). Polyubiquitination acts as a protein tag marking a target substrate for recognition by ubiquitin-binding proteins and the inherent variety of their ubiquitin chains (length and composition) modulates the specificity of this interaction. Adaptors often serve as a link between the substrate and a functional mediator in addition to delivering ubiquitinated proteins to their appropriate localization. Although it has been shown that multiple types of lysine linkages are indeed possible, two specific chains (on lysine 48 and lysine 63) have been identified as the main linkages in the primary ubiquitination pathways leading to protein degradation. Lysine 48 linkages are involved in the Ubiquitin-Proteasome System (UPS), while lysine 63 linkages are connected to another method of protein clearance, autophagy/lysosomal degradation.
1.1.1 Ubiquitin Related Function: UPS

The Ubiquitin Proteasome System is the primary method for cellular maintenance of protein homeostasis. It plays a key role in regulating many crucial processes including: the cell cycle, DNA repair, apoptosis and transcription by mediating the elimination of relatively short-lived regulatory proteins when they are no longer needed. In addition to modulating cellular activities, it also controls the degradation of unfolded or misfolded proteins. This system involves the ubiquitination of target substrates and their subsequent trafficking to the 26S proteasome for degradation into peptide fragments followed by further degradation into their component amino acids by cytoplasmic peptidases. This process occurs in the cytoplasm or nucleoplasm of the cell. As stated above, ubiquitin moieties are conjugated onto target substrates by E3 ubiquitin ligases that create ubiquitin chains of variable length and composition. Multiple ubiquitin linkages will target proteins to the proteasome, the most common being lysine 48 (Chau et al. 1989) (Finley 2009). Mass spectroscopy data in yeast has shown that upon proteasome inhibition, all ubiquitin chains accumulate with the clear exception of lysine 63 (Xu et al. 2009). The 26S proteasome is a multimeric complex consisting of ~31 different proteins. It is comprised of two different subcomplexes, the 19S regulatory complex and the 20S proteolytic core (Voges, Zwickl & Baumeister 1999). The 19S regulatory cap is responsible for recognizing ubiquitinated substrates (via ubiquitin binding protein SP5/RPN10), removing their ubiquitin chains by complex component deubiquitinating enzymes, RPN11 and UCH37 or a transiently associated DUB, USP14 (Deveraux et al. 1994) (Koulich, Li & DeMartino 2008). Next, bound proteins are unfolded and fed into
the barrel-shaped 20S proteolytic core requiring the physical action of ATP hydrolysis by the proteins at the 19S and 20S interface (Saeki, Toh-e & Yokosawa 2000). Once inside the 20S proteolytic core, the substrates are sequentially cleaved into smaller peptides. The end result is the recycling of small component peptides of variable length (4-25 amino acids) and free ubiquitin (Ehring et al. 1996) (Nussbaum et al. 1998) (Figure 1).

Figure 1. The Ubiquitin-Proteasome System (from Rubenstein et al. 2006) Misfolded substrate proteins are recognized by E3 Ubiquitin ligases and are subsequently conjugated with ubiquitin chains. These chains serve as recruitment signals for subsequent trafficking to the 26S proteasome for degradation into peptides and component amino acids.
1.1.2 Ubiquitin Related Function: Autophagy

Autophagy is a key pathway in regulating cell and organelle health. In conditions where cells are placed in an energy-limiting state, autophagy leads to the engulfment of whole organelles such as the mitochondria (mitophagy), to mediate its lysosomal degradation and sustain cellular viability through the release of nutrients and cellular building blocks (Lemasters 2005). In addition, autophagy acts to protect the cell by degrading organelles, such as the endoplasmic reticulum and the mitochondria when damaged by toxic macromolecules or bacterial/viral infection (Tanaka et al. 2010) (Narendra et al. 2008) (Ding et al. 2007a). It has even been shown to be involved in the clearance of these invading bacteria themselves by engulfment and destruction by the lysosome (Ligeon, Temime-Smaali & Lafont 2011) (Zheng et al. 2009). Peroxisomes and ribosomes in yeast have also been shown to undergo selective autophagy (van Zutphen, Veenhuis & van der Klei 2011) (Nakatogawa et al. 2009). This large scale autophagy is termed macroautophagy. Autophagy is largely seen as a macro-destructive process, however, it also plays a crucial role in the clearance of individual proteins for maintaining cell health (Kraft, Peter & Hofmann 2010). Chaperone-mediated autophagy involves the specific chaperone-driven degradation of cytosolic proteins, while microautophagy is more of a passive diffusion of small cytoplasmic components into the autophagosome/lysosome (Dargemont, Ossareh-Nazari 2011). The autophagy pathway also mediates the clearance of aggregate-prone, insoluble proteins inaccessible to the unfolding machinery necessary for degradation by the 26S proteasome. In autophagy, misfolded proteins with mostly lysine 63 linkages can form perinuclear inclusions termed aggresomes (Wooten et al.
These large inclusions serve as a pooling site before trafficking or complete engulfment by the autophagosome. These ubiquitinated proteins are recognized by Ubiquitin Binding Domain (UBD) containing proteins p62 (also called sequestome 1/SQSTM1) and HDAC 6 (Histone De-acetylase 6) through direct interaction between their carboxy-terminal UBA (Ubiquitin Association) or BUZ (Binder of ubiquitin Zinc finger) domains and the ubiquitin chain (Isogai et al. 2011) (Kawaguchi et al. 2003). p62 then traffics its ubiquitinated cargo to the site of immature autophagosomal vesicle formation, marked by autophagosomal membrane protein LC3 (microtubule-associated protein 1 light chain 3). LC3 undergoes a conformational change upon the induction of autophagy. LC3-I is conjugated by Atg7 and Atg12-Atg5-Atg16L multimers to a highly lipophilic phosphatidylethanolamine (PE) group generating LC3-II which shows an accelerated migration in western blot analysis (Barth, Glick & Macleod 2010) (Kabeya et al. 2000). The origin of this double membrane vesicle has been the subject of much debate. Early work searching for autophagosomal markers identified the endoplasmic reticulum as a potential source. A key element of autophagosome membranes, (PtdIns(3)P (phosphatidylinositol 3-phosphate), was shown to accumulate to an ER-related structure. This suggested the ER as the site of vesicle budding (Axe et al. 2008). This datum was further supported by groups showing double membrane vesicles forming out of the ER (Hayashi-Nishino et al. 2009) (Yla-Anttila et al. 2009). However, the mitochondria has also been shown to be an important source of lipid membranes for autophagosome vesicle formation. In conditions of starvation-induced autophagy, it has been shown via Immuno Electron microscopy that the mitochondrial membrane stains positive for autophagosome markers LC3 and Atg5 at distinct puncta, theorized to be the
site of membrane budding. It was also shown that through photobleaching of the associated mitochondria, autophagosome markers are depleted showing an interplay between the two compartments (Hailey et al. 2010). These data coincide with starvation-induced mitophagy. The mitochondria’s own membrane serves as its autophagosomal vesicle. LC3 is present in both the inner and outer membranes of the autophagosome and is attributed to recruiting and binding key adaptors of the immature vesicle via their LC3 recognition sequence (LRS) (WXXL/I). It has been shown that p62 is one such adaptor with an LRS. Interaction data initially suggested that p62 was recruited directly to the building autophagosome by LC3 interaction, however recent work has shown that p62 does traffic to the autophagosome formation site in the absence of functional LC3 suggesting that LC3 only maintains p62/cargo localization at the forming vesicle (Itakura, Mizushima 2011). Upon full formation of a double membrane autophagosome, this still immature vesicle fuses with the lysosome in a Rab-7 dependent event through its recruitment of tether and SNARE proteins forming the autolysosome (Gutierrez et al. 2004) (Jager et al. 2004). SNARE proteins partner between opposing lipid bi-layers creating channels and mediating their fusion (Jena 2011). More than 50 lysosome resident acid hydrolases then go to work on the internalized substrates leading to their destruction and subsequent recycling of their building blocks for new protein translation (Saftig 2006). Defects in autophagy have been shown to contribute to a number of neurological disorders. Poly-glutamine (PolyQ) expansion diseases such ALS, Alzheimer’s, Parkinson’s, and Huntington’s Diseases are caused by the accumulation of different mis-translated proteins with long stretches of glutamine amino acid repeats. These proteins are not properly degraded and form cytoplasmic aggregates leading to cell
disfunction and death (Meriin, Sherman 2005). p62 serves as an excellent marker for ubiquitin-mediated autophagy as it is degraded in the autolysosome along with its bound substrates. Additionally, immunofluorescence and western blot analysis of LC3 serve as great markers for the progression of autophagy. As an autophagosomal membrane protein, LC3 serves as a strong marker for early to late progression through autophagy. A novel tandem GFP/RFP tagged LC3 is used to show the progression of autophagy pre to post fusion with the lysosome (Kimura et al. 2009) (Kimura, Noda & Yoshimori 2008) (Figure 2).

![Diagram of the Ubiquitin-Autophagy Pathway](image)

**Figure 2. The Ubiquitin-Autophagy Pathway** (from Jaeger et al. 2009) (2) Pre-autophagosomal membranes (marked by LC3-II form around autophagy substrates trafficked by p62. (3) This double membrane structure traffics to the lysosome for fusion. (4) fusion with the lysosome is necessary for the degradation of the autophagy substrates, including the marker p62.
1.1.3 Ubiquitin Related Function: Localization/Trafficking

As mentioned above, ubiquitin can be conjugated onto a target substrate using a variety of different linkages, some of which serve as a signal for a specific traffickiing event. K48 linkages direct proteins to the proteasome, while K63 linkages primarily drive proteins to the autophagosome. Polyubiquitination has also been identified as playing a key role in endocytosis and intracellular trafficking. Unlike the UPS and Autophagy pathways, monoubiquitination and multiubiquitination (multiple sites of monoubiquitination on a target substrate) are primarily involved in non-degradative pathways. It is not necessary to form long chains for ubiquitin to serve as a signal. Like phosphorylation, monoubiquitination serves as a more traditional post-translational modification, reversibly marking a protein for an interaction with a specific adaptor, leading to its intracellular relocalization and/or endocytosis. The monoubiquitination of hDCNL1, a key protein in activating Cullin-RING family E3 ubiquitin ligases, is essential to mediated its nuclear export. This trafficking is driven by E3 ligase Nedd4-1 monoubiquitination at three different lysine residues (K143, K149, K171) (Wu et al. 2011). In addition to intracellular trafficking, ubiquitin plays a key role in endocytosis, most notably in receptor tyrosine kinases and G-Protein coupled receptors (Hislop, von Zastrow 2011). Ubiquitination was first shown to function as a signal mediating endocytosis in yeast. This work showed that upon α factor antigen presentation to the G protein-coupled plasma receptor Ste2p, a signal transduction pathway is initiated leading to the ubiquitin-dependent endocytosis of the activated receptor, trafficking it into the endosomal/lysosomal pathway for degradation (Mukhopadhyay, Riezman 2007) (Hicke,
Since this initial discovery, ubiquitin has been identified as a key component in the ligand driven endocytosis of a variety of plasma membrane proteins including the Epidermal Growth Factor Receptor (EGFR) and the Notch Receptor. EGFR was shown to undergo two different mechanisms of endocytosis upon Epidermal Growth Factor (EGF) presentation. In conditions of low EGF, ubiquitin-independent, clatherin-dependent endocytosis occurred, while upon treatment with about ten fold more EGF, the EGFR underwent both clatherin-dependent and ubiquitin-mediated endocytosis (Sigismund et al. 2005). Activation of the Notch Receptor also requires monoubiquitination, however, this ubiquitination occurs on its ligands (Delta and Serrate) mediating their import into the cell (Lai et al. 2005). As described above, ubiquitination plays a key role in many cellular processes. A simple post-translational modification with a variety of substrate-specific conformational switches drives a very tightly regulated set of functions. These specific modifications serve as signals for adaptors with ubiquitin binding domains (UBDs) driving their appropriate downstream processing.

### 1.2 p97/Cdc48/VCP

Throughout evolution cells have developed common methods of driving complex and specific functions. Signal transduction pathways leading to protein phosphorylation and dephosphorylation are one such example. They represent very common protein modifications, yet they somehow lead to very specific functional consequences in different signalling pathways. These events, although similar, become tightly regulated processes through the specificity of their mediators and adaptors. Only specific
kinases/phosphatases will act on certain substrates at the appropriate times and contexts. The AAA (ATPase associated with various activities) family of ATPases represent a group of proteins performing similar functions in highly specific contexts based on adaptor-driven specificity. Type I AAA proteins are characterized by one conserved AAA ATPase domain, while type II proteins have two. The AAA family of proteins perform a wide variety of functions including substrate remodelling, translocation across membranes, maintaining cellular structure and mediating access to DNA to drive transcription (Jha, Dutta 2009) (Deichsel, Mouysset & Hoppe 2009) (Duderstadt, Berger 2008) (Numata et al. 2008) (Ballar, Pabuccuoglu & Kose 2011). They mediate these functions using the mechanical action of ATP hydrolysis to exert physical force on a substrate (Numata et al. 2008) (Gallant 2007). p97 or VCP (cdc48 in yeast) is one member of the type II AAA family of ATPases that primarily functions in ubiquitin-related pathways. It is a highly conserved and expressed eukaryotic protein representing as much as 1% of total cytosolic protein. This protein is largely cytosolic, although it has been found in the nucleus in addition to association with the membranes of the Endoplasmic Reticulum and Golgi Apparatus (Madeo et al. 1998) (Acharya et al. 1995) (Latterich, Frohlich & Schekman 1995) (Uchiyama et al. 2003). It performs a diverse group of functions including ubiquitin-mediated protein degradation and trafficking, homotypic membrane fusion, reassembly of the nuclear envelope, and effecting apoptosis and cell cycle progression (Latterich, Frohlich & Schekman 1995) (Wang, Song & Li 2004) (Asai et al. 2002) (Woodman 2003). p97 is able to mediate these varied functions through its interaction with specific adaptors. As will be shown in greater detail, p97’s function in ubiquitinated protein binding and trafficking is especially dependent on its
adaptors as it has no known ubiquitin-interaction domains and subsequently has not been shown to bind to ubiquitin at a high affinity.

1.2.1 Structure

p97 contains four structural domains, a flexible amino-terminal region (N domain), comprised of a double Ψ barrel superfold followed by a four-stranded β-barrel, two central, conserved AAA domains named D1 and D2 responsible for ATP hydrolysis (a prerequisite for all AAA ATPase family members) and a carboxy-terminal tail (C domain). The amino and carboxy terminal regions are responsible for adaptor binding while the central AAA domains contain ATP-binding motifs: Walker Boxes A & B. In cells, p97 exists as a homohexameric double ring complex ~160 Å in diameter with a central pore of about 15 Å in diameter (Zhang et al. 2000) (DeLaBarre, Brunger 2003). The D1 and D2 domains are stacked head to tail with the N domain protruding outward from the central pore at ~30°, giving the complex the appearance of a six-pointed star while the C domain protrudes from the other side of the double ring structure (Zhang et al. 2000). Althought it would be easy to surmise that p97, like the proteasome, feeds target substrates through its central pore, unfolding them before delivery to the proteasome, structural analysis suggests this is not the case. At its thinnest, the central pore is 15 Å across, just wide enough for a largely unfolded peptide string. If p97 is using its ATPase activity to unfold substrates within the pore, they would not fit unless previously unfolded. Additionally, crystallographic data of the full p97 hexamer suggest that this region of the pore is also the site of an interaction between p97’s His317 in the
D1 domain and a ZN$^{2+}$ ion, subsequently blocking the pore. This ion is unlikely to interact with all His317 residues in the D1 domains of the hexamer simultaneously, but could potentially be involved in hexamer stabilization (DeLaBarre, Brunger 2003). The functional relevance of this interaction has yet to be determined given the changes in physical state that the p97 hexamer undergoes upon ADP/ATP binding and hydrolysis. Additionally, crystallographic work was done either with p97 alone or in the presence of only one adaptor. With the high likelihood of p97 hexamers interacting with a mixed variety of adaptors working in concert or opposition to regulate p97 specificity and function, it is possible that structural data are not completely accurate in representing the in vivo conformation. However, unless adaptor/substrate binding removes this ZN$^{2+}$ ion and causes a substantial widening of the central pore, it is unlikely that substrates could fit inside the hexamer. Therefore, it has been suggested that adaptor binding to the amino and/or carboxy termini bring substrates along the side of the p97 hexamer instead of through it.

1.2.2 Biochemical Function

As stated above, AAA ATPases generally function by changing the physical conformation of substrates upon ATP hydrolysis. p97 is a well studied member of this family, with structure/function studies helping to illuminate its mechanism of action. It is known that the N domain is mostly responsible for the binding of cofactors/adaptors including UBXD1 and the well studied adaptors Ufd1-Npl4 and p47. In addition to the N domain, adaptor UBXD1 has also been shown to also interact with the C domain (Kern et
As the sites for adaptor association, the N and C domains are crucial for maintaining an interaction with p97’s poly-ubiquitinated substrates (Meyer et al. 2000) (Kern et al. 2009) (Dai, Li 2001). Adaptor binding to the N domain has been thought to be mutually exclusive, since dose-dependent competition experiments have suggested a common interaction domain between adaptors (Meyer et al. 2000) (Nagahama et al. 2009). However, recent work has shown that FAF1 and UBXD7 only interact with p97 hexamers already bound to Npl4/Ufd1 adaptors, suggesting a hierarchy in adaptor association (Hanzelmann, Buchberger & Schindelin 2011). Through structure/function and crystallographic studies, a number of models have been developed to explain how p97 hexamers can mechanically effect the conformation of substrates associated via these adaptors at the N domain. The ATPase domains are crucial in inducing a conformational change in the p97 hexamer mediating its function. A dominant-negative, catalytically-dead mutant of p97 (p97E305Q/E578Q) creates mutations in both D1 and D2 ATPase domains, blocking their ability to hydrolyze ATP (Weihl et al. 2006). The contributions of the two ATPase domains and the mechanism by which their binding and hydrolysis of ATP to ADP leads to an overall conformational change is up for debate. One model suggests a functional equivalence between the D1 and D2 domains, while the second suggests a primary requirement for the D2 domain to maintain p97 function. The molecular ratchet model suggests that the D1 and D2 domains are interdependent with binding of ATP at the D1 hexameric ring leading to the subsequent release of ADP bound at the D2 ring. While this occurs, the D1 ATP-bound ring moves inward toward the core, while the D2 ADP-bound ring moves outward. Upon ATP hydrolysis at the D1 ring, the roles are reversed and the process continues. This creates a large conformation change
throughout the protein up into the N-D1 linker region where physical force can be applied to bound substrates changing their conformation (Zhang et al. 2000) (Ye, Meyer & Rapoport 2003). Like a well-balanced game of see-saw, this model relies upon the notion that both the D1 and D2 domains bind to and hydrolyze ATP equally. Another model is based upon biochemical data and supported by the full crystallographic structure of p97 showing that the ATP binding activity of the D2 domain far surpasses that of the D1 domain potentially throwing the see-saw heavily out of balance (Song, Wang & Li 2003) (DeLaBarre, Brunger 2003). In vitro ATPase activity experiments showed mutation of the D2 domain severely inhibited p97’s hydrolysis function while D1 domain mutation had little effect. These data are further supported in vivo, in trypanosomes, showing the D2 is essential for viability while the D1 is not (Lamb et al. 2001). As mentioned above, crystallographic work seems to suggest that the physical force generated on substrates does not occur within the hexameric pore, but along the outside. As with most proteins, p97 also undergoes post-translational modifications such as phosphorylation and acetylation, which have been shown to effect its function and localization, providing still more layers of specific direction of p97 function in addition to its adaptor-driven effects. Forty sites of phosphorylation and 22 lysine residues capable of acetylation have been identified throughout the p97 monomer. 90% of all phosphorylation events identified occur at tyrosine 805 (Egerton, Samelson 1994). Phosphorylation of tyrosine 805, located in the C domain, has been shown to abolish interactions with the PUB and PUL domains in p97 adaptors, such as UBXD1 (Zhao et al. 2007) (Mullally, Chernova & Wilkinson 2006). Tyrosine 805 has been shown to undergo phosphorylation in response to multiple signals including: sperm capacitation, ER assembly in vitro, and upon T cell
receptor activation (Ficarro et al. 2003) (Lavoie et al. 2000) (Egerton et al. 1992). In addition to effecting adaptor binding, p97 phosphorylation has been shown to effect its localization to centromeres during mitosis as well as its migration to the ER to facilitate homotypic membrane fusion events (Madeo et al. 1998) (Lavoie et al. 2000). Acetylation events have also shown strong effects on p97 function. An acetylation mimic mutant of Lys696, K696Q, has shown an increase in p97’s ATPase activity. This lysine is located within the D2 domain. Although the functional significance of some sites of post-translational modification have been elucidated, the consequences of most of these modifications have not yet been identified (Mori-Konya et al. 2009). As with all AAA family members, p97 performs a variety of functions in the cell, using its physical exertion on substrates to drive an event.

1.2.3 Role in ERAD

Protein degradation is a crucial function throughout the cell in the moderation and elimination of short lived regulatory proteins as well as in the destruction of misfolded proteins to maintain overall cell health. Some organelles, like the mitochondria and lysosomes, have their own pools of proteases responsible for removing these non-cytoplasmic substrates while others require their export into the cytoplasm where they are eliminated through either the UPS or the autophagy/lysosome pathway (Leidhold, Voos 2007) (Rep et al. 1996). As the main site for chaperone-mediated post transcriptional folding and initial export of about ¼ of the cell’s proteome, misfolding in the Endoplasmic Reticulum (ER) is thought to occur in as high as 80% of all new proteins
generated (Alberts et al. 2002). One would expect the ER to be rife with proteases to quickly eliminated these misfolded products. Surprisingly, this is not the case. Because the ER lacks the ability to eliminate terminally misfolded proteins internally, a unique and efficient mechanism exists to recognize these substrates, ubiquitinate them, retro-translocate them out of the ER, and traffic them to the 26S proteasome, termed Endoplasmic Reticulum Associated Degradation (ERAD). p97 mediates the retrotranslocation of ubiquitinated substrates across the ER membrane, and trafficking to the 26S proteasome, while using physical force to assist in their unfolding to prepare them for proteasomal degradation. ERAD is initiated upon incomplete or incorrect folding of ER resident proteins. As a key site for protein folding and trafficking, the ER first imports water-soluble and transmembrane proteins co-translationally through direct attachment of the synthesizing ribosome through the narrow SEC61 translocon channel to the rough ER (Schaletzky, Rapoport 2006). The water-soluble proteins are either newly synthesized residents of the ER or are subsequently secreted to other organelles or the extra-cellular space. Likewise, imported membrane proteins will either reside in the ER or be exported for later insertion into the plasma membrane or the surface of another organelle (Alberts et al. 2002). As stated above, the ER is the site of chaperone-mediated protein folding and disulfide bond formation by disulfide isomerases to help maintain correct conformation. This occurs prior to their export to Golgi Apparatus or other target organelles. Immediately upon insertion of these unfolded polypeptides, a HSP70 chaperone protein, BiP (Binding Immunoglobulin Protein), binds to hydrophobic residue patches of the sequence Asn-X-Ser/Thr, leading to asparagine glycosylation (Glc$_3$Man$_9$GlcNAc$_2$) by an oligosaccharyl transferase complex (Matlack et al. 1999)
(Eisele, Schafer & Wolf 2010). This serves as a chaperone association signal marking a peptide’s progression through proper folding machinery. While associated with the BiP folding complex, glucosidase I/II remove the innermost glycans. This then allows for the release of the partially folded peptide allowing it to then associate with the lectin-binding chaperone system (calnexin & calreticulin). Upon release from this chaperone complex, glucosidase II removes the remaining inner glycan leaving Man$_9$GlcNAc$_2$, subsequently blocking the folding peptide’s further association with either types of chaperones. Correctly folded proteins are then released from the ER for targeted trafficking (Aebi et al. 2010). Another family of chaperones, the PDI (protein di-sulfide isomerase) family of enzymes drives di-sulfide bond formation, further helping to assemble a correctly folded protein (Appenzeller-Herzog, Ellgaard 2008). A delicate balance is necessary to allow enough time to correct mistakes yet prevent the ER machinery from being overwhelmed by futile attempts at refolding immature proteins. However, if misfolded proteins cannot be corrected, it is crucial to quickly and efficiently identify and eliminate these junk proteins before they accumulate or are incorrectly exported to their target locations. Upon initial completion of chaperone-mediated folding, quality control mechanisms evaluate substrates before trafficking them out of the ER. Poorly folded proteins are recognized by a UDP-glucose: glycoprotein glucosyltransferase (UGGT) and are re-glycosylated to allow lectin-binding chaperones more time to correct any errors. Repeated cycling to the lectin-binding complexes allows slow-acting α-1,2-mannosidases, such as EDEM1/Hrd1 (ER Degradation-enhancing α-mannosidase-like protein 1), to cleave two mannose saccharides from the substrate leaving an N-glycan degradation signal (α-1,6 terminal mannose) (Hosokawa et al. 2001) (Oda et al. 2003)
Jakob et al. 1998) (Aebi et al. 2010) (Quan et al. 2008). The PDI family of di-sulfide isomerases also serve as a quality control mechanism recognizing misfolded proteins and reducing their di-sulfide bonds to better mediate their retrotranslocation out of the ER. Some PDI enzymes (ERdj5) associate with the quality control mannosidases (EDEM1), acting in concert to efficiently break misformed di-sulfide bonds (Hagiwara et al. 2011) (Appenzeller-Herzog, Ellgaard 2008). These proteins are trafficked to specific E3 ligases and poly-ubiquitinated, by glycan-binding lectins such as OS9 and XTP3-B, marking them for export from the ER and proteasomal destruction collectively termed ERAD (Kim, Spear & Ng 2005) (Szathmary et al. 2005) (Hosokawa et al. 2009) (Christianson et al. 2008). Based on the type of misfolded proteins as well as the location of the misfolding signals, three different subcategories of ERAD substrates have been delineated:  ERAD-L (luminal proteins), ERAD-M (transmembrane proteins) and ERAD-C (cytosolic proteins). Work done in yeast has shown that ERAD-L and ERAD-M strongly rely on the Endoplasmic Reticulum integral membrane E3 ligase, Hrd1, while those proteins with cytosolic signals (ERAD-C) require the E3 ligase Doa10 to be ubiquitinated (Taxis et al. 2003) (Swanson, Locher & Hochstrasser 2001). It appears that these requirements are less stringent in mammals supporting the increased complexity and redundancy often seen in higher organisms (Eisele, Schafer & Wolf 2010) (Bernasconi et al. 2010). Once these misfolded proteins are poly-ubiquitinated, it is essential that they be retrotranslocated out of the ER through the phospholipid membrane and delivered to the 26S proteasome. p97 has been shown to use its adaptor-specific ability to exert the physical force required to mediate exit of these ubiquitinated substrates. Multiple adaptors work in concert to mediate p97’s ERAD function.
Erasin/UBXD2 is an ER membrane resident adaptor that is involved in anchoring the p97 hexamer to the site of retrotranslocation on the ER membrane. Work done in yeast has also shown that UBXD2 provides a crucial link between p97 and the membrane bound E3 ligases, Hrd1 and Doa10, potentially through its direct association with the ubiquitin chains on the E3 ligase substrates (Neuber et al. 2005) (Schuberth et al. 2004). The Npl4-Ufd1 heterodimer is a key component coupling p97 to its ubiquitinated substrates (Bays et al. 2001). Yeast strains containing mutants of these proteins showed a significant defect in the degradation of a MHC Class I protein as well as misfolded luminal protein substrate, CPY* (Ye, Meyer & Rapoport 2001) (Ye, Meyer & Rapoport 2003). In addition to interaction mediators, there are at least two substrate modifying adaptors, Png1/NGLY1 and Ufd2 involved in ERAD. The first is involved in deglycosylating substrates for efficient processing by the proteasome, while the second is an E3 ligase involved in further poly-ubiquitination of substrates (Kim et al. 2006) (Nakatsukasa et al. 2008). Two other adaptors have been shown to interact with p97 and effect ERAD, the UBX domain containing adaptors YOD1/Otu1 and UBX4, however, neither have yet been tied to a specific function in ERAD. The former is a deubiquitinating enzyme, which theoretically could be involved in modulating the extent of substrate ubiquitination, while the other has no known function and also lacks a known ubiquitin association domain (Ernst et al. 2009) (Albers et al. 2009). Although a lot is known about the specific adaptors involved in ERAD, it should be noted that the stoichiometry of the multi-protein complex is not. The composition and numbers of adaptors on each p97 hexamer has not been determined. Some of these adaptors may be present at the same time on the p97 hexamer, or signalling events could lead to their
temporary association in mixed complexes. It is expected that some of these associations are indeed cyclic as some of these UBX domain containing adaptors bind to common sites on p97, making a balanced hexamer with both adaptors, unlikely. In yeast, the adaptors UBX2 and UBX4 cannot be isolated in the same complexes suggesting a shuttling on and off the hexamer as it is required (Alberts et al. 2009). P97 functions in ERAD as the center for an ER-membrane periphery complex with variable adaptors functioning in concert to direct ubiquitinated substrates to the ER membrane and utilize p97’s physical conformational changes upon ATP hydrolysis to physically pull substrates out of the membrane into the cytosol for trafficking to the 26S proteasome.

1.2.4 Causative Mutation in Disease: IBMPFD

P97 has been identified as the causative mutation in a hereditary syndrome, IBMPFD (Inclusion Body Myopathy, Paget Disease of the Bone, Frontotemporal Dimentia) and has recently been identified as a cause in 1-2 % of familial ALS (Amyotrophic lateral sclerosis) (Watts et al. 2004) (Johnson et al. 2010) (Shaw 2010). Both IBMPFD and ALS’ neurological pathologies are characterized as FTLD-U disorders, frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions. These disorders are genetically complex with many factors leading to disease. IBMPFD is a hereditary syndrome of mixed pathologies in multiple tissues (the bone, muscle and brain) defined by an accumulation of ubiquitinated conjugates in the cytoplasm. It is a familial autosomal dominant disease marked by three main clinical symptoms with variable penetrance. In a study of 49 individuals with IBMPFD from nine families, patients
exhibited muscle disease (87%), inclusion body myopathy (39%) and frontotemporal dementia (27%) with a mean age of diagnosis at 57 years old (Kimonis et al. 2008). Muscle disease is marked by slowly progressive muscle weakness and atrophy leading to immobility, while inclusion body myopathy is defined as the accumulation of p97/ubiquitin positive aggregates forming in the cell cytoplasm of multiple tissues including the muscle, bone and brain (neurons) (Hubbers et al. 2007). IBMPFD affects the frontal and temporal sectors of the brain which are majorly responsible for the control of personality, behavior and language. Some neurodegenerative diseases are very hard to detect, diagnose and identify. Frontotemporal dementia is diagnosed through a variety of neuropsychological tests to illustrate extreme behavior alterations in personality, as well as memory and language disfunctions and mobility control problems. It is often misdiagnosed as Alzheimer’s disease, but has an earlier onset, at a mean age of 57 years compared to Alzheimer’s disease’s average age of detection at around 80 years of age (Kovach et al. 2001) (Kimonis et al. 2008) (Anonymous 2011). The similar molecular outcome of p97 mutation in FTLD-U disorders IBMPFD and ALS, is the accumulation of ubiquitinated conjugates, most notably, the heterogeneous nuclear ribonucleoprotein (hnRNP), TDP-43 (Liscic et al. 2008) (Ritson et al. 2010). TDP-43, or TAR (trans-activating response region) DNA-binding protein 43, is a DNA/RNA binding protein predominantly localized to the nucleus that continuously shuttles to the cytoplasm in a transcription-related manner. TDP-43 functions to negatively regulate transcription, splicing and RNA stability of as many as 4000 RNA targets by binding to introns, exons or the 3’UTR, interrupting appropriate post-transcriptional processing. This leads to RNA degradation and subsequent protein loss. (Ayala et al. 2008) (Sephton et al. 2011).
In FTLD diseases, TDP-43 accumulates and mislocalizes in the cytoplasm to form large aggregates (Liscic et al. 2008). Genetically, IBMPFD is caused by an autosomal dominant missense point mutation of the p97 protein. As many as 12 different amino acid changes have thus far been identified throughout the protein. These mutations occur primarily in the N domain, however two have been identified in the Linker 1 region and 1 causative mutation has been found in the AAA D1 domain. The most common amino acid residue mutated is arginine 155 with 3 different substitutions (cystine, histidine and proline), the p97\textsuperscript{R155H} mutation being the most common mutation. However, in the most aggressive cases of disease, alanine 232 is mutated to glutamic acid. This mutation lies in the junction between the Linker 1 region and the first AAA domain, D1, responsible for binding ATP/ADP (Fanganiello et al. 2011). The most common mutation in IBMPFD, p97\textsuperscript{R155H}, has also been found in familial ALS in addition to the p97\textsuperscript{R191Q} mutation. Also, two novel p97 mutations have been identified in ALS that have yet to be seen in IBMPFD patients (p97\textsuperscript{R159G} and p97\textsuperscript{D592N}) (Johnson et al. 2010). (Table 1)
Transgenic mouse models that closely recapitulate the human disease have been made of two of the most interesting IBMPFD mutants, p97^{R155H} and p97^{A232E}. By 9-10 months of age, both models exhibit muscle weakness, reduced bone density, decreased body mass and the development of neurological defects. Histologically, mouse tissues show myogenic patterns of muscle myopathy, loss of nuclear TDP-43, resulting in its cytoplasmic accumulation in skeletal muscle and brain tissues, in addition to ubiquitin accumulation in vacuoles and multivesicular inclusion bodies (MVB). The end result of these abnormalities is an increased mortality in the p97 mutant mice when compared to their wild-type counterparts (Badadani et al. 2010) (Custer et al. 2010). As p97 is a highly conserved protein throughout eukaryotes, both of the above disease-relevant mutants were tested for their effect in Drosophila. Similar to the mentioned mouse models, p97^{R155H}, and p97^{A232E} showed enhanced toxicity as measured by eye necrosis.
In addition, TDP-43 cytoplasmic accumulation and neuronal defects were seen to be greatly enhanced as measured by nuclear condensation assessment (Ritson et al. 2010). Autosomal dominant mutations of p97 lead to disease through protein accumulation. Unlike ERAD, this accumulation is due to a blockage in protein degradation of largely insoluble protein aggregates. As stated above, the primary mechanism for preventing cytoplasmic protein accumulation is autophagy.

1.2.4.1 IBMPFD and Autophagy

In addition to ERAD, p97 has also been implicated in the elimination of proteins that are polyubiquitinated primarily with lysine 63 linkages and are largely insoluble and form disordered cytoplasmic protein aggregates. (Wooten et al. 2008) (Tan et al. 2007) (Tan et al. 2008). This pathway, as stated previously, is an alternative protein degradation program to the ubiquitin proteasome system. Initial implications that p97 plays a role in autophagy were derived from studies that discovered p97 as the causative mutation in IBMPFD in patients. As mentioned previously, IBMPFD is marked by the accumulation of large, insoluble cytoplasmic protein aggregates. Immunohistochemical analysis of these IBMPFD patients showed that mutant p97 exhibited a strong localization to these variably sized cytoplasmic aggregates in muscle tissue (Watts et al. 2004). This was initially compared to the localization of p62, a key component of autophagy found mutated in multiple protein-aggregate diseases (Donaldson et al. 2003). As mentioned above, p62 is crucial for the recognition and trafficking of ubiquitinated substrates to the autophagosome. This was the first indication that p97 had a ubiquitin-proteasome
independent function in protein degradation. As mentioned previously, substrates of the aggresome/autophagy pathway include polyQ expansion proteins, whose accumulation causes a variety of neurological diseases. Interaction work with p97<sup>WT</sup> and polyQ expansion proteins showed a strong association and co-localization in PC12 (rat neuronal) cells (Hirabayashi et al. 2001) (Ju et al. 2008). Further work expanding on the effects of IBMPFD-relevant mutants of p97, notably p97<sup>R155H</sup> and p97<sup>A232E</sup>, showed a loss in this association and accumulation in aggresomes (Ju et al. 2008). Strong links between p97 mutation and the accumulation of autophagy markers LC3 and p62 were identified in patient samples in addition to identifying an increase in protein aggregates including TDP-43, the primary protein accumulated in IBMPFD (Tresse et al. 2010) (Ju, Weihl 2010) (Ju et al. 2009). Immature LC3 positive autophagosomes accumulate in IBMPFD p97 cells. Through a yet to be discovered mechanism, mutant p97 blocks the ability of these autophagosomes degrade upon fusion with the lysosome, preventing the completion of autophagy. p97 has been shown to be crucial for mediating the autophagic processing of specific substrates, however, its effect on macroautophagy via nutrient deprivation is not as clear. Research has shown that starvation-induced autophagy progresses normally in p97 mutant cells, while other work has maintained that autophagy markers will accumulate under conditions of starvation. (Tresse et al. 2010) (Ju et al. 2009). This suggests a more specific role in chaperone-mediated autophagy. It is this defect in p97’s ability to recognize and correctly traffic these substrates through the autophagy pathway (aggresome to autophagosome ending in lysosomal fusion) that underlie IBMPFD disease. Although p97 has clearly been shown to be involved in autophagy progression through the study of p97 mutant over-expression in transformed
cells as well as primary IBMPFD disease patient cells, a direct mechanism has not been elucidated.

1.2.4.2 p97 & Trafficking

p97 has been implicated in many crucial adaptor-driven functions such as ERAD, autophagy and in the mediation of ER/Golgi membrane fusion events. These events all involve polyubiquitin as a substrate marker. Monoubiquitination serves as a marker on a large number of plasma membrane proteins mediating their endocytosis and intracellular transport to endosomes or vacuoles. The Epidermal Growth Factor Receptor and other Receptor Tyrosine Kinases have been shown to be endocytosed in this manner (Levkowitz et al. 1998) (Thien, Langdon 2005) (Lee et al. 1999) (Miyake et al. 1998). Generally, upon ligand binding, receptors are activated through the phosphorylation of specific tyrosine residues. E3 ligases with tyrosine-kinase-binding domains associate with these activated receptors and recruit E2 conjugated enzymes mediating their monou or multiubiquitination. Cbl is one such E3 known to play a role in the ubiquitination and endocytosis of a variety of receptor tyrosine kinases (Thien, Langdon 2005) (Haglund, Di Fiore & Dikic 2003). Monoubiquitin-mediated endocytosis not only plays a role in modulating signalling, and degradation/recycling of activated receptors, but has also been shown to regulate the endocytosis and degradation of other membrane proteins. Caveolin-1 protein is a structural component of caveolae, large lipid rafts that form bulb-shaped pits similar to clatherin-coated pits. Upon caveolae dissassembly, Caveolin-1 monomers are known to undergo monoubiquitination, endocytosis and degradation.
through endosome-mediated delivery to the lysosome. Very recently, work has been published showing that p97 plays a key role in the post-endocytic trafficking of caveolin-1 to the endolysosome. IP/MS work looking for differential interactions between p97WT, IBMPFD mutant p97R155H and a substrate-trapping, ATPase deficient mutant p97E578Q, identified monoubiquitinated caveolin-1 as a potential substrate that preferentially interacted with wild-type p97 and the p97E578Q mutant. Authors additionally isolated UBXD1, a known p97 adaptor with no identified function, to also co-precipitate with monoubiquitinated caveolin-1. UBXD1-p97’s interaction with Caveolin-1 leads to its proper transport to the lysosome for degradation. IBMPFD mutant p97R155H and p97E578Q, were shown to block the delivery of Caveolin-1 to the lysosome. Enlarged late endosome vesicles positive for LAMP-1 (late endosome marker) and Caveolin-1 accumulated throughout the cytoplasm. Caveolin-1 was localized to the limiting membrane of these vesicles suggesting a block in processing and transport. p97 has also been shown to co-localize with the early endosome marker EEA1, and knockdown of endogenous p97 mimics the effect seen with IBMPFD mutants; the accumulation of enlarged early endosomes (Ramanathan, Ye 2011). These results were expanded upon using a p97 small molecule inhibitor, DBeQ, and the siRNA-mediated knockdown of UBXD1. Furthermore, ALS and IBMPFD patient-derived fibroblasts verified the mutant over-expression work using the muscle-specific Caveolin-1 homolog, Caveolin-3. In addition, EGFR transport was effected using the above mentioned systems, suggesting that p97, with UBXD1, plays a more generalized role in endocytic cargo sorting (Ritz et al. 2011). This work suggests that not only is autophagic transport strongly effected in
IBMPFD mutants, but that a more general defect in many protein trafficking events could lead to this severe disease.

1.3 p97 Adaptor Proteins

As illustrated repeatedly above, p97 is capable of acting in such a wide array of ubiquitin-mediated functions without having a strong affinity to ubiquitin. This is possible through its interaction with an array of adaptor proteins. A growing number of p97 interacting proteins harbor a variety of p97 interaction motifs (Elsasser, Finley 2005) (Schuberth, Buchberger 2008) (Yeung et al. 2008) (Dreveny et al. 2004b). Most adaptor/accessory proteins harbor only one p97 interaction motif, however some interact with p97 through multiple points of contact (Kern et al. 2009) (Hitt, Wolf 2004). There are seven known p97 interaction domains: the UBX domain (Ubiquitin Regulatory X), the PUB domain (Peptide:N-glycanase/UBA or UBX-containing protein), the UBX-like domain, the PUL domain (PLAP, UFD3 and Lub1), the Shp box, the VBM domain (VCP binding motif) and the VIM domain (VCP interacting motif) (Allen, Buchberger & Bycroft 2006) (Iyer, Koonin & Aravind 2004) (Hitt, Wolf 2004) (Morreale et al. 2009) (Ballar et al. 2006) (Madsen et al. 2009). The major p97 interaction motif is the Ubiquitin Regulatory X domain (UBX). As p97 is well known to operate in ubiquitin-related functions even though it does not bind ubiquitin itself, the presence of a ubiquitin binding domain is a common characteristic of most p97 adaptors/accessory proteins. The p97 adaptors/accessory proteins are divided into two groups; those that have a UBX domain and those that do not.
1.3.1 Non-UBX Containing Adaptors & Known Functions

While the UBX domain may constitute the major p97 interaction domain, the bulk of the functional work deciphering the specific roles/substrates of p97 adaptors/accessory proteins has been done with the non UBX containing interactors. p97 adaptors can be further divided into two subgroups, adaptors and accessory proteins. Adaptors are required for substrate binding, while accessory proteins may use p97 as a docking site to perform a specific enzymatic function on the already associated substrate. The majority non-UBX p97 interactors have been shown to perform a variety of key roles in mediating ERAD. Arguably the two best studied and most important p97 adaptors, Npl4 (nuclear protein localization 4) and Ufd1 (ubiquitin fusion degradation 1), form a heterodimer crucial for binding p97 at a 1:1 ratio (Meyer et al. 2000). Ufd1 was first discovered in yeast screens of *Saccharomyces cerevisiae* defective in ubiquitin degradation (Johnson et al. 1995). Both Npl4 and Ufd1 contain p97 interaction sites, but have not been shown to bind to p97 exclusively (Meyer, Wang & Warren 2002). They function as essential substrate recruiting factors binding to ubiquitinated substrates at the ER membrane linking p97’s physical conformational change upon ATP hydrolysis to the translocation of these proteins into the cytosol (Kim et al. 2006) (Ye, Meyer & Rapoport 2003) (Bays et al. 2001) (Raasi, Wolf 2007). In addition to ERAD, p97 has been shown to function as an ATP-dependent segregase with the Npl4/Ufd1 heterodimer adaptor. They were shown to separate a polyubiquitinated membrane-tethered transcription factor away from its unmodified partner (Shcherbik, Haines 2007). p97-Npl4/Ufd1 is also strongly suggested to play a role in the removal of Aurora B kinase from chromatin during mitotic exit.
It seems clear that even though p97 adaptors do promote specific functions, they are not limited to a single substrate, as they can act in similar manners as in Npl4/Ufd1, in the nucleus, and at the ER membrane as a segregase and ERAD mediator. p97 interacting proteins consist not only of adaptors, but also accessory proteins that use p97 as a docking site to localize for their specific functions. They are called substrate-processing cofactors. They include the deglycosylase, PNGase I, the E3 ubiquitin ligases Hrd1, gp78, and Ufd2, and the deubiquitinase Ataxin-3. PNGase I contains a PUB domain responsible for maintaining the interaction with p97’s carboxy-terminal tail (Zhao et al. 2007). Functionally, PNGase I serves as an accessory protein, deglycosylating misfolded p97 bound glycoproteins after their translocation out of the ER into the cytosol (Li et al. 2005) (Kim et al. 2006). As PNGase I interacts with the more carboxy terminal regions of p97, this allows for the likely possibility that its interaction with p97 is not exclusive. Other, amino-terminal adaptors may bind to p97 recruiting substrates for PNGase I to act upon. The E3 ubiquitin ligases, Hrd1, gp78 and Ufd2 have all been identified as being tied to substrate ubiquitination in ERAD. Hrd1, as mentioned above, is an integral ER membrane E3 ligase involved in primary ubiquitination of ERAD substrates. Gp78 and Ufd2 bind to p97 and drive the further ubiquitination of the associated substrates through different methods. Gp78 is an ER membrane-resident E3 ligase, while Ufd2 has been shown to act as an E4 ligase, which acts as a catalyst bringing E1, E2 and E3 enzymes together on a smaller ubiquitin chain driving its elongation (Kostova, Tsai & Weissman 2007) (Koegl et al. 1999) (Richly et al. 2005). Counter to the aforementioned E3 ligases, the DUB, Ataxin-3 (AT3) acts to remove ubiquitin chains from p97-bound substrates. Ataxin-3 has been shown to bind to
p97 via its VBM (VCP binding motif) and upon over-expression can compete off the Ufd1 adaptor, however extended studies have called this competition into question (Zhong, Pittman 2006) (Wang, Li & Ye 2006). It is likely that this is only evident upon high over-expression and does not actively regulate p97-Npl4/Ufd1 function in this manner. It is more likely that Ataxin-3 docks onto p97 and removes ubiquitin chains as proteins translocate out of the ER. It is suggested that this is in preparation for feeding substrates into the 26S proteasome (Wang, Li & Ye 2006). HDAC6 (histone deacetylase 6) is a p97 interactor that acts to negatively regulate p97-mediated protein turnover, instead driving the accumulation of ubiquitinated substrates at the aggresome or inclusion bodies, for their later processing by the autophagy pathway. It is believed that HDAC6 acts as a negative regulator of p97 function in protein degradation. It has an incredibly high affinity for ubiquitin, and was shown to prevent ubiquitin chain degradation. Additionally, p97-HDAC6 interaction was lost upon HDAC6’s association with ubiquitin, suggesting that HDAC6 acts in a preventative manner separating ubiquitinated proteins away from p97 (Boyault et al. 2006). Further functional work supports this theory as HDAC6 was shown to rescue aggresome formation defects in IBMPFD mutant cells (Ju et al. 2009). p97-independent studies have shown that HDAC6 is crucial for autophagosome maturation as well (Kawaguchi et al. 2003) (Lee et al. 2010). The roles of Ataxin-3 and HDAC6 as p97 interactors are not completely understood. In general, the UBX-containing adaptors are an even a greater mystery.
1.3.2 UBX Containing Adaptors & Known Functions

Proteins that contain a UBX domain make up the majority of known p97 adaptors. Thus far there are 14 identified UBX containing adaptors (UBXD1, UBXD2, UBXD3, UBXD4, UBXD5, UBXD7, UBXD8, FAF1, SAKS1 ASPL, p37, p47, VCIP135 and YOD1) (Schuberth, Buchberger 2008). This group can then be further divided into two subgroups, those that have a UBA (Ubiquitin Association) domain (UBXD7, UBXD8, FAF1, SAKS1 and p47), and those that do not (UBXD1, UBXD2, UBXD3, UBXD4, UBXD5, ASPL, p37, VCIP135 and YOD1). As previously mentioned, the majority of p97 adaptors have a UBA domain or similar, and this domain is crucial for tying p97 to the ubiquitin pathway. Interestingly, only 5 out of the 14 UBX-containing proteins have such a domain. This creates a very exciting area of research: discovering how these non-UBA containing proteins function as p97 adaptors. The UBX domain is an 80 amino acid region found primarily at the carboxy terminus of eukaryotic p97 adaptor proteins. This domain is structurally very similar to ubiquitin although it does not share any significant sequence homology. The three dimensional structure of the FAF1 UBX domain was solved via NMR spectroscopy. Both the UBX domain and ubiquitin share a common three dimensional β-GRASP fold consisting of a β–β–α–β–α–β secondary structure in addition to other structural elements suggesting an evolutionarily similar origin (Buchberger et al. 2001) (Lo Conte et al. 2000). One major difference between ubiquitin and the UBX domain is that the UBX domain is incapable of forming lysine chains as it lacks a required carboxy-terminal, exposed di-glycine conjugation domain for substrate conjugation. The UBX domain also lacks equivalent or alternative lysines
through which ubiquitin chains are formed: K6, K29, K48 and K63 (Buchberger et al. 2001). The first suggestion of UBX domain function came from work with the well-studied p97 adaptor and UBX containing protein p47/Shp1. It was first identified as a co-precipitant with p97 in rat liver cytosol at a ratio of 3 molecules of p47 per p97 hexamer. Subsequent mapping of the p97 binding site in p47 led to the identification of the UBX domain. Through X-Ray crystallography, this domain was shown to interact with the N domain of p97 via an S3/S4 loop that is highly conserved in UBX proteins, but absent in ubiquitin (Yuan et al. 2001) (Shin et al. 2010) (Dreveny et al. 2004a). p47 was shown to promote p97-mediated, post-mitotic, Golgi Apparatus reformation in an in vitro assay (Kondo et al. 1997). p47 mediates the reassembly of post-mitotic Golgi fragments into Golgi cisternae through its interaction with Syntaxin-5 a SNARE protein important for membrane fusion (Rabouille et al. 1998). p47 maintains a nuclear localization until after interphase, playing a key role in limiting p97’s function to this specific time (Uchiyama et al. 2003). p47 has also been implicated in autophagosome biogenesis through deletional studies in *Saccharomyces cerevisiae* (Krick et al. 2010). Two other UBA-containing adaptors of p97, UBXD7 and UBXD8/ETEA have been implicated in promoting the degradation of specific target proteins, hypoxia-inducible factor 1α (HIF1α) and neurofibromin respectively (Alexandru et al. 2008) (Phan et al. 2010). Evidence suggests that the UBA-containing adaptor FAF1 negatively regulates degradation of ubiquitinated substrates including IκBα (Song et al. 2005). Some of the non-UBA containing members of the UBX family of proteins have been shown to play important roles in ERAD, including UBXD2/Erasin, UBXD4, VCIP135 and YOD1 (Haines 2010). UBXD2 is an ER trans-membrane protein that is responsible for
anchoring p97 during substrate translocation (Neuber et al. 2005) (Schuberth et al. 2004). Through deletion work done in yeast, UBXD4 has been shown to be an important component of the p97 ERAD complex, however, its specific function is unknown (Alberts et al. 2009). p37 is a p47 paralogue that, like p47, is involved in mediating proper post-mitotic ER and Golgi membrane fusion, but lacks a UBA domain. Ubiquitin-binding to p47 is essential for p97/p47 function in Golgi reformation, while ubiquitin plays no known role in p97/p37’s identical function. p37 appears to act in a parallel pathway to p47, interacting with a different membrane-associated SNARE protein, GS15 (Uchiyama et al. 2003). The UBX family also contains p97 accessory proteins involved in substrate processing. VCIP135 and YODI are deubiquitinases involved in the trimming of ubiquitin chains in membrane reassembly and ERAD translocation respectively (Totsukawa et al. 2011) (Ernst et al. 2009). Only a few of the UBX family members have strongly been associated to known p97 functions. As mentioned above (Section 1.2), p97 plays a very important role in a largely diverse group of functions. Most of these functions are ubiquitin-mediated. Interestingly, many of the UBX family of adaptors have no identified ubiquitin-association domain, and no other functional domain to suggest a function. The diversity within the UBX family suggests an array of potential effects on cellular processes and it is of high interest to determine the function of other known p97 adaptors, such as UBXD1.
1.4 Non-UBA-Containing p97 Adaptor: UBXD1

UBXD1 (UBX domain-containing protein 1) or UBXN6, was first discovered in 2001 through the Human Genome Project-EUROIMAGE Consortium. It is a highly conserved protein of 441 amino acids with 80% identity and 87% similarity between human and mouse forms. It is expressed in a wide variety of tissues and is present at higher levels in the brain, more specifically the neuronal cells in the rat (Carim-Todd et al. 2001) (Madsen et al. 2008). The first hint of a function for this protein was discovered through a yeast two-hybrid screen using p97 as bait. Structural studies then identified two conserved regions, the PUB and UBX domains. As mentioned above, the PUB domain has been shown to mediate interaction with p97 in the PUB-containing protein N-glycanase (PNGase I) (Li et al. 2005) (McNeill et al. 2004). Unlike many UBX-containing adaptors, which bind to p97’s amino-terminus, the PUB domain has been shown to bind at the carboxy terminus, specifically, the last 10 amino acid residues of the C domain (Zhao et al. 2007). It has been shown in PUB domain-containing proteins, PNGase I, Ufd3 and UBXD1, that this interaction is abolished upon phosphorylation of p97’s highly conserved penultimate tyrosine Y805 (Zhao et al. 2007) (Madsen et al. 2008). This tyrosine makes up 90% of all p97 post-translational modifications, and has been shown to undergo phosphorylation under various stimuli (Egerton, Samelson 1994) (Ficarro et al. 2003) (Lavoie et al. 2000) (Egerton et al. 1992). The PUB and UBX domains are known to both serve as p97 interaction sites, however the UBX domain in UBXD1 has not been shown to contribute to the p97 interaction. This is due to the absence of a phenylalanine-proline-arginine conserved motif in between β-strands 3 and
within the p97 interaction site (Madsen et al. 2008). In addition to the PUB domain, UBXD1 maintains another, more amino terminal, p97-binding site (within the first 150 amino acid residues). This domain has very recently been identified as a minimal VIM (VCP Interacting Motif) within amino acids 52 to 63 nearly fitting the sequence requirement of R-X$_3$-A-A-X$_2$-R (Kern et al. 2009). UBXD1’s almost complete VIM sequence for binding with p97’s N domain is A-O-M-A-A-A-A-L-A-R; mutation of A58,59 and R62 result in a large reduction of UBXD1-p97 interaction (Stapf et al. 2011).

Functionally, not much is known about UBXD1 until recently. Most of what has been previously hypothesized has been inferred from p97’s primarily studied function in ERAD and research has yet to show a direct and specific function of this unique p97 adaptor. Initial work has suggested that UBXD1 plays a role in ERAD through moderate defects in the clearance of an ERAD substrate, CFTRΔF508 upon UBXD1 over-expression (Nagahama et al. 2009). In addition, UBXD1 was shown to co-precipitate with a known member of the ERAD pathway, the E3 ubiquitin ligase Hrd1, which is one of two main E3’s involved in ubiquitinating ERAD substrates. The authors theorize that this interaction is likely indirect, instead mediated by p97 (Madsen et al. 2008). Recent work, however, has provided some convincing evidence that UBXD1 does play an important role in directing p97’s function, in endolysosomal sorting, not ERAD. As mentioned above (Section 1.2.4.2) UBXD1 and p97, have been implicated to be key mediators in the internalization and post-endocytic trafficking to the lysosome of membrane protein Caveolin-1. This work showed that IBMPFD disease relevant p97 mutants were unable to maintain associations with Caveolin-1 leading to its accumulation in late endosomes (Ritz et al. 2011). UBXD1 is one of a number of relatively unstudied
adaptors/accessory proteins for p97 that although linked to a function, has yet to be clearly delineated. It is of particular interest to study due to its lack of any ubiquitin binding domain, in addition to the unique differences within its UBX domain (lack of phenylalanine-proline-arginine motif essential for p97 interaction). Additionally, it is one of a few adaptors that have been shown to bind both at the N-terminal and C-terminal domains of p97. Work presented here will show detailed interaction work characterizing UBXD1’s interaction with a novel partner, ERGIC-53, that, with functional data, will suggest a novel role in vesicle trafficking.
CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture & Treatments

The following transformed, immortal cell types were used throughout this work. H1299 human non-small cell lung carcinoma cells, U2-OS human osteosarcoma cells, and HEK 293T human embryo kidney cells, transformed by SV40 large T antigen (293T). These cells were routinely cultured in DMEM media supplemented with 10% FBS and 100 µg/ml Penicillin/Streptomycin at 37 °C with 5% CO₂. Transfections were performed with Fugene 6 (Roche) in accordance with the manufacturer’s recommendation. H1299 stably-transfected pSUPER-UBXD13 cells were selected in 300 µg/ml Hygromycin. H1299 and U2OS miR30 stably infected cells were selected and maintained in 200 µg/ml Hygromycin. H1299 pIND cell lines were selected in 300 µg/ml Neomycin, 300 µg/ml Zeocin and 3 µg/ml Puromycin and maintained with Neomycin, 200 µg/ml Zeocin and 0.5 µg/ml Puromycin. For starvation-mediated autophagy induction, cells were washed 2 times in PBS, then incubated in Hank’s Buffered Salt Solution (HBSS) for the required times. Cells were harvested with trypsin which was neutralized with standard media during centrifugation. All cell pellets were washed in Phosphate-Buffered Saline (PBS) prior to freezing and storage at -80 °C.
2.2 Lentiviral Infections

Lentivirus production was performed as follows. 293T cells were seeded at a density of 3x10^6 cells per 10 cm dish. The following day (Day 1) cells were transfected via Fugene 6 (as per product instructions) with 3.5 µg pSlikHygro viral carrier vector in addition to viral packaging and production plasmids: 0.5 µg pCAG4-Ampho (Envelope), 0.5 µg pCAGG-Hivgpco (Gag Pol), and 0.5 µg pCAG4-RTR2 (Rev TAT). One day post-transfection (Day 2) media was removed and replaced with 4 ml complete DMEM (see cell culture 2.1). The next day (Day 3) virus was harvested 3 times (removing virus containing media to a 4 °C cooled conical tube and replacing with 4 ml fresh media) every 5 hours, leaving 4 ml of media on the cells overnight. The virus collection was repeated the following day (Day 4) two additional times, pooling the virus-containing media. For viral infection of target cells: Cells were seeded at a density of 2x10^5 cells per 10 cm dish. The following day, viral supernatant was filtered through a 0.45 µm nylon filter. Media was aspirated and replaced with 4 ml virus/media + 6.25 µg/ml polybrene (direct to plate). New virus/media + polybrene was added to current media every 3 hours for 2 addition infections. Three hours later, media was removed and 4 ml virus/media + polybrene was added for a final 3 hours. Following this last incubation, polybrene was diluted by the addition of 6 ml complete DMEM. The following day, media was replaced with 10 ml fresh complete DMEM. Allowing the cells a day of rest post-infection, they were then put into antibiotic selection (300 µg/ml Hygromycin) or harvested for western blot analysis.
2.3 Plasmids

pcDNA3.1(-)NEO was a kind gift from Dr. Scott Shore. Plasmids: GFP-Ub (11928) and ptfLC3 (21074) were purchased from Addgene. hERGIC-53 expression plasmid, Lma1-pCMV-sport6 (5167524) was purchased from Open Biosystems. Doxycycline-inducible knockdown constructs, pEN_XmiRc2, pEN_TTmiRc2, pSLIKhygro and NS-pSLIKhygro, p97-pEN_XmiR2, and p97-pSLIKhygro were kind gifts from the Deshaies Lab (Cal Tech). In addition, the Deshaies lab provided the UBX adaptor constructs (FLAG)_UBXD1-pCEP, UBXD5FLAG, UBXD6FLAG, UBXD7FLAG, UBXD8FLAG, p47FLAG, AsplIFLAG, FAF1FLAG, and SAKS1FLAG in pCEP plasmid).

The following constructs were created in house: p97 expression constructs (p97FLAG-pCEP, p97FLAGR155H-pCEP, p97FLAGA232E-pCEP), NPL4FLAG-pCEP, and UbHA-pCEP). Full length, carboxy-terminal FLAG tagged UBXD1 was initially cloned using human cDNA as a template. UBXD1 was cloned into the PCEP vector using primers UBXD1FL-F(KpnI) and UBXD1FL-R(BamHI) (Table 2). Internal domain deletion mutants were made through a two step cloning process using the appropriate primers below (UBXD1FL, UBXD1-1, UBXD1-2, UBXD1-3) (Table 2). Amino-terminal deletion mutants were cloned using the UBXD1FL-R (BamHI) primer and the appropriate forward primer. Inducible constructs were subcloned into the pIND expression vector using KpnI and BamHI restriction sites.
**TABLE 2**

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<th>Name</th>
<th>Primer Sequence (5'-3')</th>
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<tr>
<td>UBXD1FL-R(BamHI)</td>
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<tr>
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<tr>
<td>UBXD1-43-F(KpnI)</td>
<td>GGGGTACCCACCATGCCGCCGAGGACC</td>
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Site directed mutagenesis was used to create the UBXD1\textsuperscript{43-53} (Table 3). Briefly, site-directed mutagenesis was performed as follows. Each reaction contained 5 ng of plasmid DNA, 20 mM combined NTPs, 25 µM primers (each), 10X Pfu Buffer and 1 µl Pfu Taq polymerase. PCR was run with the following program: 95 °C for 30 seconds, 55 °C for 1 minute, 68 °C for 8 minutes repeated 20 times, then held at 4 °C. Successful amplifications were cut with DPN I restriction enzyme for 1 hour to cut up template DNA. After digestion, reactions were transformed into XL-2 competent *E. coli*. Clones were picked and sequenced for proper mutation creation. To create the amino-terminal point mutant UBXD1 constructs, site-directed mutagenesis was again utilized with the appropriate primers (Table 3).
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<tr>
<th>Name</th>
<th>Primer Sequence (5’-3’)</th>
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For all in vitro binding reactions, p97 constructs (GST-p97, GST-p97<sup>R155H</sup>, GST-p97<sup>A232E</sup>) were created in house. GST-p97 was subcloned into the pGEX-6P-1 vector using BamHI and NotI restriction sites. Subsequent cloning of point mutants (GST-p97<sup>R155H</sup>, GST-p97<sup>A232E</sup>) were created via site directed mutagenesis (Table 3). UBXD1-pYES2-bbv was cloned via PCR amplification followed by digestion with BamHI and EcoRI restriction sites (Table 2)
Lenti-viral miR-driven knockdown constructs were made by first creating a double stranded DNA insert via primer annealing (100 °C for 10 minutes and allowed to cool to room temperature), then subsequent subcloning into the lentiviral packaging construct, pSLIKHygro using BfuA1 restriction sites (Table 4). Nonsensical shRNA control and p97 shRNA plasmids were obtained from Dr. Ray Deshaies lab at the California Institute of Technology.

**TABLE 4**

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| miR-ERGIC-R         | GGCATTTTACATGGAAGAAATACATCTGTGGCTTCACTATTTCATCATGTCAAAAG
2.4 Preparation of cell lysates

After harvesting, cell pellet was either thawed on ice, or if fresh, washed with 1 ml PBS, then resuspended in appropriate amount of protocol-specific lysis buffer. Routine lysates for western blot were prepared in standard RIPA Buffer (150 mM Sodium Chloride, 1% Nonidet-P-40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate, 50 mM Tris pH 8.0) supplemented with protease inhibitors (1 µg/ml Pepstatin, 1 µg/ml Aprotinin, 1 µg/ml Trypsin Inhibitor, 1 mM Phenylmethanesulfonyle fluoride [PMSF]). Samples were rotated at 4 °C for 30 minutes. Following lysis, insoluble debris was removed by centrifugation at maximum speed at 4 °C and lysates were quantified using a BSA curve / Biorad Protein Assay (Biorad). For immunoprecipitations, cell pellets were lysed as above except in EBC Buffer (50 mM Tris-HCl pH7.5, 120 mM Sodium Chloride, 1% Nonidet-P-40) plus protease inhibitors (1 µg/ml Pepstatin, 1 µg/ml Aprotinin, 1 µg/ml Trypsin Inhibitor, 1 mM Phenylmethanesulfonyle fluoride). Quantified lysates were resuspended in 1X Protein Loading Buffer (50 mM Tris pH 6.8, 2% Sodium dodecyl sulfate, 0.1% bromophenol blue, 10% Glycerol, 5% β-mercaptoethanol). For soluble/insoluble protein fractionation, lysis buffers were as follows: Lysis Buffer I (50 mM Tris-HCl pH 8.8, 100 mM Sodium Chloride, 5 mM Magnesium Chloride, 0.5% Nonidet-P-40 + Protease Inhibitors), Lysis Buffer II (20 mM Tris-HCl pH 8.0, 15 mM Magnesium Chloride, 0.5 mg/ml DNase I + Protease Inhibitors). For IP/MS work cells were lysed in Maltosidase-based Lysis Buffer (50 mM HEPES pH 7.5, 70 mM Potassium Acetate, 5 mM Magnesium Acetate, 0.2% n-dodecyl-B-D-maltoside + protease inhibitors).
2.5 Western blot Analysis

5-15 µg of protein lysate resuspended in 1X Protein Loading Buffer were boiled for 5 minutes, then loaded onto an SDS-PAGE apparatus (Mini-Protean II – Biorad) and run for 2 hours at 100 V in running buffer (250 mM Glycine, 25 mM Tris, 0.1% Sodium dodecyl sulfate). Routinely, 7.5% to 15% gels were used. Following SDS-PAGE the gels were transferred to nitrocellulose membrane (Hybond ECL – Amersham) in methanol-containing transfer buffer (200 mM Glycine, 25 mM Tris, 20% Methanol) for 1 hour at 125 V. Following transfer, membranes were stained with Ponceau S Solution (Sigma). Membranes were then washed in PBS-Tween-20 (PBS-T) (63 mM Na₂HPO₄, 15.5 mM NaH₂PO₄, 7.5 mM Sodium Chloride, 0.1% Tween-20), following blocking in 5% milk in PBS-T for 1 hour at room temperature. Primary antibodies were then added at appropriate dilutions in 5% milk in PBS-T and rocked overnight at 4 °C. Following primary antibody, membranes were washed, then incubated with secondary antibody (at appropriate dilution) in 5% milk in PBS-T for an hour. Membranes were washed then treated with western Lightning Plus – ECL (PerkinElmer) as per manufacturer’s instructions. Chemiluminescence was detected by exposure on X-Ray film (Phenix). For quantification purposes, western blot membranes were visualized using Alpha Innotech FluorChem Q hardware and quantified with the included Alpha View Software (v3.1.1.0) according to manufacturer’s protocols.
2.6 Immunoprecipitations

Fresh cell pellets were lysed in EBC Buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 1% NP-40) plus protease inhibitors (1 µg/ml Pepstatin, 1 µg/ml Aprotinin, 1 µg/ml Trypsin Inhibitor, 1 mM PMSF) for 30 minutes at 4 °C. Cell lysate was extracted from cell debris via centrifugation at maximum speed for 5 minutes at 4 °C and quantified via a BSA curve / Biorad Protein Assay (Biorad). For endogenous immunoprecipitations, 1 mg of lysate was incubated with primary antibody at 4 °C for 3 hours with agitation. Following that, 25 µl of 50% Protein G Sepharose beads (GE Healthcare) were added for 3 additional hours at 4 °C with agitation. For immunoprecipitations using tagged, over-expressed proteins, lysate was incubated with 25 µl of 50% FLAG-tagged M2 Agarose (Sigma) or 25 µl of 50% HA-tagged Agarose (Sigma) at 4 °C for 3 hours with agitation. Beads were washed 3 times in EBC buffer, then resuspended in 40 µl of 1X Protein Loading Buffer.

2.7 Immunofluorescence

Cells were grown to about 40% confluence on #1 glass coverslips in 6 well plates. Cells were washed twice in PBS, then fixed in 4% paraformaldehyde in PBS for 15 minutes. Samples were washed twice in PBS. Samples were then blocked for 1 hour at room temperature in blocking solution (10% FBS, 0.1% Triton X-100 in PBS). After two more washes in PBS, samples were incubated in primary antibody (0.5 ml of 10% FBS in PBS) for 1 hour at 37 °C with gentle rocking. After two more PBS washes, samples were
incubated in secondary antibody (0.5 ml of 10% FBS in PBS) for 45 minutes at 37 °C. For dual-fluorescence experiments samples were treated in tandem with primary antibodies first, then secondary antibodies for the above indicated times. After a final 2 washes in PBS, coverslips were inverted onto glass slides with 1 drop mounting solution (SloFade Gold with Dapi, Invitrogen). They were allowed to cure overnight in the dark.

2.8 Microscopy

Immunofluorescence was analyzed using either an Olympus IX51 inverted fluorescent microscope or a Leica TCS SP5 confocal microscope. For confocal microscopy all scans were created using sequential capture to prevent bleedthrough or cascading fluorescence. Excitation lasers and detection ranges are as follows: DAPI: 405 nm, 415 nm - 476 nm; AlexaFluor488: 488 nm, 500 nm - 658 nm; AlexaFluor 568: 561 nm, 571 nm - 638 nm; AlexaFluor 647: 633 nm, 644 nm – 703 nm. Images were modified and analyzed with either Spot Advanced sofware or LAS AF software. Post-processing of images obtained with LAS AF software consisted of mean baseline correction and medium noise reduction.

2.9 in vitro binding assays

Recombinant GST-tagged protein production was performed as follows. pGEX-6P-1 transformed, log-phase BL21 E. coli was induced with 100 µM IPTG for 3 hours at 37 °C. Cells were lysed in CDC Lysis Buffer (150 mM KCl, 20 mM Hepes (ph7.4), 5 mM
MgCl₂, 1 mM ATP, 5% Glycerol, 0.2% Triton X-100) plus protease inhibitors (1 µg/ml Pepstatin, 1 µg/ml Aprotinin, 1 µg/ml Trypsin Inhibitor, 1 mM PMSF) and 1 mg/ml lysozyme for 1 hour at 4°C. Cleared lysate was incubated overnight at 4 °C with Glutathione 4B beads (GE Healthcare). Following GST-binding, beads were washed 3 times in Binding Buffer (50 mM Tris pH 8.0, 100 mM KCl, 5% glycerol, 1 mM ATP, 2.5 mM MgCl₂). 5 µg of bound proteins were incubated with rabbit reticulocyte lysate-mediated in vitro-translated target proteins following the manufacturer’s protocol (TnT Coupled Reticulocyte Lysate System - Promega). Binding took place in 1 ml Binding Buffer overnight at 4 °C. Samples were then washed 3 times in Binding Buffer (modified with 0.5% Triton X-100), resuspended in 1X Protein Loading Buffer and loaded onto an SDS-PAGE gel. Gels were either stained in Coomassie Stain (BioRad) and destained (40% Methanol, 10% Acetic Acid), or fixed (40% Methanol, 10% Acetic Acid), amplifed (Amplify - GE Healthcare) and vaccuum-dried before exposure to autoradiograph film. For GST-binding with human cell extract, binding of 1 mg of cell lysate and subsequent washes occurred in the EBC immunoprecipitation buffer (see 2.6 Immunoprecipitation). When necessary, recombinant protein was cleaved from Glutathione beads using PreScission Protease (GE Healthcare) in accordance with manufactures protocol in CDC Lysis Buffer.

2.10 Antibodies

Anti-ERGIC-53 (H-245), ERGIC-53 (C-6), Caveolin-1 (N-20), COPG (c-19), Ub (p4d1), SQSTM1/p62 (D-3), and sec23 (E-19) antibodies were purchased from Santa Cruz
Biotechnology. Anti-β-COP (ab2899), pan-cadherin (ab6529), anti-HA (12CA5) and anti-VCP (5) antibodies were purchased from ABCAM. Alexafluor 488 goat anti-mouse, Alexafluor 568 goat anti-rabbit, Alexafluor 546 goat anti-rat and Alexafluor 647 goat anti-mouse antibodies were purchased from Invitrogen. Anti-LC3B (D11), and anti-EEA1 antibodies were purchased from Cell Signalling. Rat anti-HA (3F10) antibody was purchased from Roche. Anti-FLAG (M5) and anti-Actin (20-33) antibodies were obtained from Sigma. Secondary antibodies Mouse TruBlot: ULTRA: Anti-Mouse Ig HRP and Rabbit TrueBlot: Anti-Rabbit IgG HRP were purchased from E-bioscience. Anti-mouse IgG HRP and anti-rabbit IgG HRP antibodies were purchased from Amersham. Anti-UBXD1 antibodies (5C3-1 & 2F8-24) were generated in-house and can be obtained through Advanced Antibodies.
CHAPTER 3

RESULTS

3.1 UBXD1 Antibody Development

This thesis focuses on UBXD1, an understudied adaptor of p97, which previously had no quality antibodies available for its study. A quality antibody will be crucial to assist in defining UBXD1 function. It must be noted that a substantial portion of the antibody quality control testing was done by Poornima Ramkumar. This first section deals with the development of a UBXD1 monoclonal antibody derived from mouse. Recombinant full-length GST-UBXD1 was produced and purified on Glutathione beads, followed by PreScission Protease (GE Healthcare) cleavage as noted (Section 2.9). Lampire Biological Products created subclones of monoclonal antibodies created in mice for quality testing. Two quality antibody clones (5C3-1 and 2F8-24) were identified and evaluated. To determine their ability to detect endogenous UBXD1, H1299 cells were co-transfected with 5 µg of pSUPER-UBXD1-3 (shRNA knockdown construct) or pSUPER-UBXD1-3M (scrambled control) plus 1 µg pBabe-puro (for selection). Cells were selected in 2 µg/ml puromycin and processed for western blot analysis. A clear protein band at approximately 54 kDa can be seen with very little background. In the UBXD1 knockdown samples, this band is clearly diminished, identifying it as the
UBXD1 protein. Both clones were capable of detecting endogenous levels of UBXD1 in cells, however 5C3-1 was stronger (Figure 3A). This result was recapitulated using 5 µg transient transfection of UBXD1-pCEP or pCEP alone over-expression (Figure 3B). The antibodies were also evaluated for their ability to immunoprecipitate endogenous UBXD1. 100 µg of H1299 protein lysate was precleared in protein G-Sepharose followed by incubation with either 250 ng or 500 ng of UBXD1 antibodies. Antibodies were allowed to bind to beads for an addition 4 hours before being washed, resuspended in 1X Protein Loading Buffer and run out on 7.5% SDS-PAGE gels (Figure 3C). An antibody-dose dependent immunoprecipitation is clearly detected again showing 5C3-1 is slightly more efficient. The ability of these antibodies to detect UBXD1 in whole, fixed cells via immunofluorescence would be a key tool in co-localization studies. H1299 cells were transfected as above (Figure 3B) and processed for immunofluorescence using 133 ng/ml of UBXD1 antibody. Cells were examined on an Olympus IX51 inverted fluorescent microscope (Figure 3D). In addition to detection by western blot and immunoprecipitation, both UBXD1 clones efficiently detected over-expressed UBXD1 in immunofluorescent analysis. However, detection of endogenous UBXD1 by immunofluorescence was not possible. These antibodies will be key tools in the further identification of UBXD1 function.
D.

<table>
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Figure 3. **UBXD1 Antibody Development** (A) 5 µg of pSuper-UBXD1 knockdown construct or empty plasmid were co-transfected with 1 µg pBabe-puro into U2OS cells and selected in 2 µg/ml puromycin. 15 µg of lysates were run out on SDS-PAGE gels and either stained with Ponceau S or analyzed by western blot with UBXD1 antibodies: 5C3-1 and 2F8-24. (B) H1299 cells were transfected with the indicated amounts of UBXD1-pCEP or empty pCEP. Cells were treated as before and blots were stained with the antibodies indicated. (C) 100 µg of cell extract was precleared with protein-G sepharose beads, followed by incubation at 4 °C for 4 hours in 250 ng or 500 ng of antibody. This was followed by incubation with protein-G sepharose beads for an additional 2 hours. Beads were washed and samples were run on SDS-PAGE gels and blotted with the antibodies indicated. (D) H1299 cells were transiently transfected with 5 µg UBXD1-pCEP and processed for immunofluorescence as stated in Section 2.7, using a primary antibody concentration of 133 ng/ml and anti-mouse AlexaFluor 488 secondary antibody (Green) with DAPI staining the nucleus (Blue).
3.2 Binding of UBXD1 to p97 and ubiquitinated proteins

Given p97’s prevalence in various ubiquitin pathways, any work done with a new potential adaptor or accessory protein should be assessed for its ability to interact with ubiquitin in comparison to a well studied adaptor. As mentioned in Section 1.3.1, Npl4 is a well-studied p97 adaptor involved in binding ubiquitinated substrates with its heterodimeric partner Ufd1 and docking it with the p97 hexamer at the ER membrane (Bays et al. 2001). Npl4 relies on its binding partner to maintain a strong interaction with p97 (Meyer, Wang & Warren 2002). Immunoprecipitation experiments with transiently transfected over-expressed UBXD1 and Npl4 were done to assess their differences in interacting with ubiquitin and p97. 500 ng of HA-ubiquitin was co-transfected with increasing amounts of UBXD1\textsuperscript{FLAG} or Npl4\textsuperscript{FLAG} (100 ng, 300 ng or 1 µg). Relatively equal amounts of protein over-expression were seen (Figure 4A). Anti-FLAG immunoprecipitations were performed incubating 25 µl of 50% anti-FLAG conjugated agarose beads with 500 µg of cell protein lysate in RIPA Buffer. Beads were washed in RIPA Buffer and boiled in 1X Protein Loading Buffer and examined by western blot analysis (Figure 4B). Clear differences can be seen between these two adaptors. UBXD1 was shown to strongly bind to p97, at higher levels than Npl4, which is not surprising as its association partner Ufd1 is likely limiting at endogenous levels. Additionally, Npl4 is capable of immunoprecipitating ubiquitin in a dose-dependent manner, while UBXD1 binds weakly to ubiquitin; this interaction does not increase with UBXD1 over-expression. This strongly suggests that, as expected, UBXD1 does not directly associate with ubiquitin. However, it is possible that this interaction is mediated through an
unidentified cofactor that is limiting at endogenous levels. Also, low level ubiquitin pulldown could be attributed to another adaptor on the p97 hexamer.
A. Inputs

- Anti-FLAG
- Anti-HA (Ubiquitin)
- Anti-p97

B. Anti-Flag Immunoprecipitations
Figure 4. Binding of UBXD1 to p97 and ubiquitinated proteins (A) H1299 cells were co-transfected with HA-Ub (500 ng) and increasing amounts of UBXD1\(^{\text{FLAG}}\) or Npl4\(^{\text{FLAG}}\) (100 ng, 300 ng or 1 µg). Lysates in RIPA buffer were run on a 7.5% SDS-PAGE gel and blotted with the antibodies indicated. (B) 500 µg of cell lysate was incubated with 25 µl 50% FLAG-tagged agarose beads in RIPA buffer at 4C overnight. Beads were washed, and resuspended in 1X Protein Loading Buffer and proteins were separated on 7.5% SDS-PAGE gels and probed with the antibodies indicated.
### 3.3 Highly over-expressed UBXD1 is ubiquitinated and insoluble

Upon increased expression of full length UBXD1 and its mutants, a corresponding decrease in Ubiquitin\textsuperscript{HA} can be seen (Figure 4A). Knowing that these samples were transfected with identical amounts of Ubiquitin\textsuperscript{HA}, and that all transfections in the experiment were done with an equal amount of DNA; it was hypothesized that a result of UBXD1 over-expression is the trafficking of ubiquitinated substrates to an insoluble compartment in the cell. As the buffer used was a common lysis buffer (RIPA), it was believed that this insoluble, ubiquitinated material was separated out with cellular debris and other insoluble material during cell lysis. To further examine this outcome, a simple cellular fractionation was performed involving a two-step lysis to analyze both the soluble and insoluble cell fractions. H1299 cells were transiently transfected with 500 ng of Ubiquitin\textsuperscript{HA} in combination with increasing amounts of full length UBXD1\textsuperscript{FLAG} or UBXD1\textsuperscript{PUB} (100 ng, 300 ng, 1 µg). Cells were lysed in Lysis Buffer I for 15 minutes at 4 °C. Lysis reactions were centrifuged at maximum for 15 minutes at 4 °C. The supernatant was collected (containing the soluble fraction). The remaining pellet was further processed in Lysis Buffer II for one hour at 37 °C. The sample was mixed well and quantified as described in section 2.4. Both samples were resuspended in 1X Protein Loading Buffer and processed for western blot analysis. A clear trend is seen between the amount of UBXD1 transfected into cells, and the movement of Ubiquitin\textsuperscript{HA}-modified proteins from the soluble to the insoluble fraction. It was also observed that the ubiquitin smear representing multiple proteins’ polyubiquitination ran differently in cells transfected with the full length UBXD1 construct, versus the smaller UBXD1\textsuperscript{PUB} mutant.
Figure 5A. It appeared that the ubiquitin banding began around the size of the transfected UBXD1 protein. This suggested that the increase in insoluble ubiquitinated proteins may not be an array of insoluble-prone target proteins, but ubiquitinated UBXD1 itself. To determine if this was indeed the case, a denatured immunoprecipitation was performed. By denaturing protein complexes prior to immunoprecipitation with an antibody against transfected ubiquitin (anti-HA antibody), all non-covalent protein-protein interactions would be severed. Only proteins modified by the covalent addition of ubiquitin\textsuperscript{HA} moieties should be precipitated with the HA antibody. Western blot analysis of UBXD1 should show uncharacteristic bands of higher molecular weight representing multi-ubiquitinated UBXD1 protein. H1299 cells were transfected with 500 ng Ubiquitin\textsuperscript{HA} and 1 µg UBXD1\textsuperscript{FLAG}. Cells were lysed in complete EBC buffer, and quantified. 1 mg of cell lysate was diluted up to 200 µl in 1X Protein Loading Buffer (minus Bromophenol Blue) and boiled for 5 minutes. Denatured proteins were then diluted with EBC buffer to 1 ml. 25 µl of 50% anti-HA conjugated agarose beads were added, and the immunoprecipitation proceeded as in Section 2.6. Immunoprecipitations were run out on a 7.5% SDS-PAGE gel and blotted with anti-HA and anti-FLAG antibodies. UBXD1 is pulled down with high affinity by the HA antibody (Figure 5B). UBXD1 displays a typical banding pattern for poly-ubiquitination, while blotting the immunopurified material with an antibody against transfected ubiquitin (anti-HA) detected multi-ubiquitin banding beginning at the molecular weight mono-ubiquitinated UBXD1 would run. These results suggest that UBXD1 is not driving ubiquitinated substrates to the insoluble fraction as a functional adaptor of p97. Instead, upon high levels of over-expression, it is recognized by the ubiquitin machinery as a substrate, gets poly-
ubiquitinated and accumulates in an insoluble section of the cell. This is likely to explain the immunoprecipitation of Ubiquitin by UBXD1 seen in Section 3.2.
A. **Lysate Fractionation**

![Lysate Fractionation Diagram]

B. **Denatured IP**

![Denatured IP Diagram]
Figure 5. Over-expressed UBXD1 is ubiquitinated and insoluble (A) H1299 cells were transfected with 500 ng of Ubiquitin^HA and varying amounts of UBXD1^FLAG or UBXD1^PUB (100 ng, 300 ng, 1 µg). Cell pellets were lysed in Lysis Buffer I to isolate the soluble fraction, followed by Lysis Buffer II for the insoluble fraction. Lysates were run on a 7.5% SDS-PAGE gel and analyzed with the noted antibodies. (B) H1299 cells were transfected with 500 ng Ubiquitin^HA and 1 µg UBXD1^FLAG. Cell lysate was denatured followed by immunoprecipitation with HA antibody-conjugated agarose beads and immunoblotted with the identified antibodies.
### 3.4 UBXD1 displays decreased binding to IBMPFD mutant p97\textsuperscript{R155H}

The p97 adaptor, UBXD1 is widely expressed with enhanced expression in neuronal cells of the brain. Considering that p97 is mutated in neurodegenerative diseases, it is worthwhile to test if UBXD1 is deficient at binding p97 mutants found in human disease. Initial work characterized UBXD1’s interaction with the most common IBMPFD p97 mutant R155H. H1299 cells were co-transfected with 3 µg of UBXD1\textsuperscript{HA} and 500 ng of p97\textsuperscript{FLAG} or p97R155H\textsuperscript{FLAG}. Cells were lysed in EBC buffer, and 1 mg of protein was incubated with 25 µl of 50% anti-FLAG conjugated agarose beads. Post washing, samples were diluted in 1X Protein Loading Buffer and processed for western blot analysis probing with anti-FLAG and anti-HA antibodies. The p97\textsuperscript{R155H} mutant is largely deficient in its ability to immunoprecipitate UBXD1 (Figure 6A). These data were confirmed in an \textit{in vitro} binding reaction to more clearly evaluate this interaction in a cell free system. 10 µg of recombinant protein (\textsuperscript{GST}p97, \textsuperscript{GST}p97\textsuperscript{R155H} and GST alone) was incubated with 5 µl of rabbit reticulocyte lysate-mediated, S\textsuperscript{35}-labelled, \textit{in vitro} translated UBXD1 (Described in Section 2.9). Samples were washed, resuspended in 1X Protein Loading Buffer and run on SDS-PAGE gels. Gels were either fixed and stained with Coomassie (to show GST proteins) or fixed, amplified, dried and exposed to autoradiograph film overnight (to examine S\textsuperscript{35} labelled UBXD1). Recominant p97\textsuperscript{R155H} displays an inability to pull-down UBXD1 at wild-type p97 levels (Figure 6B). This clearly shows that a disease relevant IBMPFD p97 mutant is defective in associating with the adaptor UBXD1. This could potentially indicate a disease-relevant role for UBXD1.
A. **Inputs**

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**Anti-FLAG IPs**

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- Anti-FLAG (p97)
- Anti-HA (UBXD1)

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- Anti-FLAG (p97)
- Anti-HA (UBXD1)

B. GST WT R155H

25 KDa
-125 KDa
-100 KDa

Coomassie

-UBXD1

Autoradiography

**Figure 6.** UBXD1 displays decreased binding to IBMPFD mutant p97\textsuperscript{R155H} (A) H1299 cells were co-transfected with 3 µg of UBXD1\textsuperscript{HA} and 500 ng of p97\textsuperscript{FLAG} or p97\textsuperscript{R155H}\textsuperscript{FLAG}, and processed for anti-FLAG immunoprecipitation. (B) 10 µg of recombinant protein (GST, p97, p97\textsuperscript{R155H}) was incubated with 5 µl rabbit reticulocyte in vitro translated UBXD1. Samples were washed, and run on SDS-PAGE gels. Gels were either stained with Coomassie and dried, or fixed, amplified and exposed to autoradiograph film overnight.
3.5 p97 adaptors interact differentially with IBMPFD-relevant p97 mutant

$p97^{R155H}$

It was determined that UBXD1 shows decreased affinity for IBMPFD mutant $p97^{R155H}$. It was of immediate interest to examine an array of other adaptors in the UBX family to determine if they too showed decreased binding to $p97^{R155H}$. The UBX-containing adaptors of p97 can be divided into two groups: those that have a ubiquitin association domain (UBA) and those that do not (Schuberth, Buchberger 2008). As p97 function has repeatedly and strongly been linked to ubiquitinated substrates through such a large group of adaptors (UBX-containing and others), it is of a high interest to determine how non-UBA containing adaptors work. Surprisingly, very little work has been done to assess any potential differences in adaptor binding with IBMPFD mutant p97. It has been shown that ubiquitin-binding adaptors, p47, Npl4 and Ufd1 have an increased association with transfected IBMPFD point mutants R95G, R155C, R155H, R155P, R191Q and A232E. This increase in binding was shown to be unrelated to the level of p97 ATPase activity, using a T761E phospho-mimic mutant of p97, that was shown to have nearly double p97 wild-type ATPase function (Mori-Konya et al. 2009). While the ATPase function increased, there was no subsequent increase in the tested adaptor association (Manno et al. 2010). This work was confirmed and extended using the R95G, R155H and ATPase dead mutant E305A. Again, adaptors p47, Npl4 and Ufd1 showed a stronger association with the R95G and R155H IBMPFD mutants, with no effect in binding with the E305A mutant. This information was extended to include three additional p97 interactors (PNGase I, Hrd1 and Ataxin-3). Non-ubiquitin binding adaptor PNGase I
and the E3 ligase Hrd1 showed equal affinity for mutant and wild-type p97. Interestingly, the p97-associated DUB, Ataxin-3 showed increased association with all tested p97 mutants over wild-type (Fernandez-Saiz, Buchberger 2010). The authors recapitulated these results in R155H, R155C and R155S IBMPFD patient-derived myoblasts. It was merely concluded, that “IBMPFD-causing mutant p97 proteins exhibit imbalanced co-factor associations” without any hypothesis as to why this occurs (Fernandez-Saiz, Buchberger 2010). Therefore, it was important to assess potential differential interactions between p97 adaptors (specifically, UBX containing) and disease relevant p97 mutants. These interactions were examined utilizing FLAG-tagged UBX-containing adaptors already on hand, with the recombinant p97 proteins utilized in Figure 6. To this end, 293T cells were transiently transfected with 3 µg FLAG-tagged UBX/UBA containing adaptors (UBXD7, UBXD8, FAF1 and SAKS1) or FLAG-tagged UBX only containing adaptors (UBXD1, UBXD2, UBXD5 and Aspl1). Cells were lysed in EBC Buffer and 1 mg of cell lysate was incubated with recombinant, purified, p97WT, in addition to the most common IBMPFD mutant, p97R155H, and GST-tag alone, bound to Glutathione beads. After overnight incubation at 4 °C, beads were washed and boiled in 1X Protein Loading Buffer. Samples were run out on 7.5% SDS-PAGE gels, and either Coomassie stained or processed for western blot analysis and probed with anti-FLAG antibody. All of the UBX/UBA-containing adaptors tested showed a very significant increased association with p97R155H when compared to wild-type (Figure 7A). For quantification purposes, western blot membranes and Coomassie stained gels were visualized using Alpha Innotech FluorChem Q hardware and quantified with the included Alpha View Software (v3.1.1.0) according to manufacturer’s protocols. Quantification
was normalized per adaptor, to the amount pulled down with p97WT (Figure 7B). Although the amounts being pulled down differ between adaptors, a clear preference in UBA adaptors for mutant p97R155H can be seen, as high as 42 fold over wild-type p97 in the case of UBXD7. In contrast to what was seen with adaptors with ubiquitin association domains, UBX adaptors without identified ubiquitin association domains, showed no preference for p97R155H protein over p97WT (Figure 7C). Quantifying results as before show an inverse effect with UBXD1 and UBXD2. A clear decrease in association with mutant p97 can be seen in the pulldown with p97R155H. A much less dramatic effect is seen with UBXD5 and ASPL1 adaptors (Figure 7D). It is highly likely that the UBA domain is mediating an increased interaction with the recombinant GST-p97 mutants. The UBA domain has not been recognized as a p97-interaction motif. It is possible that these preferential interactions are through the UBA domain’s capacity to bind ubiquitinated substrates. Mutant p97 may be misfolded, and subsequently ubiquitinated. UBA–containing proteins may bind it as misfolded substrate, and not as an adaptor. GST-bound p97 and the p97R155H mutant, in addition to p97A232E (the IBMPFD mutant with the most severe phenotype) were incubated in 1 mg of 293T lysate as before and purified. Extracts were either stained with Coomassie to visualize p97 pulldown, or analyzed via western blot using anti-ubiquitin antibody (Figure 7E). Clearly, mutant p97 is highly ubiquitinated in comparison to the low level ubiquitination seen in wild-type p97. Using recombinant proteins created in E. coli, this ubiquitination must be occurring in the binding reaction, followed by its association with the UBA domains of the UBX/UBA adaptors tested in Figure 7A. Ubiquitination is an ATP-dependent process; eliminating ATP from the binding buffer should strongly decrease
GST-p97 mutant ubiquitination. This was shown to be the case (Figure 7F). In this system, p97\textsuperscript{R155H} is ubiquitinated in 293T cell lysates and preferentially associates with UBA-containing adaptors. Collectively, these data raise the possibility that any previously defined affinities for UBA-containing adaptors, is likely due to the recognition of said mutants by the ubiquitin machinery as misfolded proteins, and not due to a specific mutation-derived preference for adaptors. The most functionally significant discovery is the decreased association between the non-UBA adaptors, UBXD1 and UBXD2 with IBMPFD mutants. This could be disease relevant and important to further explore. UBXD2 is relatively well characterized, while very little is known about UBXD1’s function (Neuber et al. 2005) (Schuberth et al. 2004). Recently published data support our conclusions. It was shown that ubiquitin-binding adaptors Npl4, Ufd1 and p47 preferentially associate with p97 IBMPFD mutants, while non UBA containing adaptor, UBXD1 shows a strong decrease in association when compared to wild-type (Ritz et al. 2011). UBXD1 was recently shown to have decreased binding capacity for p97\textsuperscript{R155H} over wild-type p97, confirming our results. Additionally, p97\textsuperscript{A232E} and p97\textsuperscript{R95G} exhibited decreased binding to UBXD1, while the ATPase deficient substrate trapping mutant, p97\textsuperscript{E578Q} did not (Hayer et al. 2010).
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B.  

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- Anti-FLAG
C. Inputs

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-D Hannity

D.

![Bar graph showing fold decrease to p97 WT]
E.

![Image of gel electrophoresis with bands labeled GST, p97 WT, p97 R155H, p97 A232E. Bands are stained with Ponceau S and anti-Ub antibodies.](image)

F.

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- Anti-p97
- Anti-HA (Ubiquitin)
Figure 7. p97 adaptors interact differentially with IBMPFD-relevant p97 mutant p97\textsuperscript{R155H} (A) 293T cells were transiently transfected with 3µg of the UBA-containing adaptors UBXD7, UBXD8, FAF1 & SAKS1. 1mg of cell lysate in EBC Buffer was incubated with 10 µg of Glutathione-bound recombinant p97\textsuperscript{GST}, p97R155H\textsuperscript{GST} or GST tag alone, overnight. Samples were washed and run out on a 10% SDS-PAGE gel and processed for Coomassie staining and Anti-FLAG western blot. (B) Bands were quantified using Alpha Innotech (Alpha View) software and normalized for GST protein input and graphed according to fold increase over p97-WT. (C) 1 mg of the non-UBA-containing adaptors, UBXD1, UBXD2, UBXD5 & ASPLI were assayed for interaction as in 7A. (D) Bands were quantified as mentioned above 7B. (E) 10 µg of Glutathione-bound, recombinant \textsuperscript{GST}p97, \textsuperscript{GST}p97R155H and \textsuperscript{GST}p97A232E were incubated overnight in 293T cell lysate. Samples were washed and run out on a 7.5% SDS-PAGE gel and processed for Coomassie staining and anti-HA (Ubiquitin) (F) 10 µg of Glutathione-bound recombinant \textsuperscript{GST}p97, \textsuperscript{GST}p97R155H and \textsuperscript{GST}p97A232E were incubated overnight in 293T cell lysate +/- 1 mM ATP. Samples were processed as above. In addition to binding reactions, an equivalent amount of unincubated GST proteins were run as an input.
3.6 The pIND system allows for controlled expression of UBXD1

UBXD1 appears to be a highly regulated protein that is quickly and somewhat uniquely dealt with upon over-expression at levels common for transient transfection experiments. Why UBXD1 appears to undergo ubiquitination upon routinely used levels and almost uniquely traffics to an insoluble fraction of the cell is unknown. It presents a large challenge when studying pathways of protein trafficking, ubiquitination and degradation. To more clearly examine its function, a tightly regulated method of stably over-expressing UBXD1 and its mutants, at lower, sustainable levels in a time-dependent manner is required. An ecdysome inducible system was chosen for this approach. It takes advantage of the heterodimerization of two different receptors upon ligand presentation leading to a tightly controlled, drug-induced gene expression. Cells are stably transfected with a plasmid containing both components of the heterodimer: the chimeric ecdysone receptor fused to a VP16 activation domain (VgECR) and the retinoid X receptor (RXR). Upon administration of ligand (ponasterone A) these receptors dimerize and are now capable of binding to a synthetic response element (5xE/GRE) on the stably transfected reporter/expression plasmid leading to transcription initiation of the target gene (Wakita, McCormick & Tetsu 2001). This system provides a very low background as the mammalian hormone receptors cannot recognize the synthetic response element. H1299 cells were first transfected with pVgRXR plasmid and stably selected with 300 µg/ml zeocin. This lead to an expression of the reporter construct by approximately ten fold. To enhance expression, a second round of transfections/selections were performed co-transfecting the pVgRXR plasmid with
pBabe-puro. Cells were selected in 0.5 µg/ml puromycin. These combined transfections lead to a ponasterone A–induced expression of up to about 46 fold (Data not shown).

UBXD1 constructs: full length UBXD1, UBXD1^{PUB}, and UBXD1^{UBX} were cloned into the pIND reporter/expression vector and stably transfected into the 46X pVgRXXR H1299 cells followed by selection in 300 µg/ml Neomycin. Time course test inductions were performed by treating the cells with 5 µM ponasterone A; adding new ponasterone A containing media daily. Samples were harvested and processed for western blot analysis. Protein extracts were run out on 7.5% SDS-PAGE gels and blotted for UBXD1 and Actin (Figure 8A). Uninduced cells clearly show little background expression; a testament to the value of this system. Target protein expression can be seen as early as 8 hours, increasing up to 48 hours. In the case of the UBXD1^{PUB} and UBXD1^{PUB/UBX} expressing cell lines, protein levels maximized at around 8 hours. The ability to help track UBXD1’s localization in the cell, in addition to being able to evaluate potential co-localization with novel interactors, would be a useful tool in defining UBXD1’s function. However, given the problems working with transfected UBXD1 (ubiquitination and trafficking to the insoluble cell fraction), this ponasterone A inducible system may be more amenable to tracking UBXD1. To ensure that induced UBXD1 expression does not lead to a ubiquitinated artifact, co-localization with a ubiquitin marker (GFP-Ub) is important to examine. H1299 inducible cells lines (pIND vector and full length UBXD1^{FLAG}) were transiently transfected with 5 µg of GFP-tagged Ubiquitin plasmid (GFP-Ub). 24 hours post-transfection, cells were split to a density of 1.0x10^5 per well in a 6 well plate containing a glass coverslip. The following day, cells were treated with 5 µM ponasterone A for 48 hours (as above). Cells were processed for
immunofluorescence using anti-UBXD1 and AlexaFluor647 secondary antibody and analyzed on a confocal microscope. Upon induction, UBXD1 displays a strong cytoplasmic localization with some concentrated cytoplasmic speckling and perimembrane localization. This will be expanded on in Section 3.11. Little to no co-localization can be seen with ubiquitin (Figure 8B). This confirms that the inducible system can be used to express a more reasonable level of UBXD1 and its mutants, allowing accurate study of its functions and localization within cells while avoiding the tight cellular regulation seen in transient transfection experiments leading to its ubiquitination and subsequent trafficking to an insoluble fraction.
A.

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- Anti-UBXD1
- Anti-Actin

B.

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Figure 8. The pIND system allows for controlled expression of UBXD1 (A) H1299 cells stably transfected with two rounds of pVgRXR plasmid and pIND expression plasmid with empty vector, full length UBXD1, UBXD1^{PUB}, UBXD1^{UBX} or UBXD1^{PUB/UBX} were induced with 5 μM ponasterone A for 0, 8, 24 and 48 hours refreshing with new ponasterone A-containing media every 24 hours. Cells were lysed and run on 7.5% SDS-PAGE gel and blotted for UBXD1 and actin. (B) H1299 inducible cell lines were transiently transfected with 5 μg GFP-Ubiquitin, followed by plating on glass coverslips in 6 well plates at a density of 1x10^5 cells/well, induced for 48 hours, then processed for UBXD1/AlexaFluor 647 (Red), GFP-Ubiquitin (Green) and DAPI (Blue) immunofluorescence. They were all visualized with a 40X lense and 2X digital zoom.
3.7 The identification of a unique UBXD1 interactor: ERGIC-53

UBXD1 has a suggested function in ERAD, and recently has been shown to help mediate endosomal trafficking of mono-ubiquitinated Caveolin-1. No direct interacting proteins have been discovered for UBXD1, with the exception of p97. Immunoprecipitation followed by Mass Spectrometry (IP/MS) is a common method for studying unknown proteins in an attempt to find novel and interesting protein interactions. Previously, this involved the excision of Coomassie-stained bands visualized after immunoprecipitation and SDS-PAGE. Currently, cutting edge methods of IP/MS are used that eliminate the requirement for visualization of potential protein bands, but instead rely on solution-based separation prior to mass spectrometry. This method is referred to solution-based Immunoprecipitation/Mass Spectrometry. Briefly, immunopurified protein samples are eluted from beads, and digested into individual peptides. These peptides are loaded onto a column that separates peptide bands based on their hydrophobicity, then are immediately sprayed into a linear ion trap spectrometer for identification. A primary scan assigns peptides a mass to charge ratio. Following that, the strongest peptide signals are further fragmented in a second scan. The data are then fed into various bioinformatics databases to identify immunoprecipitated proteins (Graumann et al. 2004).

To this end, Dr. Dale Haines utilized the ponasterone A inducible system to discover new interacting partners for UBXD1 at The California Institute of Technology in the lab of Dr. Ray Deshaies. Low-level ponasterone A-driven UBXD1 induction was initiated in H1299 cells (0.1 µM ponasterone A) and empty vector control cells. Cells were harvested and lysed in a Maltoside-based Lysis Buffer and 1 mg of cell lysate was
incubated with FLAG-conjugated beads. Post-washing, bound proteins were eluted through treatment with 10 M urea. Eluant was digested for 4 hours with first 0.1 µg Lys-C then with 0.5 µg Trypsin. The samples were desalted, lyophilized and resuspended in 0.2% formic acid. UBXD1\textsuperscript{FLAG} and vector control samples were then analyzed via Mass Spectroscopy (Table 5). The number of spectral hits correlates to the amount of protein peptides identified by MS. UBXD1 precipitated multiple proteins, the most prominent being p97 (serves as a positive control) and ERGIC-53. This experiment was repeated using a higher level of UBXD1 induction (0.3 µM ponasterone A). Again UBXD1 precipitated numerous proteins including p97 and ERGIC-53 with the highest number of spectral hits (Table 6). A final experiment was done using transient transfection of UBXD1\textsuperscript{FLAG} into 293T cells to drive over-expression to a very high level (Table 7). The other proteins identified with UBXD1 may potentially be of importance, but most are not reproduced in all three experiments and they have relatively few spectral hits in comparison to p97 and ERGIC-53. However, it is interesting to note that no ubiquitin pathway components were identified in any of the three UBXD1 experiments. This supports a ubiquitin-independent role of UBXD1/p97 in cells. Additionally, Caveolin-1 (CAV1) was identified with 5 spectral hits in the 0.3 µM ponasterone A experiment (Table 6). This confirms published work identifying Caveolin-1 as a UBXD1 interactor, although not an incredibly strong one when compared to the detection of ERGIC-53 (Ritz et al. 2011). ERGIC-53 (ER-Golgi intermediate compartment) also known as Lman1 (lectin mannose-binding protein 1) was first identified as a component of a tubulovesicular compartment also known as vesicular tubular clusters (VTCs) near the cis Golgi. It has also been shown to traffic to the cell periphery upon temperature shift,
which, together with the VTC localization, constitute a cytoplasmic compartment termed the ERGIC (Schweizer et al. 1990) (Schweizer et al. 1988) (Klumperman et al. 1998). In yeast, this protein was then shown to be involved in bi-directional trafficking between the ER and Golgi, and subsequently named ERGIC-53 (Schweizer et al. 1990). It is a 510 amino acid, single pass transmembrane protein shown to be a key member of the early secretory pathway. ERGIC-53 is the namesake and only marker for an intermediate compartment through which ER exported proteins traffic on the way to the Golgi for further processing and export. In the ER, it is concentrated in budding structures at transitional elements (Klumperman et al. 1998). ERGIC-53, with its soluble luminal substrate recruitment factor, MCDF2 (Multiple coagulation factor deficiency protein 2) is responsible for mediating the ER-export and trafficking of multiple glycoproteins including lysosomal proteases cathepsin Z, and cathepsin C, in addition to blood coagulation factors V and VIII, and a serine protease inhibitor, α1-antitrypsin (Appenzeller et al. 1999) (Vollenweider et al. 1998) (Nichols et al. 1998) (Nyfeler et al. 2008) (Nyfeler et al. 2006). In cells it forms either a homodimer or homohexamer, through disulfide linkages within its Stalk domain (Schweizer et al. 1988) (Lahtinen, Svensson & Pettersson 1999). In addition to its Stalk domain, ERGIC-53 has an amino-terminal signal sequence, S (key for ER translocation), a Carbohydrate Recognition Domain, CRD (responsible for mediating cargo binding), a small transmembrane domain, TMD, and a short (12 amino acids) cytoplasmic tail, that is crucial for recognition by cytoplasmic coatamer proteins which mediate proper membrane budding, vesicle trafficking and localization (Hauri et al. 2000) (Nufer et al. 2002) (Nufer et al. 2003) (Figure 9A). This 12 amino acid stretch contains two crucial elements for proper
ERGIC-53 function, a di-Lysine ER retention motif and a di-Phenylalanine ER exit determinant (Jackson, Nilsson & Peterson 1990) (Nufer et al. 2002). The cytosolic di-Phenylalanine motif serves as a recognition signal for the recruitment of a multiprotein complex collectively termed the COPII coatamer. Upon COPII coatamer recruitment to the ER exit site (which contain concentrated levels of ERGIC-53), COPII coatamer complexes assemble polymerizing the membrane and driving its budding out from the ER (Barlowe 2002). Once out of the ER, the COPII coated vesicle traffics to the ERGIC and dissasembles. These now naked vesicles fuse to the Golgi to deliver their cargo. Now ERGIC-53 is free to recycle back to the ER. This is mediated by another multiprotein complex closely related to COPII, the COPI coatamer, which recognizes and binds to ERGIC-53 di-Lysine ER retention motif and drives its retrograde transport and fusion with the ER (Hauri et al. 2000). Following the initial identification of ERGIC-53 as a UBXD1 interactor, it was essential to determine if this association was unique among p97 adaptors, primarily UBX family members. To this end, various FLAG-tagged p97 adaptors were evaluated for their ability to immunoprecipitate ERGIC-53. 5 µg of empty Vector, amino and carboxyl terminal tagged UBXD1, and carboxy terminal tagged UBXD5, UBXD6, UBXD7, UBXD8, p47, Npl4, Aspl1, Faf1 and Saks1 were transfected into H1299 cells and processed for FLAG-tagged immunoprecipitation (Figure 9B). As seen in lane 3 UBXD1^{FLAG} is the only adaptor tested that will immunoprecipitate ERGIC-53. Interestingly, only the carboxy-terminal FLAG-tagged UBXD1 pulled down ERGIC-53, while both constructs maintained an interaction with p97. Somehow an amino-terminal FLAG tag on UBXD1 blocks the ERGIC-53 interaction, suggesting the extreme amino terminus of UBXD1 is important for this interaction.
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**Tables 5-7. Mass-Spectroscopy Results**  
**Table 5.** Summary of UBXD1-induced (0.1 µM ponasterone A) H1299 cells followed by solution-based MS (see above). The top 3 proteins identified were: p97, UBXD1 and ERGIC-53.  
**Table 6.** Summary of UBXD1-induced (0.3 µM ponasterone A) H1299 cells followed by solution-based MS (see above). These results confirm a UBXD1-dose dependent immunoprecipitation of p97 and ERGIC-53.  
**Table 7.** Summary of transiently transfected UBXD1\(^\text{FLAG}\) (5 µg) in 293T cells followed by solution-based MS (see above). High level expression of UBXD1\(^\text{FLAG}\) further confirms p97 and ERGIC-53 immunoprecipitation.
A.

B. 

Inputs

1 2 3 4 5 6 7 8 9 10 11 12

-Anti-FLAG

-Anti-ERGIC-53

-Anti-p97

Recycling Signal

Export Signal

RSQQEAAAKKFF
C. **Immunoprecipitations**

Figure 9. **The identification of a unique UBXD1 interactor: ERGIC-53** (A) Linear structure of hERGIC-53. S: Signal Sequence, CRD: Carbohydrate Recognition Domain, Stalk: oligomerization domain, TMD: Transmembrane Domain. Cytoplasmic domain: amino acids 497-510 (B) An array of FLAG-tagged p97 adaptors (1: Vector, 2: UBXD1-NH₂, 3: UBXD1-COOH, 4: UBXD5, 5: UBXD6 6: UBXD7, 7: UBXD8, 8: p47, 9: Npl4, 10: Aspl1, 11: Faf1, 12: Saks1) were transfected (5 µg) into H1299 cells, lysed and run on 7.5% SDS-PAGE gel and probed with the noted antibodies. (C) Lysates were immunopurified using anti-FLAG antibody-conjugated beads and run on a 7.5% SDS-PAGE gel and probed with the noted antibodies.
3.8 UBXD1 amino-terminus is crucial for interactions with p97 and ERGIC-53

A differential ERGIC-53 interaction can be seen between two UBXD1 constructs; an amino-terminal FLAG tagged UBXD1 does not immunoprecipitate ERGIC-53, while a UBXD1 construct FLAG tagged on its carboxy end does (Figure 9C). As proteins were expressed at equal levels, and neither tag effected the p97 interaction, this strongly suggests that placing an amino terminal tag on UBXD1 blocks its ability to interact with ERGIC-53. This immediately directed further binding-site analysis to the extreme N-terminus of UBXD1. In an attempt to narrow down the UBXD1-ERGIC-53 binding site, stepwise deletions were made roughly every ten amino acids at UBXD1’s N-terminus up until Methionine 54 using carboxy terminal FLAG-tagged UBXD1 (Figure 10A). H1299 cells were transfected with 5 µg of empty vector or the indicated UBXD1-pCEP construct and processed for anti-FLAG immunoprecipitation. All constructs expressed at near identical levels. Lysates were processed and FLAG tagged proteins and interactors were immunoprecipitated as previously described. Samples were analyzed by western blot analysis for UBXD1, endogenous ERGIC-53 and endogenous p97 (Figure 10B). ERGIC-53 immunoprecipitated only with full length UBXD1 confirming the hypothesis that the extreme amino terminus of UBXD1 mediates this interaction. However, p97 immunoprecipitated at levels equal to full length UBXD1 with all mutants except UBXD153, which lacks the first 53 amino acids. These results suggest that the ERGIC-53 interaction is not mediated by p97 or through the same interaction site. UBXD1 is either responsible for linking ERGIC-53 and p97 in a novel complex, or functions in two independent complexes, one with ERGIC-53 and one with p97.
Figure 10. **UBXD1 amino-terminus is crucial for interactions with p97 and ERGIC-53**

(A) Schematic of UBXD1 and the sequential deletion mutants used. (B) H1299 cells were transfected with 5 µg of the indicated constructs and processed for anti-FLAG immunoprecipitation. 1 mg of lysate in EBC Buffer was bound overnight to 25 µl of 50% anti-FLAG conjugated agarose. Beads were washed and boiled in 1X Protein Loading Buffer prior to SDS-PAGE analysis and blotting with the indicated antibodies.
3.9 Defining amino acids of UBXD1 required for ERGIC-53 binding

The extreme amino terminus of UBXD1 (within the first 10 amino acids) was shown to be responsible for interacting with ERGIC-53. Although this interaction could be mediated through multiple contact points within UBXD1’s secondary structure, it may be possible to identify specific residues within this small region that are responsible. UBXD1’s amino terminus is as follows: methionine (start codon), 2 lysine residues (Basic), 2 phenylalanines (hydrophobic, aromatic), glutamine (hydrophilic, nonpolar), glutamic acid (negatively charged), a phenylalanine (hydrophobic, aromatic), lysine (hydrophobic, aromatic) and an alanine (inactive hydrophobic) (Figure 11A). As many of these residues can become charged in a pH neutral environment or contain relatively large side chains, alanine scanning, or sequentially mutating amino acids to alanine residues, should help define which amino acids are important for this interaction. Site directed mutagenesis was performed as referenced in Section 2.3. Since amino acid 10 is already an alanine, it was not changed. H1299 cells were transiently transfected with 5 µg of empty vector, full length UBXD1, UBXD1Δ10 or the above mentioned point mutations. Relatively equal expression levels were achieved (Figure 11B). Cells were lysed and processed for anti-FLAG immunoprecipitations as above and blotted for UBXD1, ERGIC-53 and p97. As shown in Section 3.8, mutations in the first ten amino acids of UBXD1 have no effect on p97 binding. However, numerous amino acids are involved in the UBXD1-ERGIC-53 interaction. K2A, F4A, F5A, F8A mutants completely lost the ability to interact with ERGIC-53. E7A also had a large decrease in binding ERGIC-53. Of the amino acids that effected UBXD1-ERGIC-53 interaction, one is basic (lysine), three are hydrophobic
(phenylalanine) and one is negatively charged (glutamic acid). Although not as accurate as X-ray Crystallography, molecular modelling programs are useful tools for predicting a protein’s 3D secondary structure. I-Tasser is an online program from the University of Michigan that uses a comparison between query amino acid sequences and those in a protein databank with sequence similarity. Protein folds, or super-secondary structures are assembled and the query protein is reassembled in its lowest energy state. This method may not be suitable for large proteins with incredibly novel sequences, but modelling the first 50 amino acids of UBXD1 could help indicate why these specific amino acid residues are important. The first ten amino acids are predicted for form an alpha helix (with high probability) (Figure 11B). The phenylalanine residues, F4, F5 and F8 are predicted to be in the internal face of the alpha helix, as shown by solvent accessibility (lower the score, more buried the amino acid). This creates a putative binding pocket for ERGIC-53. Interaction work has been done showing the cytosolic 12 amino acid residues of ERGIC-53 to be required for UBXD1 interaction (unpublished data, Dane Kyle MS thesis). It is therefore likely that ERGIC-53’s small cytosolic tail interacts in this putative binding pocket structured by the hydrophobic amino acids F4, F5 and F8.
A. MKKFFQEFK

B. Inputs

C. Anti-FLAG Immunoprecipitations
D.

Figure 11. Defining amino acids of UBXD1 required for ERGIC-53 binding (A)

Schematic of UBXD1 and the first 9 amino acids. (B) H1299 cells were transfected with pCEP vector, carboxy FLAG tagged full length UBXD1, UBXD1<sup>Δ10</sup>, or UBXD1<sup>K2A</sup>, UBXD1<sup>K3A</sup>, UBXD1<sup>F4A</sup>, UBXD1<sup>F5A</sup>, UBXD1<sup>Q6A</sup>, UBXD1<sup>E7A</sup>, UBXD1<sup>F8A</sup> or UBXD1<sup>K9A</sup>. (C) Anti-FLAG immunoprecipitations were performed with 1 mg cell lysate in EBC Buffer and 25 μl of 50% FLAG-conjugated agarose, followed by western blot analysis with the antibodies indicated. (D) Molecular modelling of the first 50 amino acids of UBXD1 by I-Tasser. Alpha helical secondary structure is indicated in the first 10 amino acids. The strength of prediction is indicated by a 0-9 scale, 9 being highly likely. Solvent accessibility (an indication of amino acid exposure) is indicated by a 0-9 scale, 9 being highly exposed.
3.10 p97 specifically interacts with βCOPI of the COP I Coatamer complex

ERGIC-53 is a single pass transmembrane protein with a short 12 amino acid tail protruding into the cytoplasm. This tail is responsible for directing the trafficking of cargo proteins within ERGIC-53 positive vesicles. ERGIC-53 is known to mediate the coating of its vesicles with either COPI or COPII coatamer complexes through direct interaction with primary coatamer proteins βCOPI and Sec23/Sec24 (Tisdale et al. 1997) (Jensen, Schekman 2011). These two multi-protein complexes recognize different amino acids on the ERGIC-53 tail and are responsible for assembling a polymerized coat that directs the vesicle to a variety of subcellular localizations including the ER, ERGIC, Golgi and endosomes (Aridor et al. 1995) (Hauri et al. 2000) (Nufer et al. 2003) (Klumperman et al. 1998) (Aniento et al. 1996) (Figure 12). The COPII coatamer complex is involved in the export of glycoproteins from the ER and trafficking to the ERGIC and possibly the cis-Golgi (Sztul, Lupashin 2009). The COPII coatamer consists of 5 proteins: the coatamer initiating protein Sar1, the inner core proteins (heterodimers of Sec23 and Sec24) and the outer proteins (heterotetramers of Sec13 and Sec31) (Jensen, Schekman 2011). Sar1, a small GTPase is first recruited to ER exit sites (home to its guanine nucleotide exchange factor Sec12) and is activated upon GTP binding. Sec23/Sec24 homodimers are then recruited to ER exit sites through the concerted binding of Sar1 and membrane cargo receptor (di-Phenylalanine motif of ERGIC-53) by Sec23 and Sec24 respectively. Subsequent recruitment and assembly of the outer core members Sec13 and Sec31 lead to the full, polymerized COPII coatamer (Jensen, Schekman 2011). COPII coated vesicles traffic to the ERGIC or the cis-Golgi apparatus.
and undergo rapid uncoating and fusion, delivering their cargos (Aridor et al. 1995) (Sztul, Lupashin 2009) (Behnia et al. 2007). While the COPII coatamer has only been indicated in ER exit, the COPI coatamer is involved in a more complicated array of trafficking events. The core COPI coatamer consists of 7 proteins: α−, β−, β′−, γ−, δ−, ε−, ζ−COP (Malhotra et al. 1989). The first step in COPI coatamer assembly closely parallels the COPII complex. Multiple isoforms of the small GTPase, ARF (ARF1, ARF4 and ARF5) are activated when bound to GTP, which is mediated by a guanine nucleotide exchange factor protein (Popoff et al. 2011). This exposes an amino-terminally bound myristic acid, which tethers ARF to the membrane (Palmer et al. 1993) (Goldberg 1998). Maintaining the COPI coat is completely reliant upon active ARF-GTP (Sun et al. 2007). The protective COPI coat is quickly disassembled upon GTP hydrolysis by ARF catalyzed by Arf1 GTPase-activating proteins (ArfGAPs) (Malsam et al. 1999) (Tanigawa et al. 1993). Activated ARF immediately leads to the recruitment of the coatamer and direct interaction with βCOP and γCOP and subsequent binding to εCOP and δCOP (Sun et al. 2007) (Lowe, Kreis 1996). COPI coatamer binds to the di-Lysine ER retention motif of ERGIC-53 and members of the related p23/p24 family of membrane proteins mediating their retrograde transport back to the Endoplasmic Reticulum (Cosson, Letourneur 1994). While COPII coatmers mediate ER export of glycoprotein cargo bound to ERGIC-53, COPI coatmers have been implicated in directing vesicles to the Golgi, back to the ER and as a protective coat in the fusion of early endosomes (Schekman, Mellman 1997) (Letourneur et al. 1994) (Razi, Chan & Tooze 2009) (Gu et al. 1997). As COPI and COPII coatmers interact with the carboxy tail of ERGIC-53 and are crucial for ERGIC-53 positive vesicle formation and
trafficking, it would be worthwhile to examine whether any of the ERGIC-53 binding components involved were present in UBXD1/p97/ERGIC-53 complexes. Although transfected UBXD1 has been shown to have solubility issues, it is still possible to use it as a means for evaluating protein-protein interactions; a large percentage of transfected UBXD1 does appear to be localized to the soluble fraction. H1299 cells were transiently transfected with 5 µg of empty pCEP vector, full length UBXD1, UBXD1Δ53 or UBXD1Δ10. Cells were lysed in EBC buffer and 1 mg of protein was incubated in 25 µl of 50% anti-FLAG conjugated beads. Beads were washed and resuspended in 25 µl of 1X Protein Loading Buffer. Immunoprecipitated proteins were processed for western blot analysis using antibodies against UBXD1, ERGIC-53, p97, βCOPI, γCOPI, and Sec23 (Figure 13A). Interestingly, full length UBXD1 immunoprecipitated only one of the three COP proteins tested, βCOPI. The p97-binding deficient UBXD1 deletion, UBXD1Δ53 also lost its ability to interact with βCOPI, while the ERGIC-53 binding mutant, UBXD1Δ10 was still able to immunoprecipitate βCOPI. This suggests that either βCOPI and p97 bind to a similar site on UBXD1, or instead, βCOPI is immunoprecipitating with UBXD1 through direct interaction with p97. It was necessary to evaluate whether UBXD1 contributed at all to a p97-βCOPI interaction. To this end, Dane Kyle MS induced UBXD1 knockdown in the H1299 mir-30 UBXD1 cell line with 1 µg/ml Doxycycline for 96 hours (system to be described in detail in Section 3.15). Cells were then transiently transfected with 5 µg of empty pCEP vector or pCEP-p97FLAG. Cells were lysed in EBC Buffer and immunoprecipitated with anti-FLAG conjugated agarose beads, as above. Washed beads were processed for western blot analysis, blotting with the antibodies indicated. p97 maintains its ability to
immunoprecipitate βCOPI with near complete depletion of UBXD1 by shRNA, indicating that the p97-βCOPI interaction is completely independent of UBXD1.
Figure 12. Schematic representation of ERGIC-53/coatomer vesicle trafficking.

Taken from Hauri et al. 2000. ERGIC-53 cytoplasmic tail is crucial for recognition by COPII coatomer proteins mediating its budding from the ER and subsequent trafficking to the ERGIC-53. This vesicle is then uncoated and either traffics onward to the cis-Golgi delivering its cargo, or takes on a COPI coatomer coat mediating its recycling back to the ER or trafficking into the endosomal system (not pictured).
A. Inputs | IP's
--- | ---
v | wt | Δ53 | Δ10 | v | wt | Δ53 | Δ10 | UBXD1^{FLAG}

- Anti-UBXD1

- Anti-ERGIC-53

- Anti-p97

- Anti-βCOPI

- Anti-γCOPI

- Anti-Sec23 (COPII)

B. Inputs | IP's
--- | ---
Control | anti-UBXD1 shRNA | Control | anti-UBXD1 shRNA
V p97^{FL} | V p97^{FL} | V p97^{FL} | V p97^{FL}

- Anti-FLAG

- Anti-p97

- Anti-βCOPI

- Anti-βCOPI

- Anti-UBXD1
Figure 13.  p97 specifically interacts with βCOPI of the COP I Coatomer complex

(A) H1299 cells were transfected with either 5 µg of pCEP vector control, full length UBXD1, UBXD1\textsuperscript{53} or UBXD1\textsuperscript{10} and lysed in EBC buffer. 1 mg of protein was immunoprecipitated in 25 µl of 50% anti-FLAG conjugated agarose, washed in EBC buffer and ran out on SDS-PAGE gels. Western blots were probed with the antibodies indicated. (B) miR-30 UBXD1 H1299 cells were induced for knockdown with 1 µg/ml Doxycycline for 96 hours, followed by transient transfection with 5 µg of pCEP vector (V) or pCEP-p97\textsuperscript{FLAG} (p97\textsuperscript{FL}). 1mg of lysates were immunoprecipitated with 25 µl of 50% anti-FLAG conjugated agarose, washed in EBC buffer, run out on SDS-PAGE gels and blotted with the antibodies indicated.
3.11 **Upon ponasterone A-induced expression, UBXD1 forms large occlusions in the cytoplasm and along the plasma membrane**

UBXD1 has been recognized as having a diffuse cytoplasmic localization with some localization to the nucleus (Madsen et al. 2008) (Nagahama et al. 2009) (Ramkumar et al. 2009). These experiments were done using transfected UBXD1, and as seen above, this level of over-expression may have unwanted effects. It was necessary to use the ecdysone inducible system to more carefully evaluate UBXD1’s localization pattern. Because of the inducible nature of this system, it is also possible to evaluate any trafficking and accumulation over time. H1299 ponasterone A inducible cell lines were split onto glass coverslips in 6 well plates at a density of 1.0x10⁵ cells per well. They were also split into 6 cm dishes at a density of 3x10⁵ cells per dish. Both sets were induced with 5 µM ponasterone A for up to 48 hours. Cells on 6 cm dishes were harvested and lysed in RIPA Buffer. Protein extracts were run out on 7.5% SDS-PAGE gels and blotted for UBXD1 and Actin (Figure 14A). Full-length UBXD1 does indeed display a strong cytoplasmic staining which can be visualized by 8 hours post-induction. Beginning at 24 hours, small cytoplasmic speckles can be seen. Interestingly, these vesicles appear to fuse into rather large cytoplasmic aggregates by 48 hours. Full length UBXD1 can also be seen localizing along the plasma membrane within the same cells. As these cells were visualized by confocal microscopy, it was necessary to take separate images first focusing on the nucleus to best show a cross-cut of the cell for membrane localization. The focus was then adjusted -1.26 µm until clear cytoplasmic occlusions could be visualized within the same cell. As shown previously, these strong cytoplasmic
occlusions do not appear to contain ubiquitin as UBXD1 did not colocalize with GFP-Ub (Figure 8).
A. Immunofluorescence

Vector  |  UBXD1<sup>FL</sup>
---------|------------------
 0  |  8  |  24  |  48  |  0  |  8  |  24  |  48  | Time (hrs.)

- Anti-UBXD1

- Anti-Actin

B. Immunofluorescence

DAPI  |  UBXD1
---------|------------------
UBXD1  |  T<sub>8</sub>

UBXD1  |  T<sub>24</sub>

UBXD1  |  T<sub>48</sub>

DAPI  |  UBXD1<sup>V</sup>
---------|------------------
UBXD1  |  T<sub>8</sub>

UBXD1  |  T<sub>24</sub>

UBXD1  |  T<sub>48</sub>

-1.26 µm
Figure 14. Upon ponasterone A-induced expression, UBXD1 forms large occlusions in the cytoplasm and along the plasma membrane (A) H1299 cells stably transfected with two rounds of pVgRXR plasmid and pIND expression plasmid with empty vector, and full length UBXD1 were induced with 5 µM ponasterone A for 0, 8, 24 and 48 hours refreshing with new ponasterone A-containing media every 24 hours. Cells were lysed and run on 7.5% SDS-PAGE gel and blotted for UBXD1 and actin. (B) H1299 cells were plated on glass coverslips and induced with 5 µM ponasterone A for 0, 8, 24 and 48 hours refreshing with new ponasterone A-containing media every 24 hours. Cells were processed for immunofluorescence using anti-UBXD1 antibody and Alexafluor 488 secondary antibody (green), and mounted with Slowfade Gold with DAPI (blue). As these cells were visualized via confocal microscopy, it was necessary to take 2 fields of view. Cross cutting the nucleus to best show membrane localization, then adjusting -1.26 µm downward to best shown cytoplasmic vesicles. Both rows of Figure 14B are of the same field using the 40X optical lens and 2.5X digital zoom.
3.12 UBXD1 directs the localization of ERGIC-53 positive vesicles

ERGIC-53 is localized to tubulovesicular clusters close to the Golgi Apparatus and has been seen in yeast to traffic to the cellular periphery, marking an intermediate compartment that plays a key role in protein trafficking between the ER and the Golgi, termed the ERGIC. As discussed above, ERGIC-53 positive vesicles are coated with either COPII or COPI coatomers depending on their destination, be it trafficking to the ERGIC, trafficking back to the ER or trafficking onward to the Golgi or endosomes. Ultrastructural analysis of ER export and trafficking of the glycoprotein of the vesicular stomatitis virus (VSV-G) showed ERGIC vesicles colocalizing with COPI, COPII or both coatomers, as seen by βCOPI and Sec13 markers respectively (Hauri et al. 2000) (Aridor et al. 1995) (Scales, Pepperkok & Kreis 1997). As shown above in Figure 13, UBXD1 is involved in only ERGIC-53/COPI coatomer protein βCOPI containing complexes. As UBXD1 induced over-expression forms large cytoplasmic aggregates (Figure 14), and ERGIC-53 positive vesicles are functionally involved in ER/ERGIC-53/Golgi/Endosome fusion events, it was necessary to determine if UBXD1 effected ERGIC-53 localization (Hauri et al. 2000) (Scales, Pepperkok & Kreis 1997). H1299, ponasterone-A inducible lines (empty vector, wild-type UBXD1, UBXD1Δ10, UBXD1PUB, and UBXD1Δ53) were split into 6 well plates containing a glass coverslip at a density of 1x10^5 cells per well. For protein analysis, they were plated on 6 cm dishes at a density of 3x10^5 cells per plate as well. The following day they treated with 5 µM ponasterone A and refreshed at 24 hours. Cells were processed in parallel for western blot analysis and UBXD1/ERGIC-53 co-immunofluorescence (Figure 15A & B). Fluorescent signals were produced using
AlexaFluor 568 anti-rabbit secondary antibody for ERGIC-53 (Red) and Alexafluor 647 anti-mouse secondary antibody for UBXD1 (Green), in addition to DAPI staining, staining the nucleus (Blue). In the absence of UBXD1 over-expression, endogenous ERGIC-53 maintains a strong perinuclear tubulovesicular localization (Figure 15B). Upon full length UBXD1 over-expression, ERGIC-53 appears to be sequestered away within the UBXD1 positive cytoplasmic occlusions. These localizations are spread throughout the cell, and appear to also be located near the plasma membrane. This seems to be a phenotype unique to full length UBXD1, as UBXD1 mutants without ERGIC-53 interaction domains (UBXD1\(^{10}\)), p97 interaction domains (UBXD1\(^{PUB}\)), or both (UBXD1\(^{53}\)) show no noticeable effect on ERGIC-53 localization.
A. Vector

- UBXD1 FL
- UBXD1 ΔL0
- UBXD1 ΔUB
- UBXD1 Δ53

PonA

-Anti-UBXD1

-Anti-Actin

B. Vector

DAPI  UBXD1  ERGIC-53  MERGE

Pon -

Pon +
Figure 15. UBXD1 directs the localization of ERGIC-53 positive vesicles

(A) H1299 ponasterone-inducible lines were plated in 6 cm dishes at a density of $3 \times 10^5$ and treated with ponasterone A as described above for 48 hours. Cells were the processed for western blot analysis and probed with the indicated antibodies. (B) Cells were also plated on coverslips in 6 well plates at a density of $1 \times 10^5$ cells per well, treated with 5 µM ponasterone A for 48 hours and processed for UBXD1/ERGIC-53 co-immunofluorescence. UBXD1 row 3 was further zoomed to 6X as seen in UBXD1 row 4. All images were taken using the 40X optical lense and 2.5X digital zoom unless otherwise noted. (Vector row 2, 2.0X, UBXD1 row 4, 6X, UBXD1Δ10, 2.0X)
3.13 UBXD1 co-localizes with Caveolin-1 at the membrane, but not in the cytoplasm

Recent work has identified a very specific role for UBXD1-p97 in the internalization of mono-ubiquitinated Caveolin-1 and subsequent trafficking to early endosomes for degradation. The UBXD1-p97 complex was shown to interact with mature, oligomeric Caveolin-1, post Golgi exit, at or near the plasma membrane (Ritz et al. 2011). Interestingly, the authors only saw very minor cytoplasmic co-localizations between over-expressed UBXD1-GFP and Caveolin-1-mCherry. Immunofluorescent co-localization was examined in H1299 ponasterone A inducible cells for full length UBXD1 and endogenous Caveolin-1. Cells were plated and induced as previously described. Cells were harvested for western blot analysis for UBXD1 over-expression (Figure 16A). Cells grown on coverslips were processed for immunofluorescence as described using UBXD1 primary antibody with anti-mouse AlexaFluor 647 and Caveolin-1 primary antibody with anti-rabbit AlexaFluor 568. Caveolin-1 is primarily localized at the plasma membrane, with some diffuse staining in the cytoplasm. As shown previously, UBXD1 localizes in large cytoplasmic aggregates as well as at or near the plasma membrane (Figure 14). Upon co-staining, UBXD1 and Caveolin-1 co-localize at the plasma membrane (Figure 16B). However, Caveolin-1 is completely absent from UBXD1 cytoplasmic occlusions (Figure 16B rows 3 & 4). It appears that UBXD1 does co-localize strongly with Caveolin-1 at the plasma membrane, where it likely acts with p97 in its internalization. However, UBXD1 does not appear to interact with Caveolin-1 upon its internalization.
A.

Vector Vector UBXD1 FL UBXD1 FL
- + - + PonA

-Anti-UBXD1

-Anti-Actin

B.

DAPI UBXD1 Caveolin-1 MERGE

Pon -

Pon +

Pon +

Pon +
Figure 16. UBXD1 co-localizes with Caveolin-1 at the membrane, but not in the cytoplasm (A) H1299 ponasterone-inducible lines were plated in 6 cm dishes at a density of $3 \times 10^5$ and treated with ponasterone A as described above for 48 hours. Cells were the processed for western blot analysis as previously described and probed with the indicated antibodies. (B) Cells were also plated in 6 well plates on coverslips at a density of $1 \times 10^5$ cells/well. After treatment with 5 µM ponasterone A for 48 hours cells were processed for immunofluorescence with antibodies against UBXD1 (Green) and Caveolin-1 (Red), with anti-mouse AlexaFluor 647 and anti-rabbit AlexaFluor 568 anti-mouse antibodies. All images were taken using the 40X optical lense and 2.5X digital zoom, except row 4, which used 6X digital zoom to better visualize the lack of co-localization within the cytoplasm.
3.14 **UBXD1 complex components (UBXD1, ERGIC-53 and p97) show a strong effect in basal autophagy progression**

One of the hallmarks of IBMPFD disease is an accumulation of protein aggregates in the cell cytoplasm (Watts et al. 2004). This accumulation has been attributed to a p97-mediated defect in proper autophagy progression. p97 mutation leads to the accumulation of immature autophagosomes that do not undergo appropriate lysosomal fusion. p62 recognizes ubiquitinated autophagy substrates and traffics them to the forming autophagosome. Upon proper fusion with the lysosome, p62 is degraded as a secondary effect of autophagosome processing. p62 serves as a marker for autophagy and its accumulation is an indication of a block in proper progression. In work done with IBMPFD-relevant p97 mutant constructs, as well as work done in mouse embryonic fibroblasts with siRNA-mediated knockdown of p97, p62 is seen to accumulate approximately 2 fold over control vectors (Tresse et al. 2010). Interestingly, this blockage in p62 is only seen in basal autophagy. Basal autophagy is the constant autophagic processing and clearance of misfolded proteins to prevent their accumulation within the cell. Autophagy can also be induced when cells are placed in an energy-limiting state. When cells are starved of nutrients, they will induce autophagy and engulf the cytosol and intracellular components for degradation in an attempt to reclaim deficient nutrients. This is also termed macroautophagy (Lemasters 2005). This type of autophagy does not involve specific ubiquitinated targets; it is likely to proceed in a different manner than substrate-induced autophagy. Therefore, it is not surprising that p97 loss or mutation shows no negative effect on starvation-induced autophagy (Tresse et
Given the potential role of UBXD1 and ERGIC-53, within the context of p97 function, in vesicle trafficking and autophagosomal maturation, it was very important to assess these proteins’ role in autophagy alongside p97. As p97 constitutes approximately 1% of all cytosolic protein, an efficient system of shRNA-mediated knockdown was essential to study autophagic induction. A microRNA-based short hairpin RNA system capable of regulated induction via Doxycycline treatment would allow for highly efficient knockdown in a controlled time frame. This system utilizes microRNA (miR) hairpin regions of the naturally occurring miR30, allowing for more efficient shRNA processing by RNA Polymerase II (Stegmeier et al. 2005) (Zhu et al. 2007). These miR30 flanking sequences are placed under the control of a Tetracycline inducible transcription factor (Tet-ON), that when treated with Doxycycline (Tetracycline family member), transcription of the target sequence is induced (Shin et al. 2006) (Figure 17). Lenti-virus infected, stable cell lines were created as described in Section 2.2, with constructs containing a non-silencing sequence (negative control), UBXD1 shRNA, p97 shRNA or ERGIC-53 shRNA in both H1299 and U2OS cell lines. Knockdown was induced for 4 days with 1 µg/ml Doxycycline in U2OS cells, refreshing media every 24 hours. Following 96 hour treatment (optimal time for knockdown), cells were washed and placed in Hank’s Buffered Salt Solution (HBSS) for 8 and 24 hours to initiate starvation-induced autophagy. Cells were harvested in serum-containing media and lysed for western blot analysis. UBXD1 and ERGIC-53 knockdown constructs reduced protein levels by reasonable amounts, while the p97 knockdown was less effective likely due to the large amounts of p97 protein in the cell (~1% cytosolic protein) (Figure 18A). Functionally, p62 accumulated in p97 knockdown cells to similar levels seen in published
work (2.7 fold versus 2 fold) which linked p97 to autophagosome maturation (Figure 18A, p97 T₀) (Quantified in Figure 18B) (Tresse et al. 2010). Both UBXD1 and ERGIC-53 knockdown also lead to the accumulation of p62 (5.4 fold and 3.2 fold respectively) marking an arrest in autophagy progression. Although this experiment has been repeated multiple times this quantification datum, only represents this one experiment presented in Figure 18A. Since UBXD1 is likely acting as an adaptor linking ERGIC-53 to p97, it is not surprising that its knockdown leads to the most dramatic effect. As shown previously, p97 is suggested to not be involved in starvation-induced autophagy. In conditions where p97 is lost, or IBMPFD mutants are over-expressed, autophagy proceeds normally when driven by nutrient deprivation. When miR30 shRNA knockdown cells are then placed in HBSS, p62 levels are seen to decrease by 8 hours. By 24 hours p62 cannot be detected (Figure 18B). UBXD1 and ERGIC-53, like p97 are important for the progression of basal autophagy, but do not seem to play a role in starvation-induced autophagy. These results were somewhat reiterated in H1299 cells. Upon knockdown of p97 or ERGIC-53, p62 accumulates. Upon starvation, p62 is also degraded, albeit much slower than in U2OS cells (Figure 18C). For unknown reasons, UBXD1 knockdown in H1299 cells does not appear to elicit the same level of response as seen in U2OS cells. Starvation-induced autophagy induction is likely to differ between cell types. Both of these experiments have repeated multiple times, but quantitiation in Figure 16B, only represent this one experiment. Another marker of defective autophagy progression is the accumulation of LC3-II (the highly lipophilic phosphatidylethanolamine-conjugated form present in autophagosome membranes). Again, H1299-mir30 stable lines were treated as above except they were starved for 2
and 4 hours (LC3-II has a shorter half-life than p62). In all three knockdown lines, LC3-II is seen to accumulate to higher levels than control, again degrading upon starvation-induced autophagy (Figure 18D).

Since work in IBMPFD patients has indicated that p97 mutation leads to a block in autophagy, marked by the accumulation of immature autophagosomes, it is also worthwhile to assess whether UBXD1 plays a role here as well (Tresse et al. 2010). As mentioned in Section 1.2.1, LC3 is an autophagosome membrane protein that serves as a well-studied marker of both autophagosome vesicle localization and autophagy progression. Early fluorescence work using a fluorescent protein tagged LC3 showed a loss in GFP signal upon fusion of the autophagosome to the lysosome, while the RFP tagged version did not. It was determined that upon fusion with the highly acidic lysosome the GFP tag is protonated and the signal is lost, while the RFP signal is much more stable in the lysosomal environment. Using this unwanted side-effect, a novel tandem GFP/RFP tagged LC3 (ptfLC3) is used to show the progression of autophagy pre to post fusion with the lysosome (Kimura et al. 2009) (Kimura, Noda & Yoshimori 2008) (Kimura, Noda & Yoshimori 2007). H1299 Ponasterone-A inducible full-length UBXD1 cells were transiently transfected with 5 µg of ptfLC3 plasmid. 24 hours later they were split onto 6 cm dishes and 6 well plates with glass coverslips at densities of 3x10^5 and 1x10^5 cells per well, respectively. The following day UBXD1 expression was induced with 5 µM ponasterone A for 48 hours. 6 cm dishes were then harvested for western blot analysis (Figure 18E). The cells grown on coverslips were processed for immunofluorescence as previously described using anti-UBXD1 primary antibody and
anti-mouse AlexaFluor 647 secondary antibody and analysed on a confocal microscope. In the absence of UBXD1 expression, ptfLC3 showed a largely diffuse cytoplasmic localization with some concentrated speckling often occurring at the aggresome (single perinuclear spot). Although different intensities, the RFP and GFP signals largely colocalized. The ptfLC3 construct also shows clear nuclear staining, possibly an artifact of over-expression. Upon over-expression of UBXD1, cells lose their diffuse ptfLC3 localization, and mainly localize to larger speckles. Interestingly, these speckles are disparate from the larger UBXD1 cytoplasmic occlusions seen previously. Since these cells were transiently transfected with ptfLC3 and as the inducible system does not lead to 100% UBXD1 over-expression, it is possible to compare the effect of UBXD1 on LC3 localization within one field (Figure 18F row 3). It appears that UBXD1 plays a positive role in promoting immature autophagosome formation, but does not localize to these vesicles. It cannot be ruled out, however, that UBXD1 over-expression may have an effect in squelching ptfLC3 expression, thereby lowering its cytoplasmic levels.

Together, these data indicate that UBXD1, ERGIC-53 and p97 all play an important role in driving basal autophagy.
Figure 17. Schematic of miR30-based shRNA knockdown system. Gene-specific short hairpin RNA’s are subcloned between to miR30 flanking sequences and placed under the control of a Reverse tetracycline transactivator (rTA3). Upon Doxycycline treatment, the Tetracycline-responsive element (TRE) lead to target transcription. These transcribed hairpins are processed via RNA Polymerase II preceeding degradation of target mRNA by RISC (RNA-induced Silencing Complex).
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Starvation (hrs.)

Dox

-Anti-p97
-Anti-UBXD1
-Anti-ERGIC-53
-Anti-p62
-Anti-BCOP1
-Anti-Actin

B.

Graph showing ALU normalized to non-sense T0. The x-axis represents Starvation post Dox Treatment (hrs.), and the y-axis represents ALU normalized to Non-sense T0. The bars indicate the expression levels over time.
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Starvation (hrs.)

Dox

- Anti-p97
- Anti-UBXD1
- Anti-ERGIC-53
- Anti-p62
- Anti-BCOP1

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Starvation (hrs.)

Dox

- Anti-p97
- Anti-UBXD1
- Anti-ERGIC-53
- Anti-LC3
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Figure 18. **UBXD1 complex components (UBXD1, ERGIC-53 and p97) show a strong effect in basal autophagy progression** (A) Stably infected miR30-shRNA U2OS cells were treated with 1 μg/ml Doxycycline for 4 days. Cells were then put into HBSS starvation media for 0, 8 or 24 hours and harvested for western blot analysis, and blotted with the antibodies indicated. NS = nonsilencing sequence. (B) Quantification of (A). p62 levels were individually normalized to actin, then to p62 levels measured in the (NS) shRNA sample without starvation (T₀) and assigned relative arbitrary units. (C) H1299 miR30shRNA cells were treated as above (A) (D) H1299 miR30shRNA cells were treated as above (A) except starved for 0, 2 or 4 hours. (E) H1299 ponasterone-inducible lines were transiently transfected with 5 μg of ptfLC3 for 24 hours, then plated in 6 cm dishes at a density of 3×10⁵ and treated with 5 μM ponasterone A as described above for 48 hours. Cells were the processed for western blot analysis as previously described, probed with the indicated antibodies. (F) The above cells were also plated on glass coverslips in 6 well plates at a density of 1x10⁵ cells/well and treated with 5 μM ponasterone A for 48 hours, followed by immunofluorescence processing with anti-UBXD1 antibody and AlexaFluor 647 anti-mouse secondary antibody (Pseudocolored light blue). Cells were visualized using the 40X optical lense and 1X digital zoom (rows 1 & 3) or 6X digital zoom (rows 2 & 4).
CHAPTER 4

DISCUSSION & FUTURE DIRECTIONS

4.1 Summary

This thesis focuses on UBXD1, an understudied adaptor of p97, which previously had no quality antibodies available for its study. The development of a high quality monoclonal antibody against UBXD1 in mouse has proven to be a crucial tool in determining UBXD1’s function. Initial work in characterizing UBXD1 interactions with p97 and ubiquitin have unveiled an unexpected consequence of transient-transfection-mediated over-expression of UBXD1. Upon high levels of over-expression, it is somewhat uniquely ubiquitinated and trafficked to the insoluble fraction of the cell. This unexpected result created a roadblock in examining UBXD1 in cells. A ponasterone-A inducible cell line was utilized to address this issue. It has allowed dose and time-dependent expression of wild-type UBXD1 and deletion mutants at lower levels, allowing for better study of its function. Given that UBXD1 is highly expressed in the brain, and its potential functional mediator, p97 is mutated in neurodegenerative disease, it was of interest to evaluate the ability of UBXD1 to interact with a relevant p97 mutant, R155H identified in the disease IBMPFD. UBXD1 displayed decreased binding with p97\textsuperscript{R155H} when compared to wild-type p97. Evaluating other UBX domain-containing
adaptors for differential interactions with p97R155H yielded results that suggest the UBA containing adaptors preferentially bind to mutant p97 through its UBA domain upon its ubiquitination. Using a high quality solution-based IP/MS approach, ERGIC-53 was identified as a UBXD1 interacting protein. This interaction is unique among p97 adaptors, suggesting that UBXD1 likely acts as a unique modulator in a small subset of p97 function. The first ten amino acids of UBXD1, specifically residues K2, F4, F5 and F8, were determined to be essential for ERGIC-53 binding. Examining members of the ERGIC-53 related COPI and COPII coatomer complexes identified βCOPI, as a UBXD1-independent interactor with p97. Upon ponasterone A-induced expression, UBXD1 forms large occlusions in the cytoplasm and along the plasma membrane. ERGIC-53 mislocalizes to both of these sites upon UBXD1 expression. A recently identified UBXD1 interactor, Caveolin-1 (also detected in the IP/MS approach), co-localizes with UBXD1 at the plasma membrane, but not at the cytoplasmic occlusions. As p97 mutation/loss has been strongly associated with defects in autophagy progression, it was of interest to evaluate whether UBXD1 and ERGIC-53 played a role here as well. Using a highly efficient miR30-based shRNA knockdown system, UBXD1, p97 and ERGIC-53 loss lead to a block in basal autophagy as measured by p62 and LC3-II accumulation. Ponasterone A-induced UBXD1 also promoted the formation of immature autophagosomes as measured by immunofluorescent studies of the tandem-fluorescent tagged LC3 protein. The work presented here strongly suggests a role for UBXD1 and p97 in regulating the trafficking of ERGIC-53 positive vesicles. Data suggest that this may subsequently be important for moderating proper basal autophagy.
4.2 UBA-containing adaptors’ enhanced association with p97 IBMPFD mutants may be misleading

Mutations in p97 have been attributed as the causative factors in IBMPFD disease and approximately 1-2% of familial cases of ALS. Much of what is known about these diseases comes from studies on the downstream effects of these mutations. It is known that IBMPFD disease is marked by multiple pathologies in the brain, bone and muscle, which is largely attributed to protein accumulation through defects in autophagy progression. More descriptive work has been done identifying and characterizing the prevalence of these disease-causing p97 mutations, than in actually defining their potential effects in cofactor/adaptor association. 9 out of 14 p97 mutations found in IBMPFD or ALS are localized to p97’s N domain, which is the interaction site for a majority of p97 adaptors (Table 1). It is logical to attribute p97-mediated disease to differential interactions with known adaptors at this domain. Some published work has shown differential adaptor associations with wild-type p97 and IBMPFD mutant p97. Fernandez-Saiz and Buchberger have recently published work showing that p97 adaptors, p47, Ufd1, Npl4 and Ataxin-3 bind stronger to the N domain in IBMPFD mutants p97R95G and p97R155H compared to wild-type. In contrast, they have shown that the E4 ligase E4B (UFD2) and the E3 ligase, HRD1 show no preferential binding, or a decrease in association with the IBMPFD mutants respectively. The authors fail to notice that those proteins with enhanced binding to mutant p97 (Npl4, Ufd1, p47, Ataxin-3) all bind ubiquitin chains through ubiquitin interaction motifs or UBA domains, while E4B and HRD1 do not. The authors only speculate that this differential adaptor binding in
IBMPFD mutants may have disease relevance (Fernandez-Saiz, Buchberger 2010). Manno et al. have also shown that Npl4, Ufd1 and p47 exhibit increased binding to exogenously expressed IBMPFD p97 mutants. They have also shown an increase in ubiquitin immunoprecipitation with these p97 mutants. This is attributed to the increase in adaptor binding, although it may be, at least in part, ubiquitinated mutant p97 (Manno et al. 2010). Work presented here indicates a less interesting theory, although one that must be considered when analyzing p97 cofactor associations with mutant proteins. As shown in Section 3.5, recombinant p97\textsuperscript{R155H} is strongly ubiquitinated in cell lysate. This ubiquitination leads to an enhanced association with only the UBA-containing p97 adaptors tested (UBXD7, UBXD8, FAF1, SAKS1), while the non UBA-containing adaptors showed decreased binding to p97\textsuperscript{R155H} (UBXD1, UBXD2) or no difference (UBXD5, ASPL1) (Figure 7). We believe that the enhanced association between UBA-containing adaptors and mutant p97 can be attributed to its recognition as a misfolded substrate by the ubiquitination machinery, followed by it’s recognition by the UBA-containing adaptors as a ubiquitinated substrate instead of as a functional cofactor. The system employed here uses recombinant p97; our results may not accurately represent what happens to p97\textsuperscript{R155H} \textit{in vivo}. However, Fernandez-Saiz and Buchberger also showed increased association between UBA-containing adaptors (Ufd1, Npl4 and Ataxin-3) and p97\textsuperscript{R155H} in IBMPFD-derived myoblasts, supporting cell based conclusions (Fernandez-Saiz, Buchberger 2010). Work presented here suggests that a more careful examination of this association is warranted to claim an enhanced, functionally relevant association between adaptors and p97 mutants. To confirm and expand these data, and clearly link IBMPFD disease to differences in UBXD1 and UBXD2 interaction with p97,
the remaining 13 identified p97 mutations should be evaluated. The system used herein (recombinant p97 with transfected adaptors in 293T cells) does not accurately reflect in vivo interactions. To clearly answer questions about differential interactions between adaptors and IBMPFD mutants, IBMPFD patient-derived myoblasts should be examined for endogenous interactions between adaptors and mutant p97. If sufficient primary antibodies do not exist, over-expressed adaptor studies in IBMPFD myoblasts would still better represent in vivo conditions.

4.3 Examining UBXD1/ERGIC-53 involvement in ALS

As mentioned above, p97 is the causative mutation in IBMPFD, and has been shown to also be mutated in a small percentage (1-2%) of familial ALS, a fatal neurodegenerative disease marked by protein aggregate accumulation (one component being TDP-43, also seen in IBMPFD). This leads to motor neuron dysfunction, paralysis and then death. Familial ALS is thought to be attributed to a large variety of gene mutations. The predominant gene involved, superoxide dismutase 1 (SOD1) accounts for only 15% of all familial cases (Chio et al. 2008). The remaining 85% are attributed to mutations in other genes including TDP-43 (~1-2%) and FUS (~1-2%) (Kabashi et al. 2008) (Chio et al. 2009). UBXD1 and ERGIC-53 have been shown to interact with p97 and may have disease relevance in the neurodegenerative disease IBMPFD by positively mediating autophagy and the subsequent degradation of accumulating proteins. It is possible that UBXD1 or ERGIC-53 mutation may play a role in a small minority of these familial ALS
cases. It would be worthwhile to evaluate familial ALS patient samples for UBXD1 or ERGIC-53 mutation.

4.4 Further examination of UBXD1 localizations

Upon ponasterone-A induced expression, full length UBXD1 is seen to accumulate in large cytoplasmic vesicles, and to segments along the plasma membrane. The identity of these UBXD1 positive cytoplasmic occlusions has yet to be determined. It is likely that they are enlarged endosomes involved in trafficking cargo throughout the cytoplasmic space and to/from the plasma membrane. Evaluating their co-localization with markers for endosomal components would aid in their identification. Early endosome markers (EEA1, Rab4 and Rab5) should be examined in addition to markers for the late endosome (Rab7, Rab9 and Mannose 6 Phosphate Receptor). As UBXD1 localizes to the plasma membrane, evaluating markers for recycling endosomes (Rab11) which are required for membrane protein internalization and trafficking must be considered as well. Given the knowledge that ERGIC-53 is a cargo receptor for lysosomal proteases cathepsin C and Z, the possibility that these vesicles are lysosomes should also be examined using Lamp1 and Lamp2 as markers.

4.5 UBXD1/p97 effect ERGIC-53-mediated intercellular trafficking

The work presented here strongly suggests a role for UBXD1 and p97 in regulating the trafficking of ERGIC-53 positive vesicles. Data suggest that this is subsequently
important for moderating proper basal autophagy. ERGIC-53 was initially identified as a
glycoprotein cargo receptor, mediating the transport of newly folded proteins out of the
ER towards the Golgi Apparatus. Identified cargo includes lysosomal proteases
cathepsins Z, and C. Upon COPII mediated budding from the ER, ERGIC-53 positive
vesicles were shown to shed their COPII coats and acquire COPI coatamer coats that
would direct their recycling back to the ER. More recent work has suggested that post-
ER trafficking of ERGIC-53 positive vesicles is far less linear than previously thought.
COPII-mediated trafficking of ERGIC-53 positive vesicles has also been shown to go to
the Golgi to deliver their glycoprotein cargo, and most interestingly, to serve as a
protective coat in the fusion of early endosomes (Schekman, Mellman 1997) (Razi, Chan
& Tooze 2009) (Gu et al. 1997). Interaction work has shown a clear association between
the extreme amino terminus of UBXD1 and the carboxy tail of ERGIC-53. Investigation
into the possible involvement of coatamer proteins identified the primary COPI coatamer
component, βCOPI as a UBXD1-independent interactor of p97. Interestingly, γCOPI or
Sec23 (primary COPII coatamer protein) were not immunoprecipitated, suggesting a
targeted interaction with this primary COPI protein. Immunofluorescence work further
suggested a role for UBXD1 in ERGIC-53 positive vesicle trafficking (Section 3.12).
UBXD1 sequestered ERGIC-53 to large cytoplasmic aggregates and near the plasma
membrane, while the UBXD1Δ10 (deficient in ERGIC-53 interaction) formed no
cytoplasmic occlusions and had no noticeable effect on ERGIC-53 localization. A
potential model begins to unfold when combining the UBXD1 interaction and
localization work done here with what is already known about p97, ERGIC-53 and COPI
coatamers in endosomal/autophagosomal trafficking. As mentioned previously, p97 loss
or mutation leads to an accumulation of immature autophagosomes; the mechanism for this blockage in autophagy progression is still undetermined (Tresse et al. 2010) (Ju et al. 2009). Studies on the effects of COPI component loss has shown a similar accumulation of immature autophagosomes. This was attributed to a defect in early endosome fusion (Razi, Chan & Tooze 2009). The absence of functional COPI coatamer components have also been shown to inhibit early to late endosomal trafficking resulting in vesicle clustering (Aniento et al. 1996) (Gu et al. 1997). This work supports a model by which UBXD1 acts as an adaptor for p97, driving its localization to ERGIC-53 positive vesicles (through UBXD1’s interaction with ERGIC-53’s cytoplasmic tail). At the site of coatamer formation, p97 may bind to members of the COPI coatamer (βCOPI), and through its physical action of ATP hydrolysis, mediate a structural change or potentially its dissolution from the vesicle. These now altered vesicles are able to fuse with endosomes/Golgi Apparatus, delivering their cargo. Upon increased UBXD1 expression, ERGIC-53 positive vesicles may undergo enhanced uncoating leading to their mislocalization.
Figure 19. Speculative model of UBXD1/p97 in modulating ERGIC-53 positive vesicle trafficking UBXD1 acts as an adaptor for 97, driving its localization to ERGIC-53 positive vesicles via direct interaction between UBXD1’s amino terminus and the ERGIC-53 cytoplasmic tail. p97 uses its mechanical force upon ATP hydrolysis to bind COPIβ and remodel the coatamer complex. This newly remodelled coat on ERGIC-53 positive vesicles allows their trafficking and fusion either with endosomes/lysosomes or the plasma membrane, delivering ERGIC-53 cargos, like lysosomal proteases. Alternatively, ERGIC-53 vesicles may traffic to the plasma membrane first before recycling back into the endosome/lysosome system.
4.6 UBXD1/p97’s function in ERGIC-53 trafficking is necessary for basal autophagy progression

This thesis suggests that UBXD1/p97 effect general ERGIC-53-mediated fusion events. Subsequent studies on the effects of UBXD1, p97 and ERGIC-53 depletion have identified a role in promoting proper autophagy progression. Individual shRNA-mediated knockdown of all three components resulted in the accumulation of autophagy markers p62 and LC3-II. Upon nutrient-deprivation, autophagy proceeded normally. The levels of p62 accumulation seen here are comparable to those seen in the work initially identifying an essential role for p97 in basal autophagy progression (Tresse et al. 2010). UBXD1 was also shown to play a positive role in the formation of immature autophagosomes. Upon ponasterone-A induction, UBXD1 promoted the trafficking of RFP-GFP tagged LC3 (a marker for immature autophagosomes) from a diffuse cytoplasmic localization to distinct cytoplasmic spots (autophagosomes). The most well known purpose of ERGIC-53 is in ER export and delivery of newly synthesized glycoproteins. Loss or mutation of UBXD1, p97 or ERGIC-53 could negatively effect this cargo delivery and subsequently effect autophagic progression. As previously mentioned, two of ERGIC-53’s identified cargos are lysosomal proteases Cathepsin C and Z. Their mistrafficking and absence from the lysosome could possibly lead to a defect in autophagy progression. Lysosomal storage diseases (LSDs) are a collection of 50 or more diseases typically caused by the loss of a single lysosomal enzyme. They are often marked by large, distended lysosomes, and the progressive accumulation of undigested lysosomal substrates. Because these diseases are caused by the loss/mutation
of different lysosomal proteins, they vary in severity in multiple organ systems. However, ~75% of LSDs involve significant central nervous system impairment (Hawkins-Salsbury, Reddy & Sands 2011). Cathepsins C and Z have not been identified as a cause of LSDs. Diseases of vesicle trafficking and fusion share phenotypic commonalities with LSDs; mucolipidosis type IV (a defect in the mucolipin-1, a cation channel protein in endosomal membranes) (Raychowdhury et al. 2004). It is possible, that upon UBXD1, p97, ERGIC-53 loss or mutation, Cathepsins C and Z are not efficiently trafficked to the lysosome. Knocking down Cathepsins C and Z could provide evidence of their impact in basal autophagy. However, their absence alone may not necessarily lead to the defect seen in autophagy progression. ERGIC-53 is likely responsible for the trafficking of other yet to be identified proteins. Defective ERGIC-53-mediated trafficking of multiple cargos to the endosome/lysosome pathway could cumulatively create a defective lysosome, subsequently limiting the degradation of substrates and markers p62 and LC3-II in the fused autolysosome. It is also possible that substrate trafficking is redundant and not solely mediated by ERGIC-53. Upon starvation, alternative methods of cargo trafficking may be initiated to handle the increased requirements of macrophagy. As mentioned above, showing the colocalization of UBXD1 positive cytoplasmic occlusions with lysosome markers would support this theory. Differential co-staining of lysosomes with lysosomal membrane markers (LAMP1) and antibodies against lysosome resident degradative enzymes upon UBXD1 over-expression would help determine if there is a difference in lysosome resident proteins. Examining purified lysosomes via IP/MS proteomics may serve as a stronger
tool for examining differences in lysosomal composition with and without UBXD1 expression.

Future work should also include studying the effect of UBXD1, p97 and ERGIC-53 knockdowns on cells put under additional forms of autophagic stress including the expression of autophagy substates (such as Huntington’s poly-glutamine expansion proteins, htt25Q and htt103Q). Additionally, blocking proteasomal degradation via proteasome inhibitors such as MG132 has been shown to drive misfolded proteins to the autophagic pathway (Ding et al. 2007b). It may be possible to identify an even stronger role for UBXD1, p97 and ERGIC-53 in autophagy upon stressed conditions. Rescue experiments should also be performed re-introducing full-length UBXD1 and ERGIC-53-binding deficient UBXD1 point mutants (K2A, F4A, F5A, F8A). Testing their ability to rescue basal autophagy will show if UBXD1’s function in autophagy is directly related to its interaction with ERGIC-53. Additionally, UBXD1 mutant ponasterone-A-inducible expression lines should be evaluated for their ability to promote autophagosome formation in comparison to wild-type (Section 3.14).

4.7 UBXD1 traffics ERGIC-53 to the plasma membrane

UBXD1/p97 has also been shown to interact with mono-ubiquitinated Caveolin-1 and mediate its endosomal sorting and degradation in endolysosomes (Ritz et al. 2011). Upon UBXD1 siRNA-mediated knockdown, Caveolin-1 was shown to accumulate at Rab5+ early endosomes. The authors show some co-localization of UBXD1-mCherry
with Caveolin-1-GFP to small cytoplasmic vesicles co-staining with markers for the early endosome, late endosome and lysosome. Little to no localization of Caveolin-1-GFP can be seen at or near the plasma membrane. We have shown that upon ponasterone-A induction in H1299 cells, UBXD1 does colocalize with endogenous Caveolin-1, however not in a few cytoplasmic vesicles, but instead robustly at the plasma membrane (Section 3.13). They maintain clearly separate localizations within the cytoplasm. Caveolin-1 is primarily localized at the plasma membrane (as high as 90%) in concentrated caveolae structures, but, upon monoubiquitination, is internalized and trafficked to early endosomes (Das et al. 1999) (Hayer et al. 2010). The previous work used transfected UBXD1 and Caveolin-1 in U2OS cells; it has been shown that upon transient transfection-mediated over-expression, both of these proteins mislocalize when compared to endogenous protein (Section 3.13) (Hayer et al. 2010). Upon over-expression, Caveolin-1 was shown to quickly traffic to late endosomes, while endogenous Caveolin-1 had nearly twice the half-life at the plasma membrane. UBXD1 accumulates at the plasma membrane colocalizing with both endogenous Caveolin-1 and endogenous ERGIC-53. It is possible UBXD1 somehow plays a yet to be identified role in trafficking ERGIC-53 cargo to the membrane which in turn positively effects Caveolin-1 internalization and subsequent movement into the endosome pathway. Alternatively, UBXD1, with p97, may traffic to the membrane specifically to promote monoubiquitinated Caveolin-1 internalization via p97’s ATPase function. ERGIC-53 may just accumulate there as a consequence of increased UBXD1 trafficking.
In addition to Cathepsins C and Z, ERGIC-53 is responsible for the export of secretory proteins: Blood Coagulation Factors V and VIII and a serine protease inhibitor, α1-antitrypsin (Nyfeler et al. 2008). ERGIC-53 was discovered as the causative mutation in Combined Factor V and VIII Deficiency, a disease related to classic hemophilia. The authors show that mutations effecting ERGIC-53’s ability to bind to Factor’s V and VIII prevents their secretion (Nichols et al. 1998). α1-antitrypsin was discovered as a substrate for ERGIC-53-mediated protein export via a protein complimentation screen using ERGIC-53 as bait. Using ERGIC-53 siRNA and ERGIC-53 knockout MEFs, the authors went on to show that α1-antitrypsin secretion relied on active ERGIC-53. Loss of ERGIC-53 resulted in secretion levels as low as 10% of control. This defect was rescued upon re-expression of full length protein (Nyfeler et al. 2008). ERGIC-53 is a single pass transmembrane protein important for the trafficking of cargos out of the ER, to the Golgi Apparatus and endosomes. Upon ponasterone-A induced induction of UBXD1, ERGIC-53 accumulates at the plasma membrane. This novel localization could suggest that ERGIC-53 plays a direct role in trafficking cargo to the membrane for secretion into the extracellular space. UBXD1/p97 may promote this movement, again through the remodelling of ERGIC-53 positive vesicle coats.

4.8 Evaluating UBXD1/ERGIC-53/p97 pathway in disease-relevant neurons

As mentioned above, UBXD1’s function is likely to be very specific, and although solid functional leads have been discovered here using transformed cell lines (H1299 and U2OS), it is very likely that UBXD1 primarily functions in a more specialized cell type.
UBXD1 is highly expressed in the brain and its functional mediator, p97, is the causative mutation in IBMPFD and in 1-2% of familial ALS, neurodegenerative diseases marked by protein aggregate formation leading to cell death in neurons. Neurons derived from IBMPFD patients would be the truest system in which to study UBXD1/p97 function at endogenous levels. However, neurons are terminally differentiated cells that do not replicate, making *in vitro* manipulations nearly impossible. The recent creation of a human embryonic stem cell derived, “long-term, self-renewing, primitive neural precursor” cell line capable of directed differentiation into a variety of sub-type specific neurons will address this issue (Li et al. 2011). Briefly, through the administration of small molecule inhibitors of glycogen synthase kinase 3 (GSK3), transforming growth factor β (TGFβ) and Notch signalling pathways, it is possible to convert human embryonic stem cells to a homogenous population of primitive neural stem cells. A variety of *in vitro* manipulations are possible in these self-renewing cells. For example, these cells could be infected with Lentivirus carrying the doxycycline-inducible mir30-shRNA knockdown plasmids and/or the pIND ponasterone A inducible over-expression system prior to their differentiation into neurons. These now-inducible lines are capable of long-term expansion prior to their drug-induced differentiation into multiple neuronal subtypes. The effects of gene knockdown or over-expression could be evaluated in this now disease relevant cell type.


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