UNDERSTANDING APOLIPOPROTEIN B'S ABILITY TO AGGREGATE THROUGH LIPID DROPLETS AND CHAPERONE HOLDASE

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Endoplasmic Reticulum (ER) associated degradation (ERAD) is the general process in which misfolded secretory proteins are monitored and degraded to protect the cell from a buildup of nonfunctioning proteins. Apolipoprotein B (ApoB), an ERAD substrate is a large hydrophobic secretory protein associated with the transport of lipids and cholesterol by lipoproteins in the body. ApoB synthesis involves cotranslational translocation through the Sec61 translocon into the ER. If properly folded and lipidated, ApoB is then retrotranslocated through the same pore. Since ApoB contains many aggregation-prone hydrophobic β-sheets, what prevents ApoB aggregation before degradation by ERAD? Initial considerations suggested that cytosolic factors, such as lipid droplets or chaperone "holdases," "foldases," and "disaggregases" may help to maintain ApoB's solubility post retrotranslocation. To test this hypothesis, I adapted our yeast galactose inducible ApoB expression system to be β-estradiol inducible and used it to investigate various chaperone candidates to determine if they affect ApoB stability. Upon large scale isolation of lipid droplets, ApoB was found not to interact with lipid droplets. Next, I investigated potential chaperones. I found that the small heat shock proteins, a family of ATP-independent chaperones, and the TRiC complex, an Hsp60 family member, do not affect ApoB stability. However, I determined that Hsp104, a AAA+ ATPase which helps to refold and reactivate aggregated proteins, is a pro-degradation factor for ApoB. ApoB degradation was slowed in the absence of this chaperone while overexpression caused faster degradation. I then investigated Rvb2, the yeast homolog of the human functional analog of Hsp104, to determine its

effect on ApoB stability. Unexpectedly, Rvb2 did not restore ApoB degradation in the absence of Hsp104. Together, my data indicate that ApoB does require chaperone disaggregase function prior to ERAD.

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PREFACE

The ability to become a scientist is not a solo journey. Fortunately, I was able to interact with a wonderful group of people, both inside and outside of graduate school, that have helped me become the person I am today. First and foremost, I am most grateful for my wonderful mentor, Dr. Jeffrey L. Brodsky. His support, encouragement, and advice have helped facilitate my growth as a scientist and as a person during my time in graduate school. He also encouraged me to pursue my passion for teaching, which allowed me to complete a teaching minor. For this, I'll be forever grateful. None of my science would have been possible if not for our wonderful lab manager, Jen Goeckeler-Fried. Our equipment and supplies were organized, which greatly helped my science go smoothly. I am forever indebted to Dr. Sarah Grubb, who served as my mentor when I first joined the Brodsky lab. Without her guidance, support, advice, and passion, my transition into my thesis work would have been difficult.

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LIST OF FREQUENTLY USED ABBREVIATIONS

ApoB – Apolipoprotein B

CAD – Coronary Artery Disease

DUB – Deubiquitinating Enzyme

ERAD – Endoplasmic Reticulum Associated Degradation

HDL – High Density Lipoprotein

Hmg CoA – 3-hydroxy-3-methyl-glutaryl coenzyme A

HSP – Heat Shock Protein

LDL – Low Density Lipoprotein

MTP- Microsomal Triglyceride Transfer Protein

PDI – Protein Disulfide Isomerase

PERPP – Post ER Presecretory Proteolysis

TRiC – Tcp1 Ring complex

vLDL – Very Low Density Lipoprotein

1.0 INTRODUCTION

Cardiovascular disease is a serious concern for human health, affecting nearly one third of all people. Hence, understanding the molecular defects underlying this disease is necessary for the development of therapeutics to combat this condition. One hallmark of cardiovascular disease is the overabundance of circulating cholesterol, which can build up on artery walls. Current treatments mainly focus on the use of statins, which block cholesterol synthesis. However, recent efforts to find alternative treatments for this condition have been focused on several factors including Apolipoprotein B (ApoB), a protein required for cholesterol delivery throughout the body. Therefore, understanding ApoB regulation is essential to identify alternative therapies for cardiovascular diseases.

ApoB protein levels are metabolically regulated by endoplasmic reticulum (ER) associated degradation (ERAD) as well as post ER degradation. ERAD is a cellular quality control mechanism, which monitors secretory proteins and selectively degrades misfolded substrates. By investigating how ERAD monitors and degrades ApoB, we can potentially identify new drug targets to treat cardiovascular disease. In this chapter, I discuss the severity of cardiovascular disease affecting humans. I further discuss how cholesterol is regulated and how it is delivered to peripheral tissues. Finally, a discussion of the current knowledge of ApoB biosynthesis and its degradation is presented.

1.1 CARDIOVASCULAR DISEASE

1.1.1 Statistics and Significance

Cardiovascular disease is one of the leading causes of death worldwide, accounting for 31% of all deaths (Roth et al, 2015). In the United States, this disease is responsible for more deaths than the next two conditions, cancer and chronic lower respiratory disease, combined. Except for 1918 due to influenza, cardiovascular disease has been the leading cause of death worldwide every year since 1900, (Roger et al, 2011). The most recent report from the American Heart Association estimates that 1 in 3 people will be affected by some form of cardiovascular disease in their lifetime and will result in the deaths of approximately 800,000 Americans annually. This is about 2,200 people per day or 1 death every 40 seconds. It is no surprise that a better understanding of pathways to combat this disease are imperative.

It is estimated that ~92 million Americans are currently living with some form of cardiovascular disease (Benjamin et al, 2017; Farvid et al, 2014). Furthermore, many Americans are at risk for developing some form of cardiovascular disease or are living with undiagnosed conditions. Cardiovascular disease encompasses a variety of disorders that affect the cardiovascular system and the heart. These conditions include stroke, heart disease, heart defects, diabetes, high cholesterol, high blood pressure, arrhythmias, arterial disease, and coronary artery disease. Coronary artery disease (CAD) is the most prevalent form of cardiovascular disease. Its levels have been steadily increasing from 20% of all cases in 2011 to the current level of 45% (Benjamin et al, 2017; Caffrey et al, 2011; Mensah et al, 2005; Roger et al, 2011). Stroke is the second most common condition, accounting for 16.5% deaths (Fang et

al, 2014; Ovbiagele et al, 2013). The third most prevalent condition, heart disease, accounts for 8.5% of cardiovascular disease related deaths (Farvid et al, 2014).

High levels of cholesterol aggravate cardiovascular risk. Reducing cardiovascular risk can be accomplished by exercise and alterations to diet. In fact through a meta-analysis study of over 120,000 people, as little as 15 minutes of moderate exercise reduce mortality rates by 22% in adults 60 years old and over (Hupin et al, 2015). However, 1 in 3 adults do not regularly perform the recommended 60 minutes of physical activity per day (Benjamin et al, 2017). Although the percentage of U.S adults who eat a healthy diet rose to 1.5% of Americans, the prevalence of obesity has risen from 30 to 37% of Americans in the same time frame (Flegal et al, 2016; Lloyd-Jones et al, 2010; Ogden et al, 2015; Rehm et al, 2016). Moreover, 12% of Americans have high cholesterol levels and 40% of Americans are living with borderline high cholesterol levels (Benjamin et al, 2017; Carroll et al, 2015; National Institutes of Health, 2012).

1.1.2 Coronary Artery Disease Pathology

CAD is characterized by atherosclerosis, which is linked to the presence of atherosclerotic plaques (see below). These plaques can accumulate on artery walls, which lead to inflammation via the generation of oxidized lipids (Han & Kaufman, 2016; Mozzini et al, 2017). As the plaques continue to grow, they reduce the artery diameter and restrict blood flow, forcing the heart to work harder to pump blood throughout the body. Parts of these plaques can potentially break off and enter the bloodstream, circulate to other areas of the body to cause heart attack or stroke if the plaque-derived particles reach the heart or brain (Insull, 2009; Stefanadis et al, 2017).

As noted above, plaques can trigger an inflammatory response due to the presence of oxidized lipids. The oxidized lipids cause the surrounding epithelial cells to secrete inflammatory signals, including adhesion molecules, chemokines, growth factors, and possibly NFκB, which recruit monocytes and lymphocytes to the artery (Boring et al, 1998; Collins et al, 2000; Deng et al, 2004; Dong et al, 1998; Gu et al, 1998; Hotamisligil, 2010; Shih et al, 1999; Watson et al, 1997). Oxidized lipids may also reduce the amount of generated nitrous oxide, further inhibiting the ability of the vasculature to widen. Furthermore, recent studies observed the upregulation of BiP, a marker of the unfolded protein response, and CHOP, a signal related to activation of the autophagy pathway and cellular apoptosis, in peripheral blood mononuclear cells in patients with CAD. These results strongly suggest that the vasculature is altered in CAD patients (Knowles et al, 2000; Lusis, 2000; Mozzini et al, 2017; Mozzini et al, 2014; Thorp et al, 2009).

Plaques are primarily derived from low density lipoproteins (LDLs) that are highly enriched in cholesterol and Apolipoprotein B (ApoB) (Fisher, 2016; Tiwari & Siddiqi, 2012). As described below, ApoB is the major structural component of LDL particles and is required for their formation (see section 1.3.2). Although LDL particles are taken up by macrophages, these cells sequester large amounts of cholesterol and ApoB in LDLs rather than digesting them. Ultimately, such macrophages become stuck on the arterial wall (Fisher, 2016; Kwiterovich, 2000). Other lipoprotein remnants, including those derived from very low density lipoproteins (see section 1.2.2), can then adhere to the vasculature wall, associating with the lipoproteins that are already present on the arterial wall. As these plaques aggregate, the rate of macrophage/LDL uptake and arterial association accelerate, which further impairs blood flow and increases the risk of heart attack and stroke.

1.1.3 Methods to Combat Coronary Artery Disease

Not surprisingly, devising treatments for cardiovascular diseases is an active area of research. Prescribed to nearly 40% of Americans, the most common treatment is the use of statins. Statins are a class of drugs that inhibit cholesterol synthesis by acting as competitive inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (Hmg CoA) reductase (Saeedi Saravi et al, 2017; Sikka et al, 2011). This prevents the conversion of Hmg CoA to mevalonate, which is a precursor to cholesterol and represents the rate limiting step for cholesterol biosynthesis (Bucher et al, 1960). These drugs can reduce LDL associated cholesterol levels up to 60% at the highest doses (Boekholdt et al, 2014; Stone et al, 2014; Weber et al, 2017). Reducing LDL cholesterol levels through statin use can prevent up to 25% of sudden cardiovascular related events (Baigent et al, 2010). Combined with Ezetimibe, a drug which prevents cholesterol uptake by inhibiting the activity of Niemann-Pick C1-like protein 1 (NPC1L1), statin treatment is enhanced by a further 6.4% in reducing LDL levels (Cannon et al, 2015). NPC1L1 is a multispan membrane protein, localized to the surface of enterocytes, which binds lipids through its sterol sensing domain. The protein is rapidly internalized in the presence of dietary cholesterol and fatty acids, and Ezetimibe blocks NPC1L1 internalization to prevent cholesterol uptake (Chang & Chang, 2008; Davies et al, 2000; Garcia-Calvo et al, 2005; Ge et al, 2008; Yu et al, 2006).

Although statins remain an attractive and well established first-line compound to reduce the amount of cholesterol synthesized in the body and have been used for 30 years, they are not without complications (Endo, 2010). First, statins are no universally effective. In a meta-analysis of over 38,000 patients, Boekholdt and coworkers discovered that statin effectiveness

varied significantly among users and that, more than 40% of patients undergoing high dose statin therapy did not achieve an optimal level of circulating cholesterol (Boekholdt et al, 2014). Moreover, certain patients simply do not respond to statin therapy, possibly due to genetic variation, or the statins cannot be combined with other drugs the patients are concurrently taking (Dadu & Ballantyne, 2014; Sikka et al, 2011). Genetic variation is often due to polymorphisms in genes related to statin metabolism, including intestinal P-glycoprotein, organic anion transporter 2, coenzyme Q10, and cytochrome P450 3A4 (Baker & Samjoo, 2008; Fiegenbaum et al, 2005; Frudakis et al, 2007; Link et al, 2008; Mulder et al, 2001; Oh et al, 2007; Zuccaro et al, 2007). Due to these deficiencies and the observed side effects, it is crucial that alternative therapies are identified to control cholesterol production. Second, it is estimated that 25-30% of patients discontinue statin use to due side effects (Raju et al, 2013; Rosenson, 2016). The most common side effects of statin therapy, affecting approximately 15% of patients, include muscle wasting, leg cramps, and myopathy (Dadu & Ballantyne, 2014; Fitchett et al, 2015; Mancini et al, 2013). Although many of these relatively minor side effects disappear by simply switching to a different statin, other more serious side effects can occur, such as acute liver failure and peripheral neuropathy (Sikka et al, 2011). Third, a meta-analysis identified a 9% increase in the incidence of diabetes associated with statin use (Preiss et al, 2011; Weber et al, 2017).

Recently, antibody therapies have been considered to control circulating cholesterol levels. The most promising therapy is one developed against proprotein convertase subtilisin/kexin type 9 (PCSK9), a serine protease of the proprotein convertase superfamily (Narasimhan, 2017; Schulz & Schluter, 2017; Weber et al, 2017). In humans, PCSK9 targets the LDL receptor for degradation via autophagy through a mechanism that has not been fully elucidated (Narasimhan, 2017; Schulz & Schluter, 2017). By inhibiting PSCK9, more LDL

receptors are present on the cell surface to bind ApoB (see below), therefore reducing circulating LDLs. These antibodies reduce LDL levels by up to 60%, and two antibody therapeutics, Evolocumab and Alirocumab, were recently approved as a treatment in the absence of a large scale study for specific patient groups (Okopien et al, 2016; Robinson et al, 2015; Weber et al, 2017; Zhang et al, 2015). The initial trials followed over 27,000 patients for 2 years and investigated if Evolocumab in combination with statins had additional value compared to statins alone. Patients taking both drugs saw a 59% reduction of LDL cholesterol, with no increase of side effects (Narasimhan, 2017; Sabatine et al, 2017). While anti-PCSK9 therapy may be a promising strategy for treating CAD, further investigation into alternative therapeutic strategies is necessary as these antibodies are cost prohibitive and are thus far approved only for specific patient groups (Weber et al, 2017).

The next most promising alternative therapy for treating CAD is reducing ApoB levels. As discussed in section 1.2.1, ApoB is the main structural component of lipoproteins, which deliver cholesterol to the peripheral tissues (Fisher, 2016). In peripheral tissues, ApoB binds to the LDL receptor, which is then endocytosed and routed to the lysosome. The internalized ApoB is degraded but the cholesterol and cholesterol esters are stored and used by the host cell (Brown & Goldstein, 1975; Brown & Goldstein, 1976). One such method to reduce ApoB levels is via anti-sense oligonucleotides, which target the ApoB message and down regulate protein levels (Liscinsky, 2013; Ricotta & Frishman, 2012; Thomas & Ginsberg, 2010; Wong & Goldberg, 2014). Mipomersen is the first ApoB-specific anti-sense oligonucleotide treatment approved for use in humans (Kynamro, 2013). Prior to its approval in 2013, phase 3 clinical trials indicated a reduction in LDL cholesterol of 25-40% (McGowan et al, 2012; Raal et al, 2010). However, Mipomersen is also only approved for specific patients, i.e., those suffering from familial

hypercholesteremia, which most often arises from mutations in the LDL receptor. These mutations either lower LDL receptor populations in the cell or prevent the plasma membrane resident LDL receptor from clearing LDLs from the serum (Austin et al, 2004; Brown & Goldstein, 1975; Brown & Goldstein, 1976; Lister Hill National Center for Biomedical Communications, 2017). Furthermore, Mipomersen has not been approved for use in Europe due to concerns with liver toxicity and cardiovascular complications (Liscinsky, 2013; Okopien et al, 2016; Weber et al, 2017; Wong & Goldberg, 2014). Further investigation is undoubtedly required to understand the long term effects of this drug in humans.

Recently, small molecule inhibitors have been developed to treat CAD. One such drug, Lomitapide, prevents lipid loading onto ApoB, which subsequently reduces cholesterol delivery. Lomitapide inhibits the microsomal triglyceride transfer protein (MTP) complex, which loads cholesterol, cholesterol esters, triglycerides, and phospholipids onto ApoB in the liver (see section 1.2.2). Lomitapide treatment lowered triglyceride levels by 65% and LDL levels by 50% (Cuchel et al, 2013). However, Lomitapide is only approved for homozygous familial hypercholesterolemia patients, has severe side effects, and may cause liver damage and steatosis (Roeters van Lennep et al, 2015; Weber et al, 2017).

Another promising drug target to reduce the incidence of CAD is the cholesteryl ester transfer protein (CETP). CETP transfers cholesterol from high density lipoproteins (HDLs) to ApoB-containing lipoproteins, thus inducing reverse cholesterol transport, which is the movement of cholesterol from peripheral tissues to the liver (see section 1.2.1) (Shapiro & Fazio, 2017; Weber et al, 2017). Thus, it was predicted that CETP inhibition would lower cholesterol levels. However during clinical trials, severe complications arose, including off target effects

which lead to increased death rates in high risk patients and no obvious effects on lipid levels (Barter et al, 2007; Kastelein et al, 2015; Okopien et al, 2016; Schwartz et al, 2012).

The relationship between CAD and autophagy is another area of therapeutic interest. As described in greater detail below (section 1.6), autophagy is the pathway in which aggregated proteins, organelles, and specific cytoplasmic proteins become sequestered into double membranous vesicles in the cytoplasm, which are then engulfed by the lysosome and degraded (Levine & Klionsky, 2017; Levine & Kroemer, 2008; Yin et al, 2016). TFEB, an activator of autophagy, stimulates the degradation of lipids and fatty acids in the lysosome (Sardiello et al, 2009; Settembre et al, 2011). This phenomenon led to the discovery that overexpression of TFEB induced cholesterol removal, as well as the degradation of ubiquitinated proteins (Emanuel et al, 2014). Moreover, TFEB overexpression not only increased degradation of aggregated proteins associated with p62, an autophagy receptor/chaperone but also reduced inflammatory signaling, resulting in smaller atherosclerotic plaques (Sergin et al, 2017). Current studies in patients with non-alcoholic fatty liver disease are being undertaken to determine if polymorphisms in autophagy related genes affect lipid storage (Yu-Cheng Lin, 2017).

Although many CAD treatments seek to reduce cholesterol levels, it should be noted that cholesterol synthesis cannot be completely halted. Cholesterol is an important component of cell membranes, facilitates membrane curvature and fusion, and is a biosynthetic precursor to multiple cellular factors, including bile acids, vitamins, sterols, and hormones (Churchward et al, 2005; Simons & Ikonen, 2000; Simons & Vaz, 2004; Stevenson et al, 2016). The absence of cholesterol stunts growth and development, and results in diseases such as abetalipoproteinemia and familial hypobetalipoproteinemia. Familial hypobetalipoproteinemia affects ~1 in 1000-

3000 individuals and abetalipoproteinemia has 100 confirmed cases worldwide (Schonfeld et al, 2005; Zamel et al, 2008). Familial hypobetalipoproteinemia most often arises from mutations in ApoB while abetalipoproteinemia is due to the inability of the MTP complex to load lipids onto ApoB. Both of these conditions cause extremely low levels of circulating vLDLs (in the 5th percentile or less) and serum cholesterol (Schonfeld et al, 2005; Wetterau et al, 1992). While these diseases are rare, they can result in ataxia, neurological defects, and improper growth and development (Medicine, 2017a; Medicine, 2017b; Schonfeld et al, 2005; Zamel et al, 2008). Therefore, cholesterol levels must be tightly regulated to maintain proper homeostasis.

Thus, too much cholesterol results in cardiovascular diseases while too little cholesterol results in a host of other problems. As discussed above, drugs against multiple targets, including PCSK9 and ApoB, have been investigated, but in each case there are limitations to these treatments. Are there other ways to specifically target the levels of cholesterol? Identifying novel factors which contribute to the regulation of ApoB levels might represent new treatments for CAD.

1.2 LIPOPROTEINS

1.2.1 Classes of Lipoprotein Particles

Lipoproteins particles are responsible for the transport of cholesterol, cholesteryl esters, and, triacylglycerols throughout the body. Lipoproteins are composed of a phospholipid outer layer surrounding a neutral lipid inner core (Dominiczak & Caslake, 2011). There are four main classes of lipoproteins: HDLs, LDLs, very low density lipoproteins (vLDLs), and chylomicrons

(Figure 1). These lipoproteins are classified based on density and size. HDLs are the most dense particles, with a density of 1.063-1.210 g/mL, while chylomicrons are the least dense, with a density of less than 0.95 g/mL (Christie, 2014; Cox & Garcia-Palmieri, 1990; Dominiczak & Caslake, 2011; Wasan et al, 2008). Each class has a distinct diameter, ranging in size from 5 nm to 1000 nm, with diameter size inversely related to density (Cox & Garcia-Palmieri, 1990; Wasan et al, 2008).

Apolipoproteins provide structural rigidity by serving as a scaffold for growing lipoproteins. Apolipoproteins are amphipathic proteins that provide structural support and act as detergents and as receptors. They also regulate the enzymes involved in neutral lipid synthesis and can be transferred between lipoproteins (Dominiczak & Caslake, 2011; Wasan et al, 2008). At least 10 apolipoproteins are found in human blood plasma. ApoB is associated with cholesterol delivery, and ApoB isoforms are present in LDLs, vLDLs, and chylomicrons. ApoB provides the backbone for the growing lipoprotein particle during synthesis and subsequently during maturation and modification, and contains a recognition motif for the LDL receptor (Dominiczak & Caslake, 2011; Wasan et al, 2008). Interestingly, LDLs, vLDLs, and chylomicrons each contain only 1 molecule of ApoB, representing the sole source of protein (Cladaras et al, 1986). ApoB is also the only essential apolipoprotein and is non-exchangeable (see section 1.4). However, other lipoproteins contain exchangeable apolipoproteins, such as apolipoprotein AI, CI, CII, CIII, and E (Dominiczak & Caslake, 2011; Wasan et al, 2008).

Cholesterol not only needs to reach peripheral tissue but also needs to be recycled. This is accomplished by HDLs, which are responsible for reverse cholesterol transport in the body (Wasan et al, 2008). During this process, primordial HDLs are made in the liver and intestine, and primarily contain the exchangeable apolipoproteins, mainly apoAI and AII (Dominiczak &

Caslake, 2011; Wasan et al, 2008). In turn, HDLs receive free fatty acids from peripheral tissues and esterify the fatty acids with cholesterol through the lecithin-cholesterol acyl transferase (LCAT) and ApoAI. The HDLs can then return to the liver and interact with the HDL receptor for cholesterol internalization (Acton et al, 1996; Chau et al, 2006). However, only about half of the cholesterol is delivered to the liver while the other half will be transferred to ApoB-containing lipoproteins by cholesteryl ester transferases, which append triglycerides onto cholesterol (Kwiterovich, 2000; Wasan et al, 2008). These molecules may be stored in the liver or are reinserted into LDL particles.

1.2.2 Very Low Density Lipoprotein Maturation and Secretion

LDLs, vLDLs, and chylomicrons are responsible for transporting the majority of fatty acids, triacylglycerol, and cholesterol to peripheral tissues in a highly controlled and regulated process. Primordial vLDLs are initially synthesized in the endoplasmic reticulum (ER) on membrane bound ribosomes as ApoB is translocated into the ER through a proteinaceous channel. As a result, ApoB is cotranslationally lipidated by the MTP complex in the ER (Mitchell et al, 1998). The MTP complex consists of an "M" subunit and a molecule of protein disulfide isomerase (PDI). The M subunit is required for lipid transfer while PDI facilitates disulfide bond formation and acts as a chaperone during vLDL synthesis, allowing ApoB incorporation into the pre-vLDL particle (Lamberg et al, 1996; Wetterau et al, 1991a; Wetterau et al, 1991b; Wetterau et al, 1990). These primordial vLDL particles are secreted in non-canonical COPII coated vesicles, which are larger than normal COPII coated vesicles (Gusarova et al, 2003; Tiwari & Siddiqi, 2012).

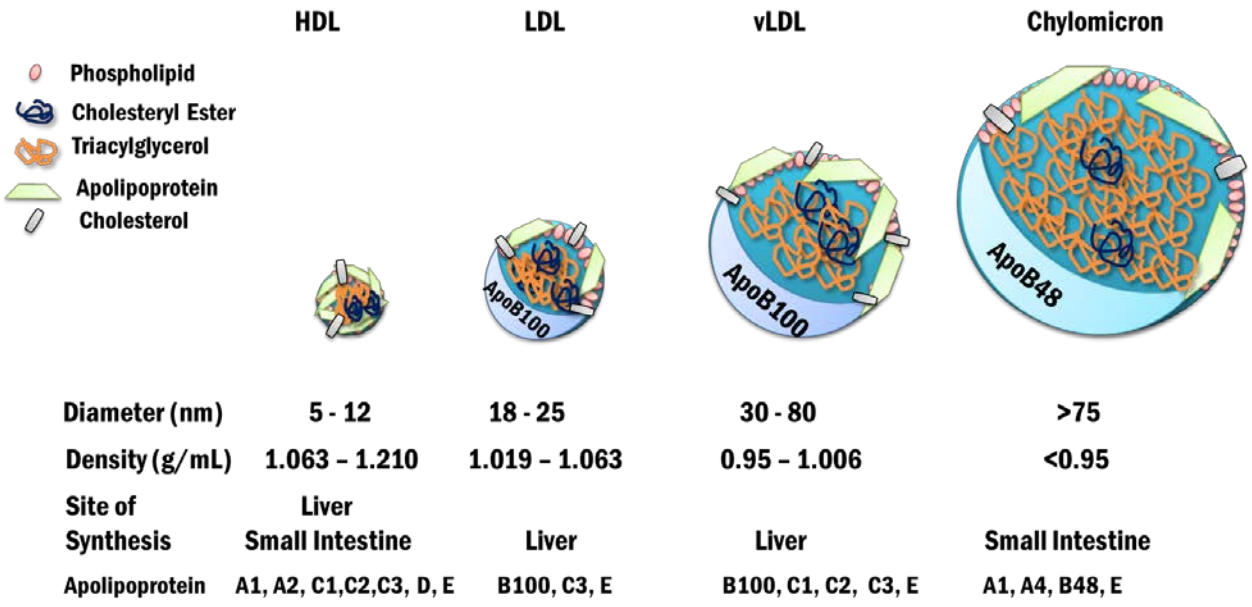


Figure 1. Lipoprotein Particles of Blood Plasma.

The four main classes of plasma lipoproteins are HDLs, LDLs, vLDLs, and chylomicrons. HDLs are the smallest particles and are the most dense. Chylomicrons are the largest particle and are the least dense. LDLs and vLDLs contain ApoB100 as their major apolipoprotein while chylomicrons in humans contain ApoB48. HDLs contain ApoAI and ApoAII as the major apolipoprotein. VLDLs are synthesized in the liver while chylomicrons are synthesized in the small intestine. LDL is a product of VLDL modifications made in circulation. HDLs are made in both tissues.

The COPII coat is a 5 member complex, consisting of the Sar1 GTPase, Sec23, Sec24, Sec13, and Sec31, which mediate transport of cargo from the ER to the Golgi (Baker et al, 1988; Barlowe et al, 1994; Kuge et al, 1994; Lord et al, 2013; Rothman & Wieland, 1996; Ruohola et al, 1988). It has been recently proposed that TANGO, a receptor for pre-collagen (another large secretory protein), and its partner TALI recruit lipids to ER exit sites to facilitate pre-vLDL secretion (Santos et al, 2016). Regardless of the mechanism, these pre-vLDL particles exit the ER and traffic next to the Golgi apparatus.

In the Golgi, the pre-vLDL particles are further lipidated and ApoB undergoes posttranslational modifications and conformational changes which result in the mature vLDL particle (Fisher & Ginsberg, 2002; Ginsberg & Fisher, 2009; Gusarova et al, 2003; Gusarova et al, 2007). After exiting the Golgi, these particles enter the blood stream and migrate to peripheral tissues. Upon reaching epithelial cells in peripheral tissue, the ApoCII component activates lipoprotein lipase on the cell surface. Through its hydrolytic activity, lipoprotein lipase releases free fatty acids and triacylglycerol, which can be taken up into the epithelial cells that line the blood stream. These molecules are in turn oxidized for ATP production or stored for future energy requirements (Dominiczak & Caslake, 2011; Kwiterovich, 2000).

Due to the reduced amount of triacylglycerol, the vLDL particle is now considered an LDL particle and must be recycled or can be used to transfer cholesterol to peripheral tissues. To this end, the LDL now exposes a C-terminal domain in ApoB which contains the recognition site for the LDL receptor (Boren et al, 1998). As noted above, this allows for receptor mediated endocytosis of LDL into tissues. After the LDL containing endocytic vesicles fuse with the lysosome and release their contents, cholesterol and triglycerides are produced from cholesteryl

esters and ApoB is degraded by the proteinases that reside in the lysosome (Brown & Goldstein, 1975; Brown & Goldstein, 1976; Kwiterovich, 2000).

1.3 APOLIPOPROTEIN B

1.3.1 Isoforms

ApoB is transcribed from a single 45 kb gene which results in two naturally occurring isoforms. This single transcript creates the ~550 kDa full length ApoB protein, known as ApoB100, as well as an isoform that contains the N-terminal 48% of ApoB, termed ApoB48 (Hussain et al, 2003; Wasan et al, 2008). To produce either isoform, the ApoB transcript is produced as one 15 kb mRNA. In the small intestine, where ApoB48 is required for chylomicron formation, the ApoB mRNA editing complex-1 (ApoBEC-1) is expressed. ApoBEC-1 is the founding member of a large family of RNA editing enzymes that deaminate nucleotides (Blanc & Davidson, 2010; King & Larijani, 2017; Salter et al, 2016). Specifically, ApoBEC-1 deaminates the ApoB100 mRNA at codon 2153. This results in a change from a CAA to UAA, which converts a glutamine into a stop codon in the mRNA transcript (Blanc & Davidson, 2010; Fisher & Ginsberg, 2002; Giannoni et al, 1994; Hadjiagapiou et al, 1994). Once translated, this edited transcript produces a protein which contains the N-terminal 48% of ApoB100 (i.e. ApoB48).

Interestingly, ApoB, the only non-exchangeable apolipoprotein, is essential for lipoprotein synthesis (Gretch et al, 1996; Hussain et al, 2003; Segrest et al, 1992). In fact, a homozygous knockout of ApoB resulted in embryonic lethality in mice, whereas heterozygous knockout mice developed normally but were protected from hypercholesteremia (Farese et al,

1995). Remarkably, over 100 coding and non-coding polymorphisms have been identified in *APOB* and several may cause disease (Benn et al, 2005; Dominiczak & Caslake, 2011). A familial defective form of ApoB arises from a glutamine to arginine substitution at codon 3500, which reduces ApoB's ability to interact with the LDL receptor, affecting ~1 in 500 Caucasians (Innerarity et al, 1990). In contrast, a rare arginine to proline substitution mutation at codon 3480, which may affect the α-helical region in which the amino acid resides, results in hypobetalipoproteinemia (Benn et al, 2005). Two other common substitutions are found in the signal peptide, which affect the ability of ApoB to enter the ER and ultimately be secreted in lipoprotein particles (Blackhart et al, 1986; Sturley et al, 1994).

Another mutation in the ApoB gene results in a truncated protein at codon 1305 by generating a premature stop codon due to a C to T nucleotide transition (Collins et al, 1988; Huang et al, 1989). Even though the resulting protein harbors only the N-terminal 29% of ApoB, lipids still associate with ApoB but lipid poor vLDL particles are produced. This "ApoB29" protein traffics through the secretory pathway as efficiently as larger ApoB variants, but smaller sized lipoprotein particles are evident (Linton et al, 1993; McLeod et al, 1994). Individuals expressing this mutated protein suffer from hypobetalipoproteinemia (Collins et al, 1988).

1.3.2 Structural Features

As ApoB is the main structural component of lipoproteins, it must interact with hydrophobic cholesterol, cholesterol esters, phospholipids, and triacylglycerol molecules to form a lipoprotein particle. However, ApoB must also be able to function in the aqueous environment of blood plasma. Although a crystal structure is currently unavailable, ApoB is believed to contain

multiple α-helical domains which give rise to its amphipathic nature (Figure 2). Based on computer predictions, ApoB is over 25% α-helical, which, is common to the exchangeable apolipoproteins (De Loof et al, 1987; Gotto et al, 1968; Scanu & Hirz, 1968; Singh & Lee, 1986) ApoB is targeted to and translocates into the ER by virtue of the first 27 amino acids, which contain a signal sequence (Sturley et al, 1994). During ApoB translocation into the ER, pause transfer sequences are present throughout the protein to help with lipid loading (see section 1.5). In addition, the N-terminal 1000 amino acids interact with the MTP complex as they form a lipid binding pocket (Dashti et al, 2002).

The ApoB domain structure is organized in a βα1-β1-α2-β2-α3 fashion (Segrest et al, 2001; Segrest et al, 1994). The N-terminus is termed βα1, as it is predicted to form a β-barrel followed by an α-helical region based upon homology to lipovitellin, an egg yolk lipoprotein (Mann et al, 1999; Segrest et al, 2001; Segrest et al, 1994). ApoB has two additional, large β-sheet domains at amino acids 827-2001 and 2571-4032. There are also two smaller α-helical domains containing amino acids 2045-2587 and 4017-4515 (Hussain et al, 2003; Segrest et al, 2001; Segrest et al, 1994). ApoB also contains 2 lipid associating domains, that span amino acids 1701-3101 and 4101-4536 (Segrest et al, 2001; Yang et al, 1989a; Yang et al, 1989b). The LDL receptor binding region resides between amino acids 3345-3381. Specifically, it was determined that once ApoB binds to the LDL receptor, a region between amino acids 2980 and 3780 could no longer bind an antibody raise to this epitope. Furthermore, it was shown that although ApoB67 (containing amino acids 1-3040) was unable to bind the LDL receptor, ApoB75 (containing amino acids 1-3387)retained this activity. By combined with a sequence comparison to the better studied ApoE receptor and ApoB from seven species, the ApoB LDL

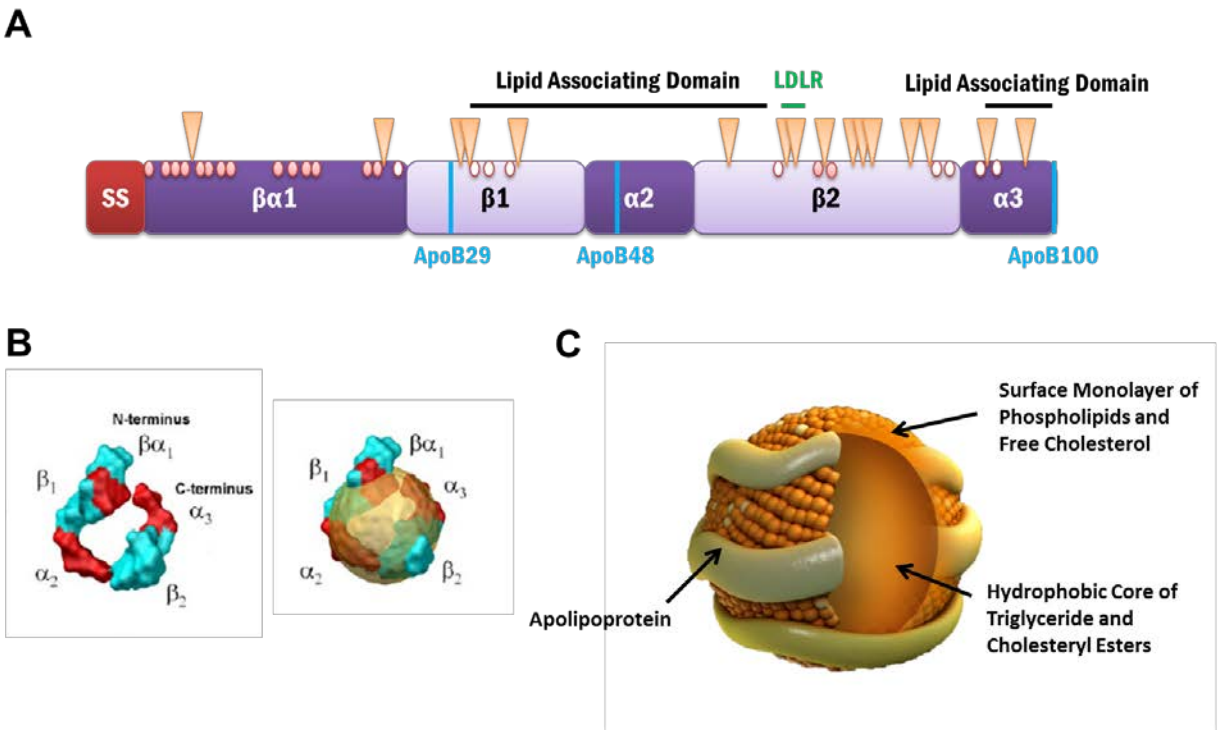


Figure 2. Proposed Structural Domains and Modifications of ApoB.

(A) The predicted ApoB domain structure contains a 27 amino acid signal sequence, which targets ApoB to the ER membrane and allows for translocation into the ER, and a β-barrel domain, which is directly followed by α-helix, β-sheet, α-helix motifs, and is interspersed by 2 lipid association domains. The naturally occurring isoforms, ApoB100 and ApoB48, and the disease causing truncation, ApoB29, are depicted using vertical blue lines. The LDL receptor binding region is found in the β2 domain and is indicated with a horizontal green line. The 16 glycosylated asparagine residues in ApoB are indicated with triangles. Of the 25 cysteine residues, only 16 participate in disulfide bond formation (red circles) while the remaining 9 are free sulfhydryls (open circles).

(B) Computer simulated model of proposed ApoB structure (left) alone and (right) in lipoprotein. Reprinted from *Johs et al. 2006* with permission.

(C) Artistic representation of lipoprotein particle. The apolipoprotein wraps around the circumference of the lipoprotein which consists of phospholipids, free cholesterol, triglycerides, and cholesteryl ester. Reprinted from (Feingold & Grunfeld, 2000)with permission.

receptor binding region was established (Boren et al, 1998; De Loof et al, 1986; Hussain et al, 2003; Krul et al, 1992; Law & Scott, 1990; Milne et al, 1989; Weisgraber, 1994; Welty et al, 1995; Yang et al, 1989a). In addition, it is thought that the N and C termini interact with one another once ApoB is completely synthesized to provide structural rigidity to the spherical lipoprotein particle (Figure 2B and C) (Johs et al, 2006).

As described earlier, ApoB is modified at several steps in the secretory pathway during lipoprotein particle maturation. The two lipid associating domains allow for the conjugation of lipids onto ApoB by the MTP complex, facilitating ApoB's incorporation into a pre-vLDL only when cholesterol and other neutral lipids are abundant. In this way, ApoB is continuously being synthesized but is only able to deliver nutrients when available (see section 1.5). Additionally, ApoB contains 25 cysteine resides but only 16 of these residues are used, to form 8 disulfide bonds (Fisher & Ginsberg, 2002; Harazono et al, 2005). The formation of these bonds help assemble the final structure of the protein and consequently the lipoprotein particle as each domain can fold co-translationally as disulfide bonds are formed. Furthermore, as with any secretory protein, ApoB can be glycosylated by the oligosaccharyltransferase in the ER. ApoB has 19 potential sites for glycosylation, which are designated by an asparagine-X-serine/threonine (where X can be any amino acid except proline), but only 16 are used (Bause & Hettkamp, 1979; Harazono et al, 2005). In total, these secondary modifications help position and stabilize ApoB in the lipoprotein particle during synthesis and while circulating in the blood. Each modification is carefully orchestrated to ensure proper synthesis, folding, and incorporation into lipoprotein particles. For example, if cysteine 4326 is mutated then, ApoB fails to become incorporated into a lipoprotein (Callow & Rubin, 1995). Furthermore, if the cysteines involved in forming disulfide bonds 2 and 4 (amino acids 51, 70, 218, and 234) are

substituted, then ApoB secretion is reduced to 3% of wildtype levels and is unable to adopt its proper conformation a lipoprotein (Huang & Shelness, 1997). Furthermore, if the 12 cysteines located in the N-terminus are mutated, then ApoB similarly fails to form a lipoprotein and the protein is degraded by the proteasome (Tran et al, 1998).

As discussed in Section 1.1.3, studies have focused on modulating ApoB levels directly, namely by antisense oligonucleotides, as a way to directly regulate cholesterol levels (Ricotta & Frishman, 2012; Thomas & Ginsberg, 2010; Wong & Goldberg, 2014). The first proof-of-principle study underlying the clinical approach of targeting the ApoB message was conducted by Zimmerman and colleagues, who used an siRNA against ApoB. The authors observed a reduction in ApoB levels as well as a reduction in circulating LDL and serum cholesterol levels in cytomogolous monkeys. However, non-ApoB containing lipoprotein particles (i.e. HDLs) remained unaffected, which supports the idea that altering ApoB levels can specifically regulate cholesterol delivery (Zimmermann et al, 2006). This and other studies ultimately led to the development of Mipomersen, as discussed above.

1.4 ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION

Nearly one third of all newly synthesized proteins enter the secretory pathway (Ghaemmaghami et al, 2003). Once these proteins enter the ER, they properly fold, establish intradomain interactions, and assemble with additional protein partners or subunits (if necessary). If secreted proteins do not achieve their proper conformations, they risk damaging the cell, which may manifest as human disease (Guerriero & Brodsky, 2012). Therefore, the cell must have a way to monitor and remove such proteins. One way to eliminate aberrant proteins in the secretory

pathway is through ER associated degradation (ERAD). ERAD is a quality control pathway that monitors and selectively degrades misfolded or misassembled secretory proteins.

ERAD is generally divided into four steps: substrate recognition, ubiquitination, retrotranslocation, and degradation (Guerriero & Brodsky, 2012; Preston & Brodsky, 2017; Stevenson et al, 2016). Substrate recognition monitors misfolded proteins which result from an amino acid change in the cytoplasmic, membranous, or luminal region of the protein, or from a protein trapped in an incorrect folding intermediate state as a result of cellular stress (Brodsky, 2007). Nascent proteins entering the ER are monitored by molecular chaperones, which prevent off pathway folding intermediates from occurring, preventing aggregation, and helping to maintain the protein in a productive folding pathway (Balchin et al, 2016; Dobson, 2003). Misfolded proteins commonly expose stretches of hydrophobic amino acids. Molecular chaperones bind these amino acid stretches and prevent hydrophobic regions from interacting and potentially aggregating in the hydrophilic environment of the ER lumen (Dobson, 2003).

Once a misfolded protein is irreversibly misfolded, it is tagged with a polyubiquitin motif, which constitutes the second step of the ERAD pathway. Ubiquitin is a 76 amino acid protein appended to protein substrates and can trigger different events including degradation, localization, and chromatin remodeling (Acconcia et al, 2009; Preston & Brodsky, 2017; Turinetto & Giachino, 2015). Ubiquitin is most commonly added to the side chain of lysine in the protein substrate or can be appended at the N-terminus. Ubiquitin itself contains 7 lysine residues, in addition to the N-terminus, which can be further modified to form linear or branched ubiquitin chains (Komander & Rape, 2012). Polyubiquitin chains built on lysine-48 of the ubiquitin moiety commonly act as signals for degradation of the modified protein substrate by the 26S proteasome (Chau et al, 1989; Komander & Rape, 2012).

Ubiquitin is added to a protein through an enzymatic cascade. First, an E1 activating enzyme activates the ubiquitin molecule through ATP hydrolysis to create a thiol-ester bond to the C-terminus of ubiquitin (Hershko et al, 1983; Pickart, 2001). The activated ubiquitin is then conjugated to an E2 conjugating enzyme which further defines target protein specificity. Finally, the ubiquitin is transferred to an E3 ubiquitin ligase, which in turn attaches ubiquitin onto the protein substrate (Komander & Rape, 2012; Preston & Brodsky, 2017). Ubiquitin chains can also be extended with the help of a class of factors termed E4 ubiquitin chain extension enzymes (Koegl et al, 1999). This cascade is opposed by the action of deubiquitinating enzymes (DUBs) that remove ubiquitin molecules from growing chains or at the proteasome immediately prior to degradation (Stevenson et al, 2016). DUBs have been suggested to play a role in substrate discrimination and the DUB may specifically amplify the difference between a folded protein and an unfolded one through ubiquitin chain amplification (Zhang et al, 2013). Ultimately, by continually adding and removing ubiquitin molecules, the conformation of a protein is monitored until at least 4 ubiquitin moieties remain, which signals protein degradation (Thrower et al, 2000).

Substrate polyubiquitin leads to retrotranslocation, the third step of ERAD. Retrotranslocation of proteins involves removal of the protein from the ER lumen or membrane into the cytoplasm. To extract the protein, mechanical force must be used to pull the protein out of the ER by Cdc48 in yeast or p97 in mammals (Rabinovich et al, 2002; Ye et al, 2001). Cdc48p is a AAA+ ATPase that translates ATP hydrolysis into mechanical force to retrotranslocate proteins into the cytoplasm. This protein forms a complex with two cofactors, Npl4 and Ufd1, which help bind ubiquitinated proteins and recruit Cdc48 at the ER membrane (Ye et al, 2001). A mechanism for the retrotranslocation of proteins was recently put forth based

on a novel reconstituted system (Bodnar & Rapoport, 2017). To retrotranslocate proteins, Npl4 and Ufd1 first bind lysine-48 ubiquitinated substrates. As a result, the substrate is locally denatured, which allows for the extension of a flexible loop of the protein into the Cdc48 cavity. Substrate binding can also stimulate ATPase hydrolysis, which further helps denature the protein as it is translocated through the central cavity. Following hydrolysis, there is a conformational change which places the protein in close contact with a Cdc48-associated DUB, Otu1. Otu1 can remove ubiquitin molecules until association with Npl4 and Ufd1 is lost and the protein is fully translocated and released from Cdc48. Interestingly, not all ubiquitin moieties need to be removed from protein substrates during translocation through the Cdc48 pore.

After the ubiquitinated protein is retrotranslocated from the ER, possibly through an integral membrane protein in the ER known as Hrd1, degradation is accomplished by the 26S proteasome (Schoebel et al, 2017). This complex protease consists of a 20S core particle and a 19S regulatory particle or "cap" (Budenholzer et al, 2017). The 20S core particle contains 4 stacked heptameric rings which facilitate protein degradation. Facing the central cavity of the core particle are the residues responsible for the trypsin-like, chymotrypsin-like, and acidic/caspase-like protease activities used to hydrolyze proteins (Budenholzer et al, 2017; Kunjappu & Hochstrasser, 2014). After recognition by ubiquitin receptors in the cap, the AAA ATPase in this particle drives proteins into the 20S core. Moreover, through their N-terminal tails, the 19S cap can open the 20S core particle to facilitate protein entry into the central cavity of the 20S particle (Smith et al, 2007). As noted above, there are also DUBs associated with the cap to remove the polyubiquitin chain and help denature the protein (Bashore et al, 2015; Elsasser et al, 2002; Shi et al, 2016). In this manner, the 26S proteasome efficiently degrades only polyubiquintinated proteins.

1.5 APOLIPOPROTEIN B IS REGULATED BY ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION

ApoB is a unique ERAD substrate as its levels are cotranslationally regulated by ERAD (Benoist & Grand-Perret, 1997; Liao et al, 1998; Mitchell et al, 1998). This means that the level of ApoB turnover by ERAD is determined during synthesis by the amount of lipids present in the cell but not necessarily by the presence of misfolded ApoB. This decision making process begins as ApoB is continuously translocated into the ER through a proteinaceous channel composed of the Sec61 complex (Mitchell et al, 1998; Robson & Collinson, 2006), which poises ApoB for a quick transition from degradation by ERAD to incorporation into pre-vLDLs (Figure 3A). To form the pre-vLDL, the MTP complex transfers cholesterol, cholesterol esters, triacylglycerols, and phospholipids to ApoB via its lipid associating domains (Hussain et al, 1997; Hussain et al, 2003). As noted above, this process is further facilitated by pause transfer sequences and β-sheets in ApoB (Chuck & Lingappa, 1992; Kivlen et al, 1997; Yamaguchi et al, 2006) (see also section 1.3.2). These features slow translocation, which confers additional time for lipids to become attached to ApoB. Once fully lipidated, translated, and translocated into the ER, ApoB enters the secretory pathway to become a mature lipoprotein particle (see section 1.2.2).

When cholesterol is not adequately supplied by the diet and therefore the presence of ApoB and vLDLs is not required (Figure 3B). In this case, cells synthesize their own supply of cholesterol. Therefore, ApoB translocation slows, and the ribosome can slightly detach from the ER, through an unknown mechanism (Dixon et al, 1991). As ApoB translation still occurs, large loops of ApoB are exposed to cytoplasmic factors, including molecular chaperones and the ubiquitination machinery (Fisher et al, 1997; Pariyarath et al, 2001; Yeung et al, 1996). However, these events can also occur if MTP complex function is compromised or if ApoB

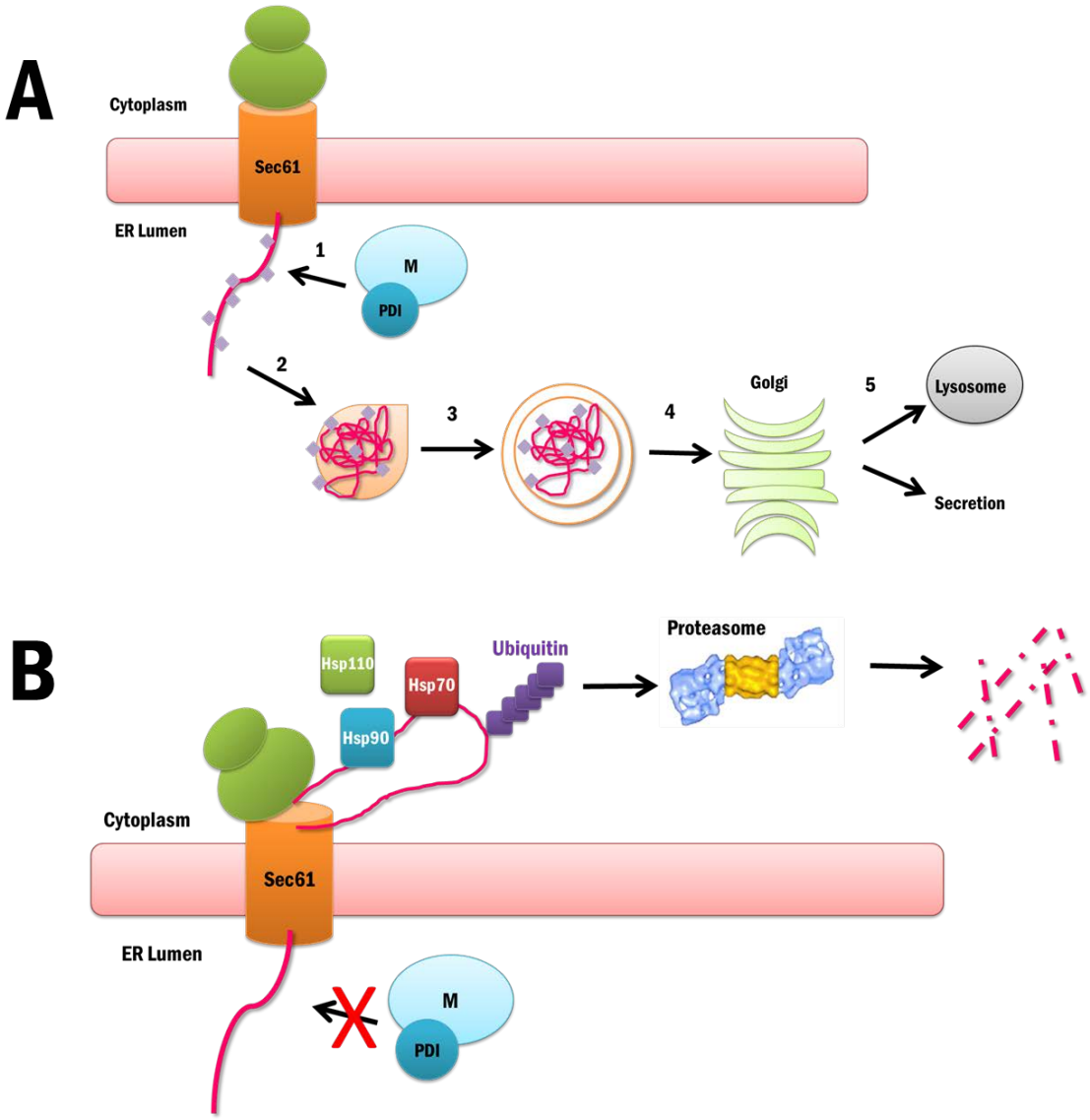


Figure 3. ApoB is Metabolically Regulated by ERAD.

(A) When lipids are in excess, ApoB is required for the synthesis of a pre-vLDL. (1) ApoB is cotranslationally translocated into the ER where the MTP complex attaches lipids (purple diamonds) to ApoB. (2) This results in a pre-vLDL particle that contains ApoB. (3) ApoB exits the ER in a non-canonical COPII coated vesicle. (4) The pre-vLDL can undergo further maturation in the Golgi apparatus. (5) If the particle fully matures, it will be secreted and enter

the bloodstream to deliver cholesterol. If the particle does not fully mature, it can also be sent to the lysosome to be degraded by post ER presecretory proteolysis.

(B) If lipids are limiting or MTP function is blunted, then ApoB does not become lipidated. Translocation slows which exposes large cytoplasmic loops of ApoB. These loops can be acted upon by chaperones and the ubiquitination machinery. Once the loops are ubiquitinated, ApoB is retrotranslocated and targeted to the proteasome for degradation.

cannot be properly lipid loaded. These chaperones that interact with ApoB include the ER luminal lectin, Calnexin, and PDI family members along with cytosolic Hsp70, Hsp90, and Hsp110, which either help to promote (Hsp70, Hsp90, PDIs) or inhibit (Hsp110) ApoB degradation (Fisher et al, 1997; Grubb et al, 2012; Gusarova et al, 2001; Hrizo et al, 2007; Liao et al, 1998). Eventually, ApoB is ubiquitinated on the exposed cytoplasmic loops by the E3 ubiquitin ligase Hrd1 (in yeast) or gp78 (in humans) (Jiang & Song, 2014; Liang et al, 2003; Rubenstein et al, 2012). Finally, ApoB is retrotranslocated by the p97 ATPase and degraded by the 26S proteasome (Cardozo et al, 2002; Fisher et al, 2008). As a result of these events, ApoB fails to enter the secretory pathway and cannot be secreted.

1.6 APOLIPOPROTEIN B DEGRADATION BY POST ER PRESECRETORY PROTEOLYSIS

ApoB is also subject to an alternate degradation pathway, post ER presecretory proteolysis (PERPP). Following translation and translocation, ApoB folding and pre-vLDL maturation may also fail in the late secretory pathway or ApoB can be oxidized by polyunsaturated fatty acids after transport from the ER. These aberrant and/or modified ApoB species are degraded by PERPP, which unlike ERAD, degrades ApoB in the presence of normal levels of triglycerides. PERPP is a specialized form of autophagy that occurs in the Golgi during maturation of the vLDL particle (Brodsky & Fisher, 2008; Fisher et al, 2001; Ginsberg & Fisher, 2009; Pan et al, 2004). As noted in section 1.1.3, autophagy can degrade proteins, cellular aggregates, and damaged organelles by delivering these particles to the lysosome through a double membrane bound vesicle (Levine & Kroemer, 2008; Yun et al, 2017).

1.6.1 N-3 Fatty Acid Induced Degradation

PERPP occurs when an immature vLDL cannot undergo complete maturation but has exited the ER. If a pre-vLDL exhibits a defect or oxidative damage and cannot mature, it may aggregate in the Golgi (Fisher et al, 2001; Pan et al, 2004). Therefore, it must be degraded, but the resulting aggregates are likely too large and complex to be targeted for proteasomal degradation. In this case, the particles are sequestered and delivered to the lysosome for degradation. Consistent with this idea, when the proteasome is inhibited (and the levels of polyubiquitinated and aggregation-prone ApoB rise), increased amounts of ApoB are found in the lysosome (Ohsaki et al, 2006). PERPP is specifically stimulated by N-3 fatty acids, such as eicosapentaenoic and docosahexaenoic acid, which cause oxidative damage and increased intracellular degradation of ApoB (Fisher et al, 2001; Pan et al, 2004; Wang et al, 1993). Interestingly, a diet rich in these N-3 fatty acids correlates with lower triglyceride level (Djousse et al, 2003; Harris, 1989). This may be a result of stimulating the PERPP of ApoB, which lowers circulating LDL particles.

1.6.2 Insulin Induced Degradation

ApoB degradation by PERPP can also be stimulated by an acute increase in insulin, which decreases vLDL and ApoB secretion (Biddinger et al, 2008; Chirieac et al, 2000; Sparks & Sparks, 1990; Taghibiglou et al, 2000). This process is mediated through MAP kinase, TNFα, and PI3-kinase (Phung et al, 1997; Qin et al, 2007; Tsai et al, 2007). Specifically, insulin activates the downstream PI3-kinase which leads to the production of PI_3P, preventing ApoB from acquiring triglycerides and forming vLDL, (Au et al, 2004; Chirieac et al, 2006; Phung et al, 1997; Sparks & Sparks, 2008). ApoB and vLDL secretion rise in insulin resistance and type 2

diabetes mellitus, suggesting that insulin resistance may be a consequence of heightened ApoB secretion (Khavandi et al, 2017).

1.7 CONCLUSION

In attempt to treat a multitude of diseases including multiple cancers, attempts to target chaperones and other pathway regulators have been attempted. One approach is the use of small molecule inhibitors, protein aptamers, and antibodies against different domains of Hsp70. For example, classes of compounds including dihydropyrimides, (e.g. MAL3-101 and SW02) and flavonoids (e.g. epigallacatchin and myricetin) target the nucleotide binding domain of Hsp70 (Chatterjee & Burns, 2017). Protein aptamers target the substrate binding domain, and an antibody therapy targets an epitope of Hsp70 (Chatterjee & Burns, 2017). The flavonoid, epigallocetchin, and one specific antibody against Hsp70 are undergoing clinical trials (Chang et al, 2011; Goloudina et al, 2012; Powers et al, 2010; Rerole et al, 2011). Similarly, inhibitors of Hsp90 have been identified and are undergoing clinical trials for treatment of cancers. These compounds appear to inhibit the ATPase activity and/or tightly bind to Hsp90 to prevent its function (Renouf et al, 2016; Wagner et al, 2016; Yong et al, 2016). Interestingly, a new class of compounds which enhances Hsp70 activity has also been recently identified (Wisen et al, 2010). One of the compounds, 115-7c, appears to bind at the hsp70-hsp40 interface, to which may regulate interaction (Wisen et al, 2010).

Both the ERAD and PERPP pathways for ApoB degradation are regulated by various factors, including lipids, post-translational modifications, molecular chaperones, and hormones.

Since ApoB levels directly correlate with circulating LDLs, vLDLs, serum cholesterol, and triacylglycerol, I hypothesize that factors that regulate ApoB could also be therapeutic targets to regulate cholesterol levels. Already, a small molecule inhibitor of Hsp90, geldanamycin, has been shown to nearly completely prevent ApoB degradation in an *in vitro* transcription/translation assay (Gusarova et al, 2001). Similarly, inhibiting the proteasome via ALLN prevented ApoB degradation while overexpression of Hsp70, which binds ApoB, promoted ApoB degradation in mammalian cells (Fisher et al, 1997; Zhou et al, 1995). Thus, these and other factors may represent new and alternative approaches to treat CAD. In this thesis, I describe a new ApoB *Saccharomyces cerevisiae* expression system that I developed and then used to identify additional regulators of ApoB degradation. Specifically, I investigated the role of lipid droplets as a site of short term storage prior to degradation. I subsequently investigated a variety of chaperones that have potential "holdase," "foldase," and "disaggregase" activity towards ApoB, including the Tcp-1 ring complex, the small heat shock proteins, and Hsp104.

2.0 HSP104 FACILITATES APOLIPOPROTEIN B DEGRADATION

ApoB is a large, hydrophobic secretory protein that translocates into the ER. ApoB levels are controlled via its degradation. If it is misfolded or not adequately lipidated, then it is degraded via the ERAD pathway. During ERAD, ApoB translation slows, exposing cytoplasmic loops which become ubiquitinated signaling for retrotranslocation into the cytoplasm and degradation by the 26S proteasome (Cardozo et al, 2002; Dixon et al, 1991; Fisher et al, 2008; Fisher et al, 1997; Pariyarath et al, 2001; Rubenstein et al, 2012; Yeung et al, 1996). Once ApoB has been retrotranslocated, it has the potential to aggregate. These aggregates are likely one of the triggers for degradation.

Previous work established that soluble, luminal ERAD substrates (mutant forms of carboxypeptidase Y, CPY*, and proalpha factor, ΔGpF) also have the potential to aggregate in the ER (Nishikawa et al, 2001). Using yeast strains that lack two ER-localized Hsp40s, Scj1 and Jem1, the authors showed that in cooperation with the ER luminal Hsp70, Kar2, the Hsp40s helped to facilitate CPY* and ΔGpF degradation. Moreover, impairment of Kar2 or Scj1 and Jem1 lead to aggregation of CPY* and ΔGpF as assessed via sucrose gradient fractionation. The authors proposed that these factors help maintain protein solubility in the ER, since aggregation could prevent retrotranslocation and result in ER stress. Interestingly, some ERAD substrates perpetually aggregate in the ER and cause diseases such as α-1-antitrypsin deficiency(Silverman et al, 2013).

To prevent ApoB aggregation during retrotranslocation, I hypothesize that cellular factors, such as lipid droplets or specific chaperones, interact with ApoB to maintain its solubility. Lipid droplets are ER-associated organelles which store lipids for the cell to use during stressful times but have also been shown to interact with aggregation prone proteins before they are degraded (Cole et al, 2002; Farese & Walther, 2009). Holdase chaperones physically interact with their substrate to help prevent protein aggregation. These chaperones include ATP dependent factors, such as TRiC and Hsp104, and ATP independent factors, such as the small heat shock proteins. TRiC is important for folding ~5% of all newly synthesized proteins, including actin and tubulin (Joachimiak et al, 2014). Hsp104 is a AAA+ ATPase that cooperates with Hsp70 and Hsp40 to remove proteins from aggregates and help them refold (Shorter, 2017). The small heat shock proteins are conserved chaperones that physically interact with their substrate by binding through their conserved α-crystallin domain (Burnie et al, 2006). Among these factors, only the small heat shock proteins have been shown to function during ERAD, and even in this case only one substrate was affected (Ahner et al, 2007). With the exception of Hsp104, all of these factors are conserved from yeast to humans. In order to investigate if these factors affect ApoB degradation, I developed a new β-estradiol ApoB expression for use in the yeast *Saccharomyces cerevisiae*. In this chapter, I discuss the development of the new expression system and then my investigation to determine if lipid droplets or the various holdase chaperones affect ApoB degradation.

2.1 MATERIALS AND METHODS

2.1.1 Yeast strains, strain construction, plasmids, and growth conditions

Unless otherwise noted, yeast strains were grown at 30°C using standard growth, media, and transformation conditions (Adams et al, 1998). Yeast strains utilized in this study are listed in Table 1.

Yeast strains were made β-estradiol inducible by linearizing pACT1-GEV (Veatch et al, 2009) (Allyson O'Donnell, Duquesne University) using EcoRV (New England Biolabs) according to the manufacturer's specifications. Linearized plasmid was transformed into log phase yeast cells and integrated at *leu2Δ0* using the standard lithium acetate protocol (Adams et al, 1998). Transformants were selected on YPD plates containing 0.1 mg/mL Nourseothricin (Werner Bioagents, Jena, Germany). Positive transformants were restruck 3 times onto YPD plates supplemented with the antibiotic to ensure successful integration.

To drive expression of ApoB29 in yeast from the GAL promoter, plasmid pSLW1-B29-HA was used (Hrizo et al, 2007). Plasmid pJJB20, which lacks the ApoB29 coding sequence, was used as a negative control vector for ApoB29 as was previously described (Hrizo et al, 2007). To constitutively express Hsp104, plasmid pRS315-Hsp104 plasmid was used (Preston *et al.* in review). To constitutively express Rvb2, pRS425-Rvb2 was used. pRS425-Rvb2 was constructed by digesting pFL44-Rvb2 (Yury Chernov, Georgia Institute of Technology) and ligating the insert into pRS425 (Mumberg et al, 1995) using the *BamHI* and *SalI* sites.

Table 1. Yeast Strains Utilized.

Strain	Genotype	Source
W303	*MAT α, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*	This lab
LDY020	*MAT α, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, ACT1-GEV-NatMX::leu2Δ0*	This study
pdr5Δ	*MAT α, ade2-1 can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdr5::KANMX4, ACT1-GEV-NatMX::leu2Δ0*	This lab
LDY001	*MAT α, ade2-1 can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdr5::KANMX4,*	This study
BY4742	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0*	This lab
LDY022	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0 ACT1-GEV-NatMX::leu2Δ0*	This study
pep4Δpdr5Δ	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0, pdr5::KANMX4, pep4::KANMX4*	This lab
LDY027	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, met15Δ0, pdr5::KANMX4, pep4::KANMX4, ACT1-GEV-NatMX::leu2Δ0*	This study
MHY500	*MAT a, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2*	Rubenstein *et al.*, 2012.
hrd1Δ	*MAT a, his3- Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, hrd1::LEU2*	Rubenstein *et al.*, 2012.
doa10Δ	*MAT a, his3- Δ200 leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, doa10::HIS3*	Rubenstein *et al.*, 2012.
hrd1Δdoa10Δ	*MAT a, his3- Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, hrd1::LEU2, doa10::HIS3*	Rubenstein *et al.*, 2012.
LDY043	*MAT a, his3- Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY044	*MAT a, his3- Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, hrd1::LEU2, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY045	*MAT a, his3- Δ200 leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, doa10::HIS3, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY046	*MAT a, his3- Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, hrd1::LEU2, doa10::HIS3, ACT1-GEV-NatMX::leu2Δ0*	This study
png1Δ	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, png1::KANMX4*	This lab
LDY026	*MAT α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0, png1::KanMX4, ACT1-GEV-NatMX::leu2Δ0*	This study

H1246 Lipid Droplet Deficient	*MAT a, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, are1::HIS3, are2::LEU2, dga1::KANMX4, lro1::TRP1ADE2*	Sandager et al. 2002.
Tgl3-GFP	*MAT a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0,* TGL3::GFP-HIS3MX6	Athenstaedt and Daum, 2003.
LDY003	*MAT a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0,* TGL3::GFP-HIS3MX6, *ACT1-GEV-NatMX::leu2Δ0*	This study
SEY6211	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL*	Haslbeck *et al.*, 2004.
hsp26Δ	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL, hsp26::HIS*	Haslbeck *et al.*, 2004.
hsp42Δ	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL. hsp42::LEU*	Haslbeck *et al.*, 2004.
hsp26Δhsp42Δ	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL, hsp26::HIS, hsp42::LEU*	Haslbeck *et al.*, 2004.
LDY002	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY016	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL, hsp26::HIS, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY017	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL. hsp42::LEU, ACT1-GEV-NatMX::leu2Δ0*	This study
LDY018	*MAT a, ura3-52, leu2-3, 112, his-d200, trp1-d901, ade2-101, suc2-d9 GAL, hsp26::HIS, hsp42::LEU, ACT1-GEV-NatMX::leu2Δ0*	This study
DUY558	*MAT a, leu2-3,-112, ura3-52, trp1-7 tcp::LEU2 (YCpMS38; TCP1::TRP,)*	Ursic and Culbertson, 1991.
LDY028	*MAT a, leu2-3,-112, ura3-52, trp1-7 tcp::LEU2 (YCpMS38; TCP1::TRP1), ACT1-GEV-NatMX::leu2Δ0*	This study
DUY326	*MAT a, leu2-3,-112, ura3-52,trp1-7 tcp::LEU2 (YCpMS38; tcp1-2::TRP1)*	Ursic and Culbertson, 1991.
LDY029	*MAT a, leu2-3,-112, ura3-52,trp1-7 tcp::LEU2 (YCpMS38; tcp1-2::TRP1), ACT1-GEV-NatMX::leu2Δ0*	This study
hsp104Δ	*MAT α, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, hsp104::KANMX4*	Preston *et al*, in review
LDY021	*MAT α, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, hsp104::KANMX4, ACT1-GEV-NatMX::leu2Δ0*	This study

To monitor protein production, yeast were grown overnight in synthetic minimal media lacking uracil supplemented with 2% glucose at 30°C. Cultures were diluted in the same media and allowed to grow to log phase (OD_{600} = 0.4-0.6). A total of 16 OD_{600}s of cells were harvested, washed once in water, and resuspended in synthetic minimal media supplemented either with either 2% galactose (to induce expression) or 2% glucose supplemented with 300 nM β-estradiol (to induce ApoB29 expression in the GEV strain; see section 2.2.1) and were incubated at 30°C. At the indicated timepoints, equal amounts of cells were harvested. Proteins were precipitated using tricholoroacetic acid (TCA) as previously described (Zhang et al, 2001) and immediately resolved by SDS-PAGE, followed by western blotting. Immunoblots were incubated with anti-HA (for ApoB) and anti-G6PD (as a loading control) antibodies as described below.

To cure strains of prions, wildtype and *hsp104Δ* yeast were grown on YPD plates supplemented with 3 mM guanidine hydrochloride and grown at 30°C (Jung et al, 2002). Individual yeast colonies were streak plated twice more onto YPD plates supplemented with 3 mM guanidine hydrochloride, for a total of 3 times.

2.1.2 Antibodies and Quantitative Western Blotting

Antibodies used in this study are as follows: horseradish peroxidase (HRP) conjugated rat anti-HA (Roche Applied Science, 3F10) used at 1:5000 dilution; rabbit anti-glucose 6 phosphate dehydrogenase (G6PD) (Sigma) used at 1:5000 dilution; HRP conjugated donkey anti-rabbit (GE Healthcare) used at 1:5000 dilution; rabbit anti-Protein Disulfide Isomerase (PDI) (Dr. Vlad Denic, Harvard University) used at 1:5000 dilution; rabbit anti-Kar2 (Brodsky & Schekman,

1993) used at 1:5000 dilution; rabbit anti-Anp1 (Hugh Pelham, MRC Laboratories of Molecular Biology) used at 1:4000 dilution; rabbit anti-Sec61 (raised again peptide: LVPGFSDLM and isolated by Cocalico Biologicals, Stevens, PA) used at 1:1000 dilution; mouse anti-Pma1 (Abcam) used at 1:1000 dilution; mouse monoclonal anti-GFP (Roche) used at 1:1000 dilution**;** HRP conjugated monoclonal anti-mouse (Cell Signaling Technology) used at 1:5000 dilution; mouse monoclonal anti-Vph1 (Abcam, 10D7A7B2) used at 1:5000 dilution; rabbit anti-Hsp104 (Dr. John Glover, University of Toronto) used at 1:1000 dilution; rabbit anti-Sse1 (Hrizo et al, 2007) used at 1:5000 dilution; rabbit anti-Ssa1 (Hrizo et al, 2007) used at 1:5000 dilution; rabbit anti-L5 (Dr. John L. Woolford, Carnegie Mellon University) used at 1:1000 dilution.

Following SDS-PAGE, proteins were transferred to nitrocellulose overnight and incubated with the appropriate antibodies. Proteins were visualized using the SuperSignal West Pico Chemiluminescent substrate kit (ThermoFisher Scientific) or SuperSignal West Femto Maximum Sensitivity chemiluminescent substrate kit (ThermoFisher Scientific). Images were obtained and quantified using either a Kodak 440CF Image station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY) or BioRAD Universal Hood II Imager and ImageJ software version 1.48v (National Institutes of Health). Unless otherwise stated, all quantitation shown describes both ApoB molecular weight species. All quantitation is done for each species individually and both bands together. Half-lives were calculated using Sigmaplot.

2.1.3 Protein Degradation Assays

ApoB degradation assays using the galactose inducible system with plasmid pSLW1-B29-HA were performed at 30°C as previously described (Grubb et al, 2012; Hrizo et al, 2007). To

determine the rate of ApoB29 degradation using the β-estradiol inducible system, yeast harboring the pACT1-GEV insert and transformed with pSLW1-B29 were grown overnight in synthetic minimal media lacking uracil but supplemented with 2% glucose at 30°C. Overnight cultures were diluted and grown to logarithmic phase (OD_{600} = 0.4-0.6) for at least 2 hours. ApoB29 protein expression was then induced using 300 nM β-estradiol for 2 hours at 30°C. Equal amounts of yeast (10 OD_{600} equivalents) were harvested and resuspended to 5 OD_{600}/mL in synthetic minimal media lacking uracil supplemented with 2% glucose and 300 nM β-estradiol. Cycloheximide chase assays were conducted at 30°C or 37°C, as indicated, similar to previously published protocols (Grubb et al, 2012; Hrizo et al, 2007). Protein synthesis was stopped using 50 µg/mL cycloheximide, and at the indicated timepoints, 2 OD_{600} units of cells were harvested. Proteins were precipitated as previously described (Zhang et al, 2001) and samples were immediately resolved by SDS-PAGE followed by western blotting. Immunoblots were incubated with anti-HA and anti-G6PD antibodies as described above.

2.1.4 Isolation of Yeast Microsomes

A total of 2 L of yeast containing plasmid pSLW1-B29-HA were grown to log phase (OD_{600} = 0.4-0.6) in synthetic minimal media lacking uracil supplemented with 2% glucose. ApoB protein expression was induced with 300 nM β-estradiol and cells were grown at 30°C for 2 hours. The yeast cells were then harvested and resuspended in 1/40th of the original volume using minimal media lacking uracil supplemented with 2% glucose and 300 nM β-estradiol and then cells were incubated at 30°C or 37°C for 1 hour, as indicated, in a shaking water bath. The cells were next harvested, washed in water, and frozen at -80°C.

Medium scale microsomes were prepared as previously described (Nakatsukasa et al, 2008). In brief, the cells were lysed in 20 mM HEPES, pH 7.4, 50 mM KOAC, 2 mM EDTA, 0.1 M sorbitol, 1 mM DTT plus protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A) by glass bead agitation on a Vortex mixer 10 times in 30 second intervals with incubations on ice in between each step. After the lysate was removed, the beads were rinsed twice with buffer, which was combined with the lysate, and the mixture was layered onto a sucrose cushion (20 mM HEPES, pH 7.4, 50 mM KOAc, 1.0 M sucrose, 1 mM DTT), and centrifuged at 6,500 rpm for 10 minutes at 4°C in an HB-6 rotor (Sorvall). The supernatant was collected and centrifuged again at 9,500 rpm for 10 minutes at 4°C in an HB-6 rotor. The pellets were washed in Buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc), recentrifuged as above, and the final pellet was resuspended in Buffer 88 to a final concentration of 10 mg/mL (OD_{280} = 40 in 2% SDS). Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

2.1.5 Biochemical Methods

To visualize the EndoH sensitive pool of ApoB, yeast transformed with pSLW1-B29-HA were grown to log phase (OD_{600} = 0.4-0.6) at 30°C and ApoB protein expression was induced using 300 nM β-estradiol for 2 hours at 30°C. Equal amounts of protein were harvested, precipitated, and resuspended in sample buffer as previously described (Zhang et al, 2001). Samples were treated in the presence or absence of Endoglycosidase H (Roche) for 2 hours at 37°C according to the manufacturer's specifications. Proteins were subsequently resolved by SDS-PAGE and

immunoblot analysis was conducted with anti-HA, anti-G6PD, and anti-PDI antibodies, as described above.

To perform limited proteolysis studies, 200 mg of microsomes, prepared as described above, were combined with Buffer 88 on ice. The “0 minute” timepoint was removed, mixed with final concentration of 30% TCA, and stored on ice. The remaining reaction was incubated with 0.04 mg/mL of Proteinase K (Sigma) on ice and samples were taken at the indicated timepoints, combined with a final concentration of 30% TCA, and stored on ice. All samples were then centrifuged at 13,000 rpm, for 10 minutes at 4°C in a microcentrifuge. The pellets were washed with acetone, air dried, and resuspended in TCA sample buffer (80 mM Tris, pH 8, 8 mM EDTA, 120 mM DTT, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue). The final samples were heated to 37°C for 20 minutes and resolved by SDS-PAGE followed by western blotting. Immunoblots were incubated with anti-HA, anti-Kar2, and anti-Sec61 antibodies, as described above.

Carbonate extraction was conducted on yeast expressing ApoB essentially as described (Buck et al, 2016). In brief, yeast cells containing pSLW1-B29-HA were grown at 30°C until log phase (OD_{600} = 0.4-0.6) in synthetic minimal media lacking uracil and supplemented with 2% glucose. ApoB protein expression then was induced using 300 nM β-estradiol for 2 hours at 30°C. The yeast were harvested and resuspended in IP Buffer I (20 mM HEPES, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.1 M sorbitol) plus protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A) and lysed using glass bead agitation on a Vortex mixer 4 times in 1 minute intervals with incubations on ice in between each step. Unbroken cells were removed by centrifugation at 2,500 rpm for 3 minutes at 4°C in a microcentrifuge. Next, the supernatant was removed and centrifuged to isolate crude membranes at 14,000 rpm for 20 minutes at 4°C in a

microcentrifuge. Membranes were resuspended in IP Buffer I plus protease inhibitors and recentrifuged as above. Finally, the resuspended membranes were incubated with either 0.1 M Na_2CO_3 or Buffer 88, both which were supplemented with protease inhibitors, and incubated on ice for 30 min. The samples were centrifuged at 50,000g for 1 hour at 4°C in a SW 55 Ti rotor (Beckman) and the supernatant and pellet samples were saved. The pellets were resuspended in 0.1 M Na_2CO_3 or Buffer 88, as appropriate, and centrifuged at 60,000g for 10 minutes at 4°C in a SW 55 Ti rotor. The final pellets were resuspended in TCA sample buffer using a mechanical pestle. The supernatant samples were mixed with a final concentration of 5% TCA and incubated on ice for 15 minutes. These samples were centrifuged at 14,000 rpm for 10 minutes at 4°C in a microcentrifuge and pellets were resuspended in TCA sample buffer using a mechanical pestle. Supernatant and pellet samples were incubated at 37°C for 20 minutes followed by SDS-PAGE and western blotting. Immunoblots were incubated with anti-HA, anti-Sec61, and anti-PDI antibodies, as described above.

2.1.6 Sucrose Gradient Sedimentation

The intracellular localization of ApoB by equilibrium density sucrose gradient analysis was examined essentially as described (O'Donnell et al, 2017). In brief, yeast containing pSLW1-B29-HA were grown overnight in synthetic minimal media lacking uracil and supplemented with 2% glucose at 30°C. Overnight cultures were diluted and grown to logarithmic phase (OD_{600} = 0.4-0.6) for at least 2 hours. Either 80 or 150 OD_{600} equivalents of cells were harvested at 3000 rpm for 3 minutes at room temperature in a clinical centrifuge. The yeast were washed in water and resuspended in twice the OD_{600} equivalent volume of YP supplemented with 2% galactose.

Expression of the ApoB29 protein was induced for 4 hours at 30°C. Next, the yeast expressing ApoB were resuspended in 400 µL of 10% sucrose and flash frozen in liquid nitrogen in a dropwise fashion. The frozen yeast pellets were ground with a mortar and pestle(15 strokes, 6 times with liquid nitrogen being added in between), and the samples were thawed and centrifuged for 2 minutes at 2000 rpm at 4°C. Five percent of the supernatant was saved for the input fraction and the remaining lysate was loaded onto the gradient (see below).

In parallel, a 70% sucrose stock solution (70% sucrose, 10 mM Tris, pH 7.6, 2 mM $MgCl_2$, 1 mM DTT) supplemented with protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A) was used to created sucrose dilutions of 10-60%. Discontinuous sucrose gradients of either 20-70% sucrose or 10%, 35%, 50%, 70% sucrose were poured. For the 20-70% gradients, 2 mL of 70%, 60%, 50%, and 40% and 1.5 mL of 30% and 20% sucrose were layered. For the 10%, 35%, 50%, 70% gradients, 1.5 mL of 10% and 70% and 4 mL of 35% and 50% were layered as appropriate. After the lysate, prepared as described above, was overlaid at the top of the gradients, the samples were centrifuged at 28,500 rpm at 4°C for 18.5 hours in a SW-41 rotor. Fractions (500 µL) were carefully removed from the top of the gradient. A 20 µL aliquot of each fraction was then combined with 5µL 5X SDS sample buffer (0.325 M Tris, pH 6.8, 10% SDS, 50% glycerol, 25 mg/mL bromophenol blue, 5% β-mercaptoethanol), incubated at room temperature for 10 minutes, and subjected to SDS-PAGE followed by western blotting. Immunoblots were incubated with anti-HA, anti-Anp1, anti-Sec61, anti-Pma1, and anti-GFP antibodies, as described above.

2.1.7 Lipid Droplet Isolation

Lipid droplets were isolated essentially as described (Leber et al, 1994). In brief, 2 L of yeast containing plasmid pSLW1-B29 were grown at 30°C to log phase (OD_{600} = 0.4-0.6) in synthetic minimal media lacking uracil and supplemented with 2% glucose. ApoB protein expression was induced using 300 nM β-estradiol for 2 hours at 30°C and cells were harvested and stored at-80°C. The cell pellets were then resuspended in 100 mM Tris, pH 9.4, supplemented with 10 mM DTT, and incubated at room temperature (~21°C) for 15 minutes. The cells were again harvested in an HB-6 rotor at 5,000 rpm for 5 minutes at 4°C and resuspended in lyticase buffer (0.7 M sorbitol, 75% Yeast extract and Peptone, 0.5% glucose, 10 mM Tris, pH 7.4) supplemented with 5 mM DTT. The cell walls were removed with 2 mL lyticase for 15 minutes at 30°C in a shaking water bath and the spheroplasted yeast were overlaid onto a sucrose cushion (0.8 M sucrose, 1.5% Ficoll 400, 20 mM HEPES, pH 7.4) and centrifuged in an HB-6 rotor at 6,000 rpm for 10 minutes at 4°C. The pelleted spheroplasts were next resuspended in wash buffer (20 mM KPO_4, pH 7.4, 1.2 M sorbitol) and recentrifuged as above. The pellet was resuspended in breaking buffer (10 mM MES-Tris, pH 6.9, 12% Ficoll 400, 0.2 mM EDTA) plus protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A) and cells were lysed with 15 slow strokes using a loose fit Dounce homogenizer on ice. Next, the homogenate was diluted with 1 volume of breaking buffer plus protease inhibitors and centrifuged in an HB-6 rotor at 5,500 rpm for 5 minutes at 4°C. The supernatant was removed and then overlaid with an equal volume of breaking buffer plus protease inhibitors and centrifuged in a SW-28 swinging bucket rotor (Beckman) at 28,000 rpm for 1 hour at 4°C. The floating layer was mixed with 1 volume of breaking buffer plus protease inhibitors, and mixed using a loose fit Dounce homogenizer with 10 strokes on ice. This sample was overlaid with an equal volume of 10 mM

MES-Tris, pH 6.9, 8% Ficoll 400, 0.2 mM EDTA plus protease inhibitors, and this mixture was again ultracentrifuged at 28,000 rpm for 1 hour at 4°C in SW-28 rotor. The top floating layer was removed and gently combined with an equal volume of 10 mM MES-Tris, pH 6.9, 0.6 M sorbitol, 8% Ficoll 400, 0.2 mM EDTA plus protease inhibitors. This sample was overlaid with an equal volume of 10 mM MES-Tris, pH 6.9, 0.25 M Sorbitol, 0.2 mM EDTA plus protease inhibitors and ultracentrifuged at 28,000 rpm for 30 minutes at 4°C in SW-28 rotor. Highly enriched lipid droplets were isolated from the top of the gradient, mixed with a final concentration of 10% TCA, incubated on ice for 20 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C in a microcentrifuge. The pelleted samples were washed with acetone, recentrifuged, and the pellets were allowed to air dry. The final pellets were resuspended in TCA sample buffer using a mechanical pestle and were run on SDS polyacrylamide gels followed by western blotting. Immunoblots were incubated with anti-HA, anti-GFP, anti-Vph1, and anti-Sec61 antibodies, as described above.

2.1.8 Coimmunoprecipitation Assays

Yeast containing pSLW1-B29-HA were grown at 30°C to log phase (OD_{600} = 0.4-0.6) in synthetic minimal media lacking uracil supplemented with 2% glucose. The ApoB protein was induced using 300 nM β-estradiol for 2 hours at 30°C and a total of 100 OD_{600} cells were harvested and stored at -80°C. Next, the thawed cells were resuspended in 1 mL lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40) plus protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A) and cOmplete EDTA-free protease inhibitor cocktail (Roche). The cells were then lysed using glass bead agitation on a Vortex mixer 6 times for 1 minute

intervals on ice in between each lysis step and the lysate was centrifuged at 5000 rpm for 2 minutes at 4°C in a refrigerated microcentrifuge. The supernatant was saved and recentrifuged at 5000 rpm for 2 minutes at 4°C in a refrigerated microcentrifuge. The final supernatant was then precleared using 30 µL of Protein G Fast Flow Sepharose (GE Healthcare) resuspended in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA) for 45 minutes at 4°C. Samples were centrifuged at 5000 rpm for 2 minutes at 4°C in a refrigerated microcentrifuge and the supernatant was isolated and 5% was reserved as the loading control. The remaining supernatant was diluted to 1.5 mL using lysis buffer supplemented with protease inhibitors and the protease inhibitor cocktail and ApoB was immunoprecipitated overnight with rotation at 4°C with 5 µL anti-ApoB (Millipore Sigma) antibody. Incubations in the absence of added antibody served as a negative control. All samples were then incubated with 50 µL Protein G Fast Flow Sepharose for 2 hours at room temperature and the beads were centrifuged at 5000 rpm for 2 minutes at 4°C. After the supernatant was removed, the beads were washed twice with lysis buffer and twice with lysis buffer supplemented with 300 mM NaCl. Both solutions were supplemented with protease inhibitors and the protease inhibitor cocktail. The final, collected beads were resuspended in TCA sample buffer. Input samples were incubated with 10% TCA, incubated on ice 15 minutes, and centrifuged for 10 minutes at 14,000 rpm at 4°C in a microcentrifuge. The final pellets were resuspended in TCA sample buffer using a mechanical pestle. Input and IP samples were incubated at 37°C for 20 minutes, and subsequently subjected to SDS-PAGE and western blotting. Immunoblots were incubated with anti-HA, anti-Hsp104, anti-Ssa1, anti-Sse1, and anti-rpL5, as described above.

2.1.9 *In Vitro* Retrotranslocation Assay

The retrotranslocation efficiency and ubiquitination of ApoB was assessed based on a protocol previously published by our laboratory (Nakatsukasa *et al*. 2008). In brief, medium scale microsomes were prepared from wildtype and *hsp104Δ* yeast expressing ApoB using the β-estradiol system, as described above, and all cells were temperature shifted to 37°C for 1 hour prior to being collected. ApoB expression was confirmed in the resulting microsomes, as described above. A 40 µL *in vitro* reaction to assess ApoB ubiquitination and retrotranslocation was set up and consisted of Buffer 88, 4.0 µL of ApoB containing microsomes, and 5 mg/mL of yeast cytosol. Where indicated, reactions included or lacked an ATP regenerating system. After a 10 minute pre-incubation at room temperature, 6 µL of ^{125}I-ubiquitin was added. The reactions were then allowed to incubate for 45 minutes at 37°C. Samples were centrifuged for 10 minutes at 4°C at 13,000 rpm in a refrigerated microcentrifuge. The supernatant was removed and the pellet was resuspended in 40 µL of Buffer 88. Next, all reactions were quenched with 125 µL of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.25% SDS supplemented with 10 mM NEM and protease inhibitors. After a 30 minute incubation at 37°C, 400 µL of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% Triton X-100 supplemented with 10 mM NEM and protease inhibitors was then added to each reaction. ApoB was immunoprecipitated overnight at 4°C using 35 µL Protein G Fast Flow Sepharose and 5 µL anti-ApoB antibody. After the beads were collected, each sample was washed 3 times using 1 mL IP Wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2%). Proteins were eluted using 40 µL TCA sample buffer incubated at 37°C for 20 minutes. Finally, the isolated proteins were resolved on duplicate 10% and 7.5% polyacrylamide gels and western blot analysis was conducted on the 10% gel using anti-HA antibody. Ubiquitination was assessed via phosphorimager analysis

using the dried 7.5% polyacrylamide gel. Images were obtained using a Typhoon FLA 7000 phosphorimager and the associated software.

2.2 INVESTIGATION OF FACTORS THAT POTENTIALLY PREVENT APOLIPOPROTEIN B AGGREGATION

2.2.1 Development and Characterization of a New ApoB Expression System

To identify factors that regulate ApoB stability, our lab previously developed a galactose inducible expression system for use in the yeast *Saccharomyces cerevisiae* (Hrizo et al, 2007). However, galactose is a non-optimal carbon source and yeast cell growth is slowed during the time of induction, potentially creating an artificial stress and altered protein homeostasis, or "proteostasis" (Adams, 1972; Balch et al, 2008). To improve upon our galactose-inducible ApoB29 expression system, I utilized the chimeric GEV transcription factor to regulate ApoB production via addition of β-estradiol (McIsaac et al, 2011). The key to this system is the GEV transcription factor, a chimeric protein containing a Gal4 DNA binding domain, an Estrogen (β-estradiol) binding domain, and a VP16 transcription factor (Figure 4A). As previously described, GEV is constitutively expressed and retained in the cytosol in an inactive form through Hsp90 binding (McIsaac et al, 2011). Upon addition of β-estradiol, Hsp90 is released and GEV enters the nucleus by virtue of the VP16 fragment, where it promotes transcription of genes under the control of the *GAL* promoter. This system can be created in any yeast strain by simply

integrating an expression vector for the chimeric protein into the genome and does not require carbon source switching. This provides the yeast with an optimal growth environment.

To test the efficacy of this system, I first determined induction conditions for ApoB expression (Figure 4B). When an HA epitope-tagged form of ApoB is induced in wildtype cells using galactose, reasonable levels of ApoB are observed after 4-6 hours of induction via western blot analysis. In comparison, ApoB protein is observed as early as 5 minutes after using 1000 nM β-estradiol was added. To determine the optimal induction conditions using β-estradiol, wildtype cells containing the ApoB vector were next grown with various concentrations of β-estradiol. In the absence of β-estradiol, no ApoB is observed via western blot analysis. However, even at the lowest concentration of added β-estradiol (100 nM), ApoB is observed as early as 30 minutes after induction. I ultimately settled on the use of a 2 hour induction with 300 nM β-estradiol, as higher concentrations of β-estradiol and a longer time course does not lead to increased protein levels. Importantly, the cells continued to replicate during these induction conditions, which is in stark contrast to the situation when the galactose inducible expression system is used.

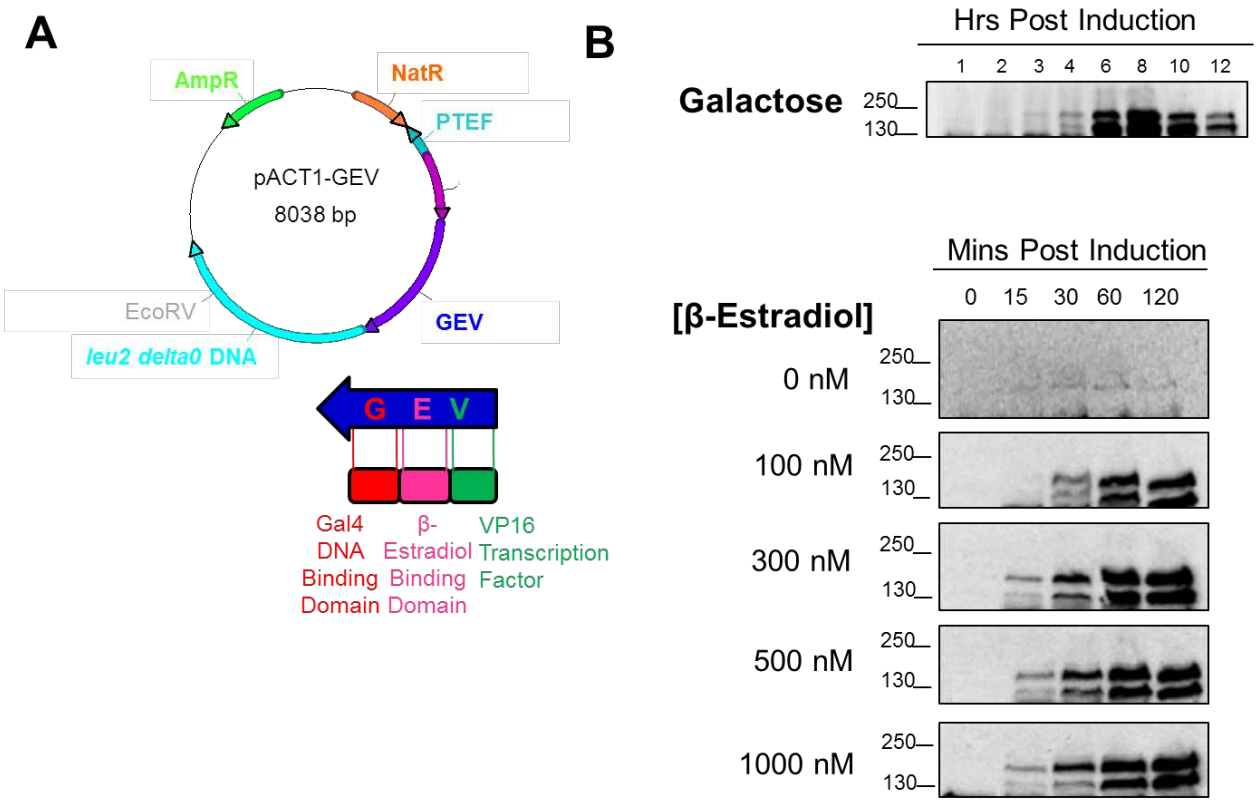


Figure 4. Comparison of ApoB29 Induction Conditions.

(A) A map of the Act1-GEV plasmid used to synthesize proteins under the control of the *GAL* promoter, which is activated in a β-estradiol dependent manner by a "GEV" fusion protein. The plasmid integrates into yeast strains at the *leu2Δ0* locus and is selected by growth on media supplemented with 0.1 mg/mL Nourseothricin. GEV consists of the Gal4 DNA binding domain, a fragment of the estrogen receptor, and the trans-activating components of the VP16 transcription factor.

(B) The ApoB29 protein was induced in wildtype yeast using either 2% galactose during a 12 hour timecourse or in the presence of various concentrations of β-estradiol over a 120 min timecourse. As no further increase was observed, 300 nM β-estradiol and a 120 min induction time were used for all further experiments.

To validate our new expression system, I first tested if ApoB29 was an ERAD substrate after β-estradiol induction. Our laboratory has previously published that this ApoB truncation , which still amasses lipids and traffics through the secretory pathway, is an ERAD substrate in yeast (Grubb et al, 2012; Hrizo et al, 2007). I therefore conducted cycloheximide chase assays in a *pdr5Δ* strain. Deletion of Pdr5 allows cells to accumulate a drug, MG132, which inhibits proteasome function and is otherwise pumped out of yeast cells (Balzi et al, 1994). ApoB was expressed in *pdr5Δ* yeast and the cells were subsequently incubated in the presence or absence of MG132 using both the galactose inducible system and the β-estradiol inducible system. In both induction systems, addition of MG132 stabilized ApoB levels relative to cells incubated in the absence of MG132 (Figure 5A). The half-life of ApoB in the absence of MG132 using the β-estradiol system is ~15 minutes while in the presence of MG132, its half-life is ~47 minutes. I also calculated the effect of proteasome inhibition on each band individual (Figure 5B). Although the higher molecular weight species was degraded to a greater extent, upon addition of MG132 the levels of both ApoB molecular weight species were stabilized compared to the cells incubated in the absence of MG132 I also found that degradation was more complete in the β-estradiol system, which degraded to ~5%, whereas only ~40% of ApoB was degraded in the absence of MG132 using the galactose inducible system, as observed previously (Hrizo et al, 2007). Therefore, ApoB degradation is proteasome dependent regardless of which expression system is used.

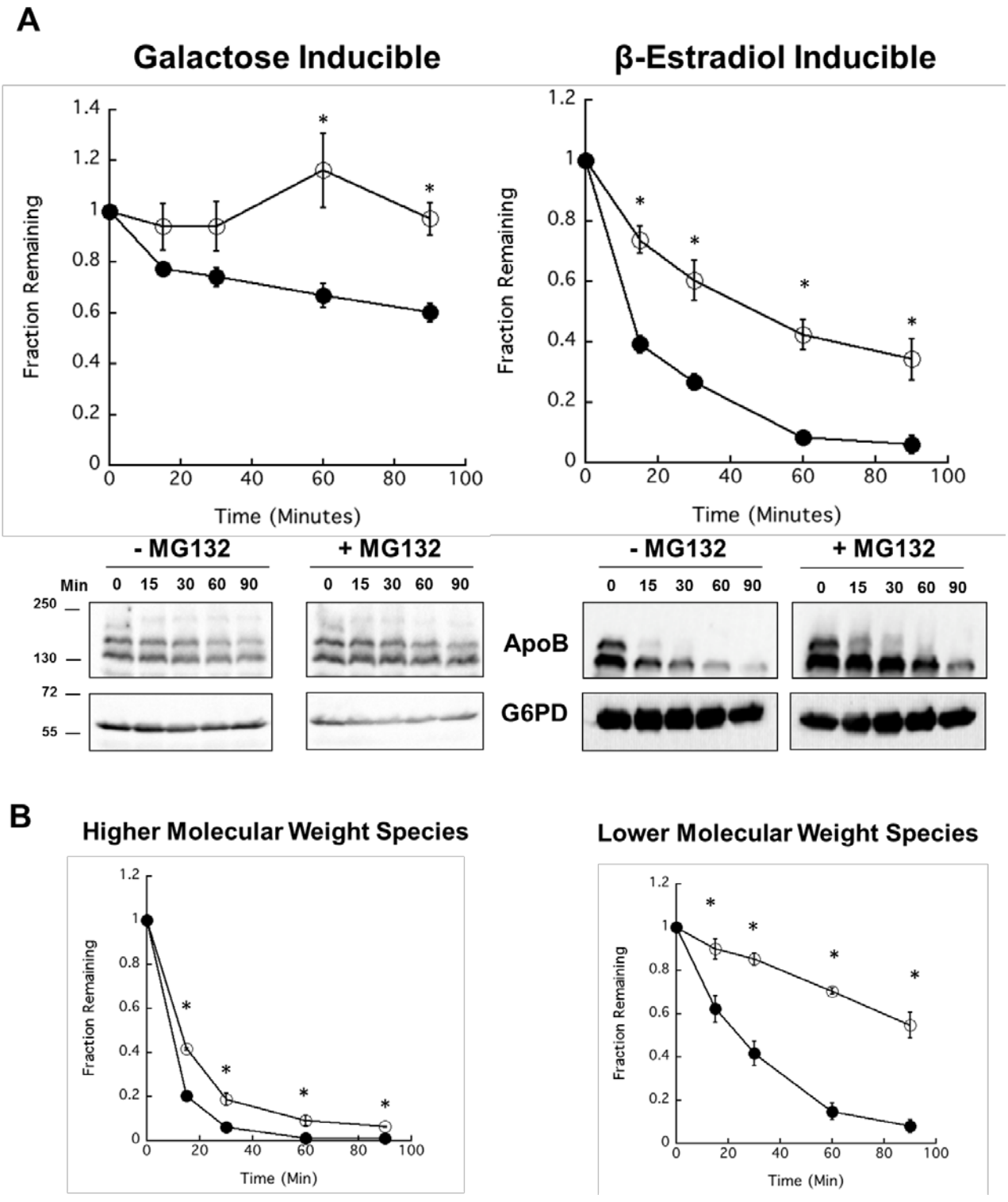


Figure 5. ApoB Degradation is Proteasome Dependent Independent of the Expression System.

(A) A cycloheximide chase analysis was conducted in *pdr5Δ* yeast expressing ApoB after either a galactose (left) (N=9) or β-estradiol (right) (N=4) induction. Yeast were incubated in the presence of MG132 (open circles) or in the absence of MG132 (closed circles) incubated at 30°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation. Asterisk denotes $p < 0.05$.

(B) Data from Figure 5A β-estradiol induction were re-calculated to show the effect of proteasome inhibition on individual ApoB species. Asterisk denotes $p < 0.05$.

An alternative method to degrade ApoB is by autophagy (Fisher et al, 2001). However since yeast lack a functional MTP complex, I predicted there would be minimal contribution by autophagy. Nevertheless, I investigated this possibility by conducting cycloheximide chase assays using yeast strains lacking both Pdr5 and Pep4 (Figure 6). Pep4 is the main vacuolar protease, which activates downstream vacuolar proteases, that together with Pep4, degrade vacuole-targeted cargo proteins (Ammerer et al, 1986; Jones et al, 1982; Woolford et al, 1986). ApoB was expressed in wildtype or *pep4Δpdr5Δ* yeast and the cells were subsequently incubated in the presence or absence of MG132. There was again profound stabilization of ApoB in *pep4Δpdr5Δ* yeast incubated with MG132 relative to cells lacking the drug, consistent with the data shown in Figure 6, however there was only modest stabilization of ApoB in *pep4Δpdr5Δ* yeast grown in the absence of MG132 compared to the wildtype cells. This effect was only apparent at early time points, and there was no significant difference in the extent of degradation by the end of the chase period. These data indicate that the vacuole plays a minimal role during the degradation of ApoB in yeast.

Because a substantial amount of ApoB appears to be degraded in both a vacuole-and proteasome-independent manner, at least with the β-estradiol induction system, it is possible that a fraction of ApoB is being secreted into the medium. This hypothesis will be tested in the near future. It is also possible that MG132 is still excreted from the cells or is being metabolized during the course of the experiment. In addition, MG132 only inhibits the chymotrypsin-like activity of the proteasome (Lee & Goldberg, 1998). It is possible that the trypsin-like and acidic/caspase-like protease functions of the proteasome can degrade ApoB, thus leading to the incomplete stabilization in the presence of MG132.

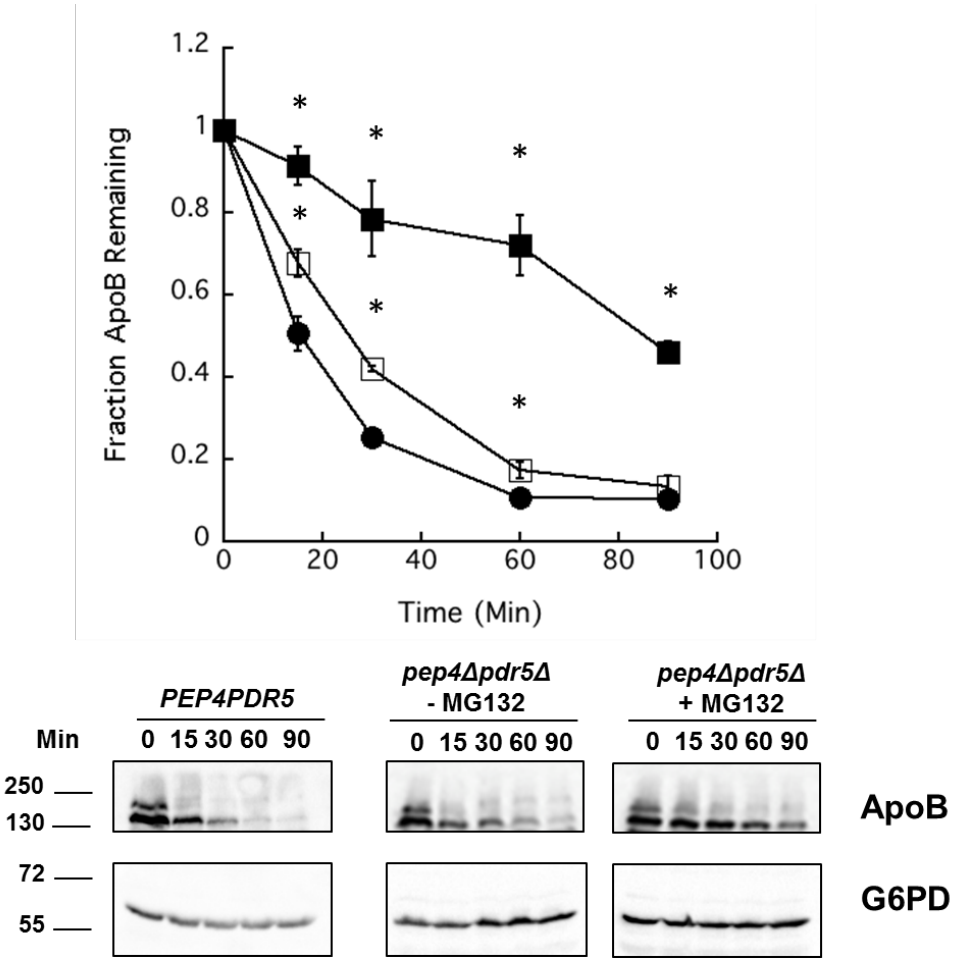


Figure 6. The Vacuole Plays a Minor Role in ApoB Degradation.

A cycloheximide chase analysis was conducted in wildtype (closed circles) (N=12) and *pep4Δpdr5Δ* yeast incubated in the presence of MG132 (closed squares) (N=12) and in the absence of MG132 (open squares) (N=12) incubated at 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation. Asterisk denotes $p < 0.05$ relative to wildtype.

The fact that ApoB is degraded at least in part in a proteasome-dependent manner when the protein is induced with β-estradiol is consistent with it being targeted for ERAD. Another hallmark of an ERAD substrate is that ubiquitin ligases are essential to degrade the protein substrate (see section 1.4). In yeast, the E3 ubiquitin ligases associated with ERAD are Hrd1 and Doa10. Hrd1 is required for the degradation of ERAD substrates with folding lesions in the ER lumen or membrane, while Doa10 is associated with ERAD substrates that have cytosolic lesions (Bays et al, 2001; Carvalho et al, 2006; Deak & Wolf, 2001; Denic et al, 2006; Swanson et al, 2001). The E3 ubiquitin ligase requirements for ApoB in yeast have been previously investigated through the use of the galactose-inducible system (Rubenstein et al, 2012). As might be predicted for an ERAD substrate whose degradation is dictated by an ER luminal "decision," ApoB protein degradation was slowed in a *HRD1* but not in a *DOA10* mutant strain. Therefore to further validate the new expression system, I conducted cycloheximide chase analyses on wildtype yeast or yeast lacking Hrd1, Doa10, or both E3 ubiquitin ligases after ApoB was induced using β-estradiol (Figure 7). As expected, ApoB degradation was slowed in *hrd1Δ* or *hrd1Δdoa10Δ* yeast while ApoB protein stability was unaffected in yeast lacking Doa10. In this experiment, the extent of stabilization was significantly greater than observed when *pdr5Δ* cells were incubated with MG132 signifying that the other proteasome activities may also contribute to ApoB degradation. Given that the β-estradiol induction system provides for a faster induction condition, does not compromise cell growth, yet still recapitulates the ERAD dependence of ApoB degradation, I used this new system for the remainder of my experiments.

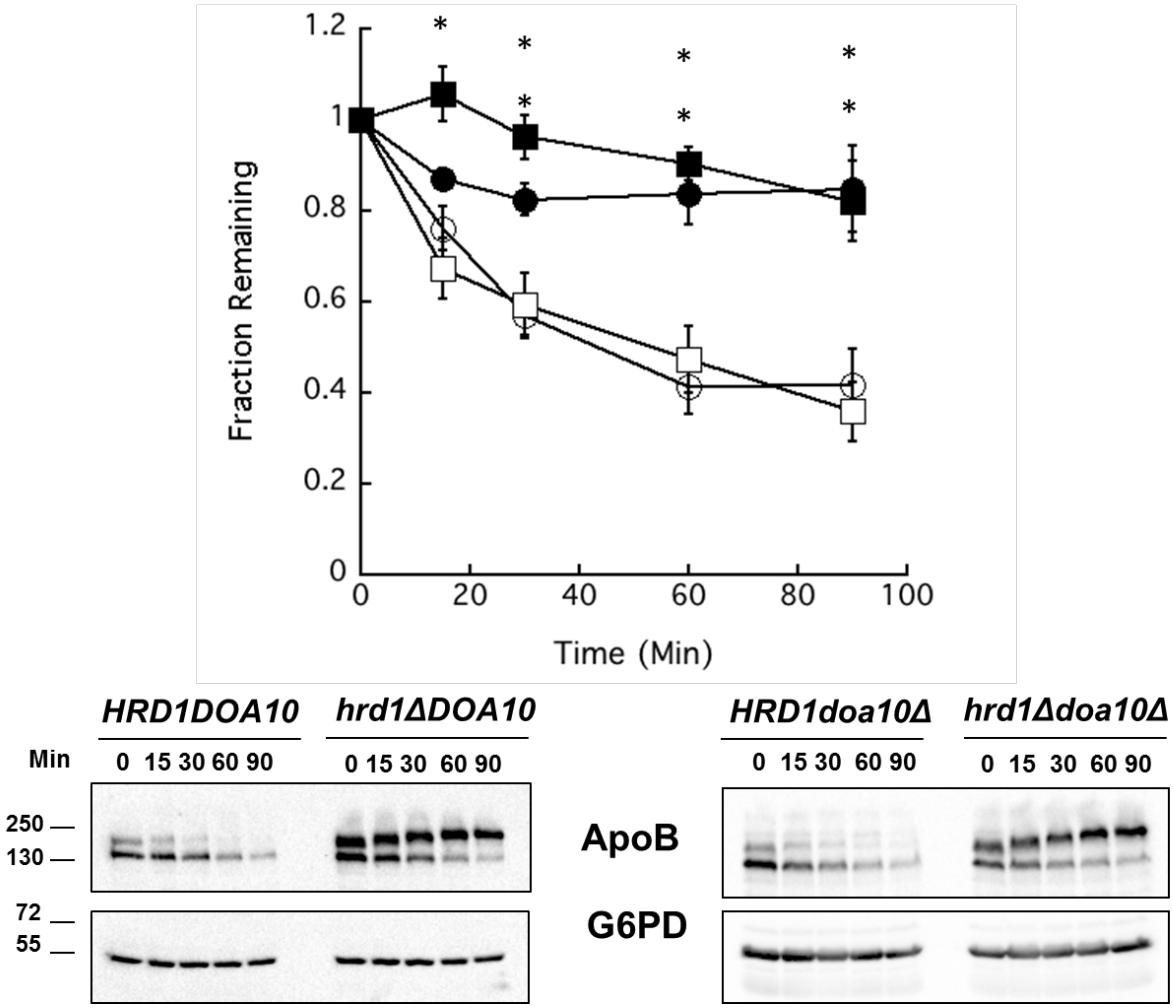


Figure 7. Hrd1 is an E3 Ubiquitin Ligase For ApoB.

A cycloheximide chase analysis was conducted in a wildtype yeast strain (N=10) (open circles), or in yeast lacking Hrd1 (N=11) (closed circles), Doa10 (N=10) (open squares), or Hrd1 and Doa10 (N=12) (closed squares) incubated at 30°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Asterisk denotes $p < 0.05$. Upper asterisks denote *hrd1Δdoa10Δ* relative to wildtype and lower asterisks indicate *hrd1Δ* relative to wildtype. Examples of representative blots are shown below the data quantitation.

2.2.2 The Two ApoB29 Species Reside in Different Cellular Locations

In both expression systems, ApoB appears as a doublet via western blot. Since most secretory proteins (including ApoB) are glycosylated as they transit into the ER, differential glycosylation may explain the nature of the doublet (Harazono et al, 2005). I first investigated this hypothesis through the use of an enzyme, EndoglycosidaseH (EndoH), which removes N-linked glycosylation from proteins residing in the ER (Maley et al, 1989). Lysates were prepared from wildtype cells expressing ApoB and incubated in the presence or absence of EndoH (Figure 8A). As a positive control, Pdi1, a glycosylated ER resident protein was also examined (Farquhar et al, 1991; Mizunaga et al, 1990). I first found that migration of Pdi1 following SDS-PAGE was faster after incubation with EndoH. In contrast, this enzyme had no effect on G6PD, which is an unglycosylated cytosolic protein. Upon incubation with EndoH, the ApoB higher molecular weight species shifted down by ~6 kDa while the lower band remained unchanged. Because there are 2 predicted glycosylation sites in ApoB29, this molecular weight shift corresponds to both sites being used (Harazono et al, 2005). Therefore, this species has entered the ER to a sufficient extent that these sites (at amino acids 158 and 956) have been post-translationally modified with N-glycans. As the lower molecular weight species was unaffected by EndoH, possible explanations include the upper band containing additional types of post-translational modifications or the lower band is a truncated form of ApoB and does not translocate far enough into the ER to be glycosylated. Because the ApoB29 used in these studies contains the HA tag at the C-terminus, the N-terminus of the protein would be absent from this lower molecular weight

species. Notably, the N-terminal truncated species would lack the signal sequence which would also explain why the protein could not be translocated into the ER.

I was then curious if ApoB required deglycosylated prior to proteasome-mediated degradation. I explored this question by conducting cycloheximide chase assays in wildtype and *png1Δ* yeast expressing ApoB (Figure 8B). Png1 is the enzyme responsible for removing N-linked glycans following retrotranslocation but before degradation, and the removal of N-glycans has been proposed to be essential to allow a polypeptide to access the 20S core of the proteasome (Hirsch et al, 2003; Huppa & Ploegh, 1997; Suzuki et al, 2000; Suzuki et al, 1998; Wang et al, 2009). However, the degradation of most ERAD substrates appears to be unaffected by the absence of Png1 (Blom et al, 2004; Hosomi et al, 2016; Kario et al, 2008; Kim et al, 2006) Consistent with these studies, deletion of Png1 did not affect ApoB degradation, except perhaps at a single early timepoint.

To better define the characteristics of the two ApoB species, I conducted limited proteolysis on ER-derived microsomes prepared from yeast expressing ApoB (Figure 9A). Microsomes were combined with a small amount of protease or buffer on ice. During a 10 minute incubation, samples were removed and processed for immunoblot analysis. Upon incubation with Proteinase K, Sec61, an ER-resident protein, and Kar2, an ER luminal chaperone, were stable during the incubations as anticipated. In contrast, the higher molecular species of ApoB was stable while the lower molecular weight species of ApoB was degraded in the presence of Proteinase K. This result indicates that the higher molecular weight ApoB species band is most likely protected inside the microsomes, consistent with its glycosylation pattern. In contrast, the lower molecular weight species was accessible to Proteinase K,

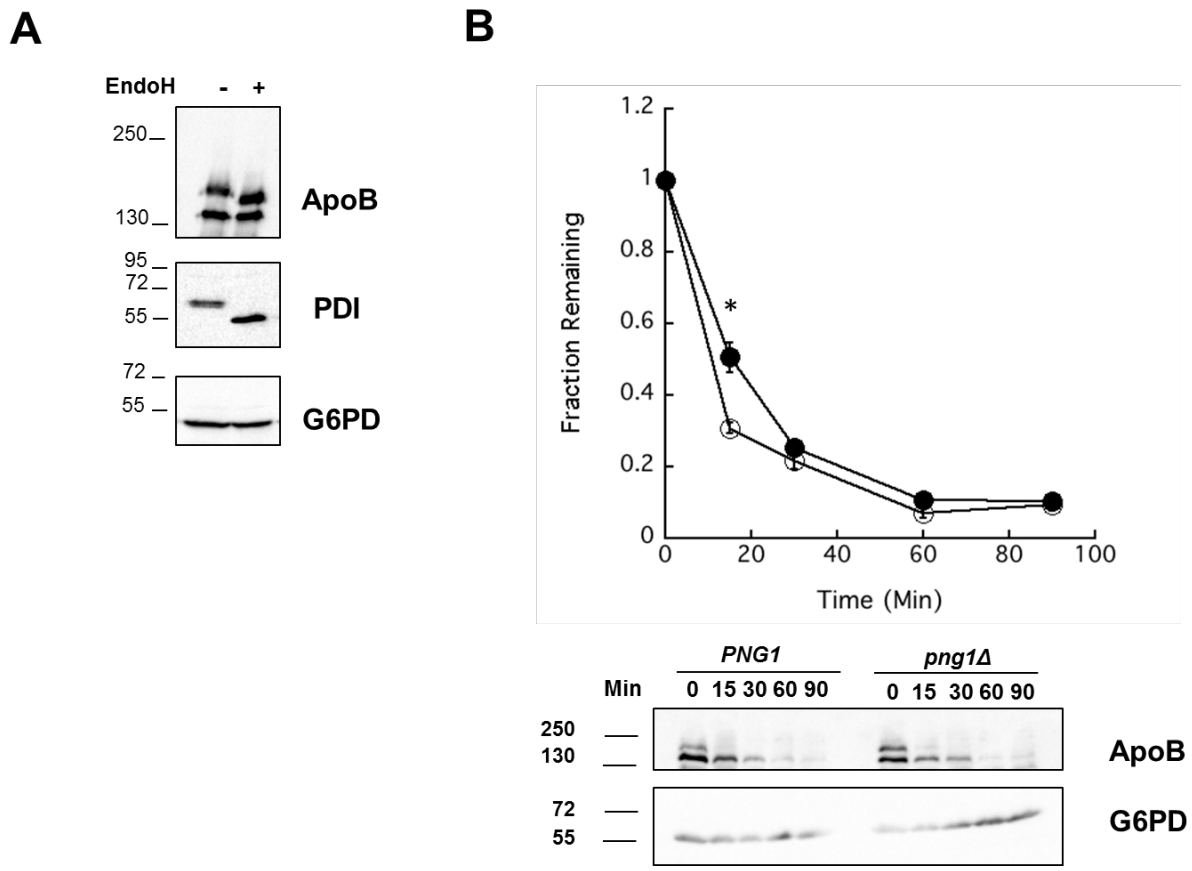


Figure 8. A Higher Molecular Weight ApoB Species is Glycosylated in Yeast.

(A) Lysate from wildtype yeast expressing ApoB was incubated in the presence or absence of EndoH. G6PD, a cytosolic protein, is unaffected by EndoH while PDI, an N-glycosylated protein, migrates at a lower molecular weight upon incubation with EndoH. The lower 166 kDa species of ApoB remains unaffected by EndoH while the upper band migrates at a slightly lower molecular weight after treatment.

(B) A cycloheximide chase analysis was conducted in wildtype (closed circles) (N=12) and *png1Δ* (open circles) (N=12) yeast expressing ApoB incubated at 30°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation. Asterisk denotes p-value <0.05.

confirming that it has not translocated into the ER and remains outside the microsomal membrane.

Next, I asked whether the lower molecular weight species might be partially aggregated since it failed to enter the ER and acquire N-linked glycans. Previous work indicated that resistance to carbonate extraction can be used to determine whether a protein resides within a lipid bilayer or is aggregation prone (Anderson & Denny, 1992; Fujiki et al, 1982; Le Parc et al, 2010). I therefore conducted carbonate extractions on ER-derived microsomes prepared from yeast expressing ApoB. The membranes were mixed with either buffer or sodium carbonate, and the mixture was then centrifuged to resolve the soluble and precipitated material (Figure 9B). When mixed with buffer, Sec61, Pdi1, and both ApoB species were found in the pellet fraction. Upon incubation with sodium carbonate, Pdi1 was found in the supernatant fraction while Sec61 was found in the pellet fraction, as expected. Interestingly, the lower molecular weight ApoB species was found in the pellet fraction while the higher molecular weight species resided in the supernatant fraction. I hypothesize that the upper band has entered the ER but a portion of the protein remains in the aqueous confines of the Sec61 translocon and is therefore accessible to the carbonate (Gilmore & Blobel, 1985). This is the topology expected for an ApoB species that is poised to either become lipid-loaded or retrotranslocated and degraded (Davis et al, 1990; Dixon et al, 1991; Fisher et al, 2008; Fisher et al, 1997; Pariyarath et al, 2001). However, the lower band is an untranslocated species that remains outside but perhaps associated with the ER and is aggregation prone. Because some disease causing mutations in ApoB similarly prevent the translocation of ApoB into the ER, this untranslocated species may serve as a model to understand the properties of these variants (Blackhart et al, 1986; Sturley et al, 1994).

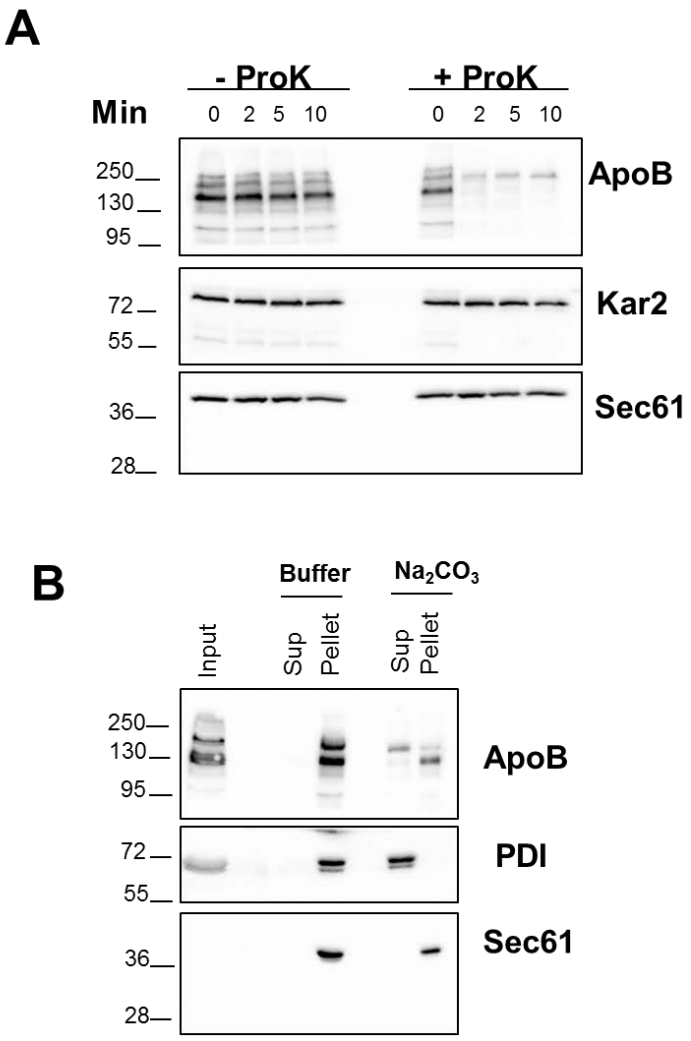


Figure 9. Different Forms of ApoB are Differentially Localized.

(A) ER derived microsomes were prepared from wildtype yeast expressing ApoB and subjected to limited proteolysis with Proteinase K (ProK) as indicated. Samples were incubated with protease at a final concentration of 0.04 mg/mL or buffer at 4°C and samples were processed at the indicated times for immunoblot analysis using anti-HA, anti-Kar2, and anti-Sec61 antibodies. The lower molecular weight species of ApoB is degraded by ProK while the upper band is protected. Kar2, an ER luminal Hsp70, and Sec61, an integral membrane protein, are protected from Proteinase K digestion.

(B) Sodium carbonate extraction was conducted with lysates from wildtype yeast expressing ApoB. Upon treatment with sodium carbonate, the ER resident and soluble protein, PDI, is found in the supernatant fraction whereas the membrane protein, Sec61, is found in the pellet fraction. In contrast, the higher molecular weight ApoB species is found in the supernatant fraction while the lower molecular weight species is found in the pellet fraction.

To establish if the lower molecular weight ApoB species was an untranslocated species, I wanted to create a mutant version of ApoB that completely lacks a signal sequence. As a first step, I conducted sequence analysis on pSLW1-B9-HA as I only had minimal details on the creation of the plasmid. Based on the methods outlined in Hrizo et al, 2007, I first compiled the predicted sequence, which should include the prepro-alpha factor signal sequence and then the full sequence of ApoB29, using the freely available databases from NCBI and the *Saccharomyces* genome database. However, upon comparing the predicted sequence with my results from direct DNA sequence analysis, a few discrepancies were identified (Figure 10). First, the ApoB29 sequence started at basepair 96 but not basepair 81 (both of which are after the signal sequence), and ended at basepair 4113 but not basepair 4122 as reported. Additionally, one of the primers used to clone *APOB29* was lacking an amino acid compared to the reference sequence. Second, the signal sequence was reported to contain nucleotides 1-300 from prepro-alpha factor but only contained nucleotides 1-212. Third, due to the missing DNA sequence, there appears to be a frameshift that would truncate the protein if both the signal sequence and ApoB29 were translated. Nevertheless, the upper band is the correct size for ApoB29 and exhibits each of the characteristics expected for the protein: it is properly glycosylated, has translocated into the ER, is recognized by a C-terminal epitope, and remains soluble. In contrast, the lower molecular weight species also harbors the C-terminal epitope but appears to lack a signal sequence since it cannot be translocated. I propose that this species uses an alternative start site found in ApoB, which produces an ApoB protein that lacks a signal sequence and may be partially compensated for by the strong promoter. In the coming months, I will be working to create a plasmid that contains the full length signal sequence properly fused to the ApoB29 sequence, as well as a form of ApoB that completely lacks a signal sequence.

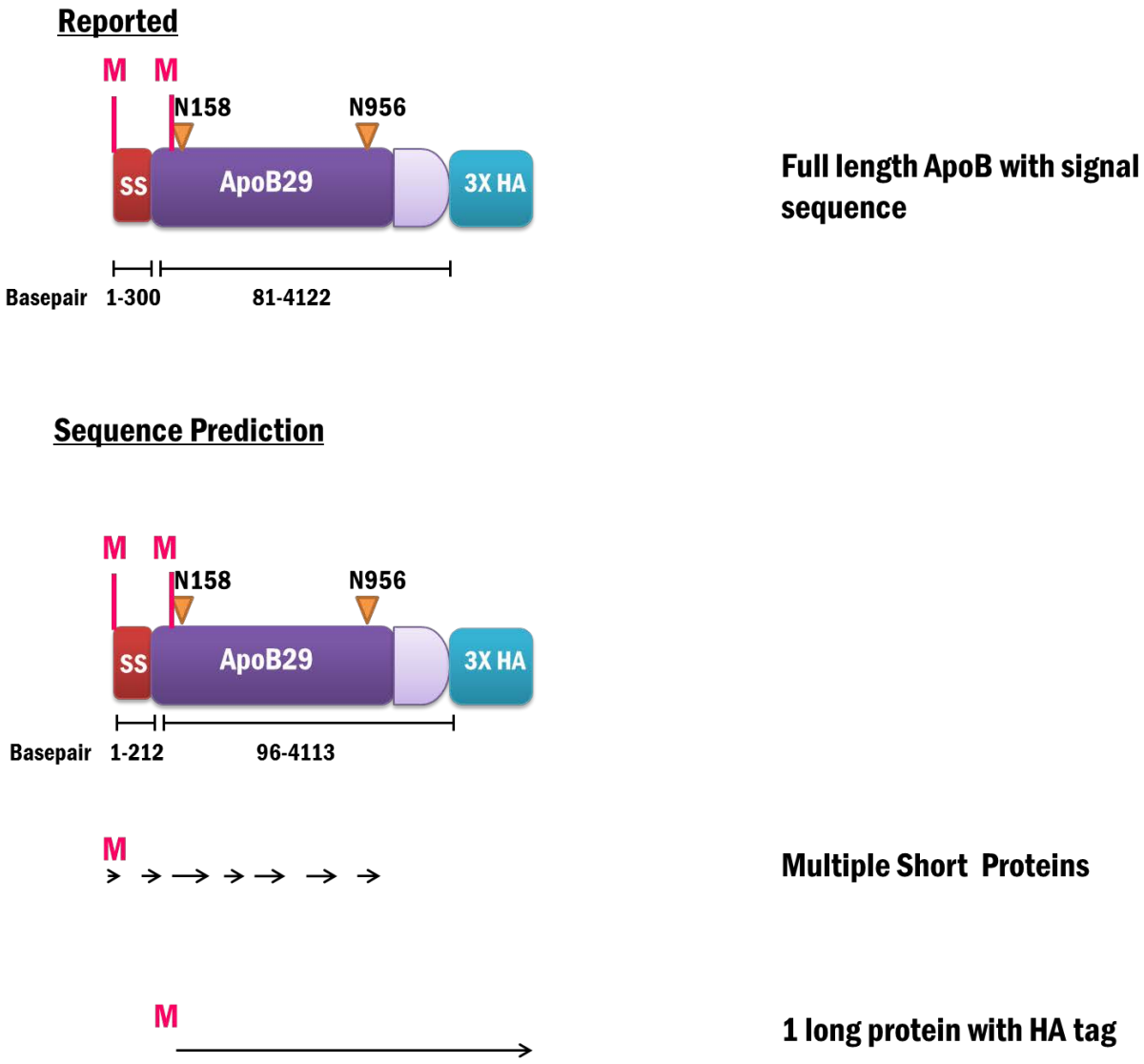


Figure 10. ApoB Sequence Predictions.

The reported ApoB29 construct contains the signal sequence from prepro-alpha factor (basepair 1-300), the N-terminal 29% of ApoB (basepair 81-4122 which lacks the ApoB signal sequence), two glycosylation sites (orange triangles), and a triple HA tag (Hrizo et al. 2007). Upon sequence analysis, the ApoB29 construct contains basepairs 1-212 for the signal sequence from prepro-alpha factor (which includes the complete signal sequence and part of the pro region), basepairs 96-4113 from ApoB, two glycosylation sites (orange triangles), and a triple HA tag. The predicted translation of the obtained sequence results using the first methionine in

the signal sequence would result in multiple stop codons in the signal sequence and ApoB29 and the generation of small protein species. The predicted translation of the sequencing results using the methionine in ApoB would result in an ApoB protein containing the triple HA tag but lacking a signal sequence. We propose that the translation is corrected in part by the strong promoter but the frameshift probably decreases translation efficiency. Since the sequencing was only conducted once on one strand, it is formally possible that these issues could arise from a wrong sequence result.

2.2.3 ApoB is Absent From Lipid Droplets

Even though the existing expression plasmids synthesize a protein that appear to lack a signal sequence or contain a few mutations relative to the native ApoB29 sequence, I propose that these proteins are still a valid model to investigate the ERAD of ApoB, as ApoB behaves as an ERAD substrate and we have previously identified factors using this ApoB29 construct in yeast that also affect ApoB in mammalian cells. Therefore, I used these proteins to investigate factors that may regulate ApoB's solubility after retrotranslocation. As ApoB is a large, amphipathic protein, I hypothesize that cytosolic factors must be present to prevent its aggregation after retrotranslocation but before degradation. I first hypothesized that lipid droplets could interact with ApoB to prevent its aggregation. Lipid droplets are highly conserved organelles that contain the largest concentration of lipids present in the cell. These lipids, which include triacylglycerols and sterol esters, are thought to be storage compartments for use during cell stress and starvation (Brasaemle & Wolins, 2012; Carman, 2012; Farese & Walther, 2009; Goodman, 2008; Murphy, 2012). Other aggregation prone proteins, such as α-synuclein which is implicated in Parkinson's disease, have been shown to interact with lipid droplets en route to degradation (Cole et al, 2002; Scherzer & Feany, 2004).

ApoB has also been proposed to interact with lipid droplets in mammalian cells (Fujimoto & Ohsaki, 2006; Ohsaki et al, 2006; Ohsaki et al, 2008; Suzuki et al, 2012). Using Huh7 cells expressing fluorescently tagged ApoB, ApoB localized to areas near the surface of lipid droplets, which were termed crescents. The authors of these studies subsequently went on to show that ApoB remained associated with these crescents until they were broken down by

proteasomal degradation or autophagy. In addition, the crescents appeared to increase under conditions that promoted ApoB degradation either by autophagy or ERAD. However, even though Huh7 cells are a hepato carcinoma cell line, they do not normally express ApoB and do not lipidate ApoB without exogenously added lipids. The majority of ApoB that is secreted is incorporated into particles with the density of LDLs and HDLs (Meex et al, 2011). Therefore, this system may not be a representative model for ApoB secretion.

Even though the yeast system similarly suffers from an inability to endogenously synthesize ApoB and assemble lipoproteins, the robust level of ERAD I observed in this model suggests that ApoB may similarly reside in lipid droplets. However, other data suggest that lipid droplets appear to be dispensable for ERAD, as model ERAD substrate degradation was unaffected by the absence of lipid droplets (Nakatsukasa & Kamura, 2016; Olzmann & Kopito, 2011; To et al, 2017)

To address this controversy, I first investigated if ApoB resides in lipid droplets through the use of sucrose gradient sedimentation. Sucrose gradient sedimentation allows for the identification of subcellular localization of proteins. By determining where proteins of known cellular location reside within the sucrose gradient, I can determine in principle where ApoB is localized in the cell. Therefore, if ApoB interacts with lipid droplets, it should co-migrate with a lipid droplet resident protein. I first conducted sucrose gradient sedimentation in wildtype yeast and lipid droplet deficient yeast. Lipid droplet synthesis occurs at the ER, as the enzymes needed for neutral lipid synthesis, Are1, Are2, Dga1 and Lro1, are localized to the ER membrane (Oelkers et al, 2002; Oelkers et al, 2000; Yang et al, 1996; Zweytick et al, 2000). Therefore, a yeast strain was created in which each enzyme is individually deleted (Sandager et al, 2002). This strain grows slowly but is viable.

Lysate from wildtype yeast and lipid droplet deficient yeast expressing ApoB using the galactose inducible expression system was placed onto a discontinuous 20-70% sucrose gradient. The gradients were ultracentrifuged, fractionated, and subjected to SDS-PAGE followed by western blot analysis. We analyzed the localization of Anp1, Sec61, and Pma1 to determine where specific membranes reside in the gradient. Anp1 is a member of the α-1,6 mannosyltransferase complex in the Golgi apparatus (Chapman & Munro, 1994; Jungmann et al, 1999; Melnick & Sherman, 1993). Sec61 is an ER resident protein and is the main component of the translocon (Deshaies & Schekman, 1987; Zhou & Schekman, 1999). Pma1 is a plasma membrane resident ATPase (Perlin et al, 1988; Serrano et al, 1986). In wildtype yeast, peak Anp1 is found in fractions 8-11, peak Sec61 is found in fractions 12-17, and peak Pma1 is found in fractions 14-19 (Figure 11A). ApoB is found throughout the gradient, in fractions 4-20. As there was a population of ApoB (fractions 4-7) that was even less dense than the Golgi, I initially hypothesized that these fractions contained ApoB that may interact with lipid droplets. In the lipid droplet deficient yeast, the Anp1 enriched fractions are 11-15, the Sec61 peak fractions are 14-19, and Pma1 is primarily found in fractions 12-17 (Figure 11B). ApoB is found throughout the gradient, in fractions 6-19. Again, there was a population of ApoB that was even less dense than the Golgi. However, this was not true of all lipid droplet deficient gradients. Some of the gradients lacked this population that was less dense than the Golgi (data not shown).

Based on these inconsistencies, I next determined lipid droplet localization in a sucrose gradient. Lysate from a wildtype strain with an integrated copy of Tgl3-GFP was placed onto a discontinuous sucrose gradient, consisting of 10%, 35%, 50%, and 70% magnesium-containing sucrose (Figure 11C). Tgl3 is a lipid droplet resident protein and is responsible for the majority of lipase activity in lipid droplets (Athenstaedt & Daum, 2003; Schmidt et al, 2013).

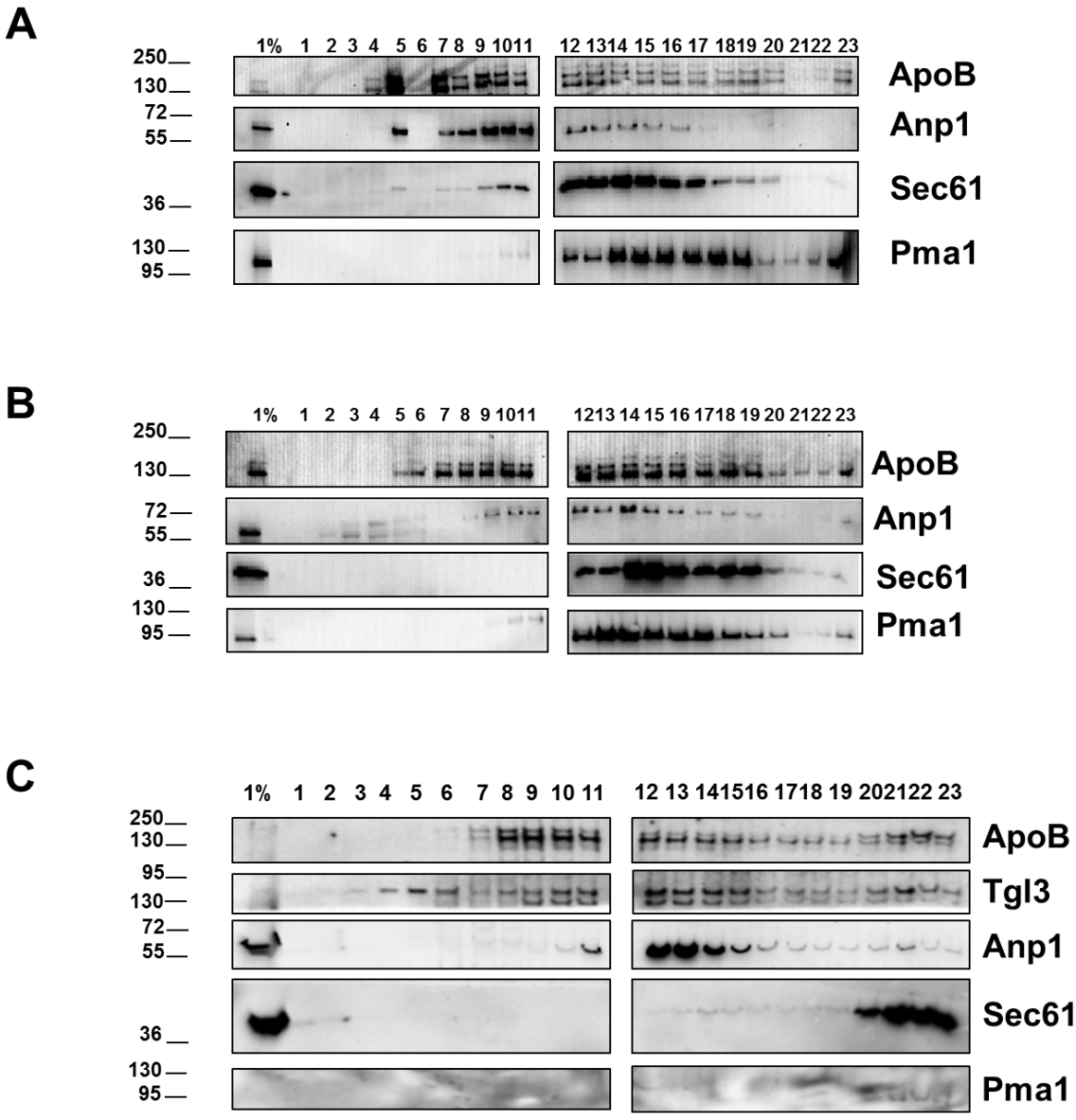


Figure 11. ApoB is Localized Throughout a Sucrose Gradient.

(A) Lysate from wildtype yeast expressing ApoB using the galactose inducible expression system was subject to 10-70% magnesium-containing sucrose gradient sedimentation. Gradients were fractionated and samples were subjected to immunoblot analysis using anti-HA, anti-Anp1, anti-Sec61, and anti-Pma1 antibodies. The identities of each protein in the gradient are shown.

(B) Lysate from lipid droplet deficient yeast (*are1Δare2Δlro1Δdga1Δ*) expressing ApoB using the galactose inducible expression system was subject to sucrose gradient sedimentation as in (A).

(C) Lysate from a wildtype yeast strain containing an integrated copy of Tgl3-GFP expressing ApoB was subject to sucrose gradient sedimentation as above. The gradient contained steps of 10%, 35%, 50% and 70% magnesium-containing sucrose. Gradients were fractionated and samples were subjected to immunoblot analysis using anti-HA, anti-GFP, anti-Anp1, anti-Sec61, and anti-Pma1 antibodies.

In this experiment, I altered the steps of the gradient to better separate the fractions containing the Golgi and the ER. In this experiment, the Anp1 peak is found in fractions 12-14, the Sec61 peak is found in fractions 20-23, and the Pma1 peak is found in fractions 21-23. The majority of Tgl3 is localized to fractions 4-15. Once again, ApoB is found throughout the gradient, but the majority is present in fractions 8-15. While these gradients were supportive of the idea that ApoB interacts with lipid droplets, the extensive spreading of the lipid droplets and ApoB throughout the gradients – perhaps because of their hydrophobic nature – made it difficult to make definitive conclusions. In addition, the data were often inconsistent and did not yield a definitive answer to where ApoB is localized.

Therefore, I next determined if ApoB localizes to lipid droplets in yeast by performing a large scale lipid droplet isolation (Leber et al, 1994). This isolation protocol involved spheroplasting yeast to remove cell walls, followed by passing the lysate over a series of Ficoll and Sorbitol containing gradients to separate lipid droplets from other subcellular compartments (Figure 12). The final gradient is especially important as vacuoles and lipid droplets are two of the least dense cellular compartments and are often difficult to separate. As the particles are heavily enriched in lipids, a TCA precipitation must also be conducted to remove the lipids in order to visualize proteins via western blot analysis.

The lipid droplet isolation was conducted using a wildtype yeast strain expressing ApoB that also contained an integrated copy of Tgl3-GFP (Figure 13). To determine the purity of select fractions, the presence or absence of several proteins was assayed. I found that Tgl3, a lipid droplet resident lipase (see above), was present in my preparation of the highly enriched lipid droplets. In contrast, Vph1, a vacuolar protein, was absent from my preparations of the

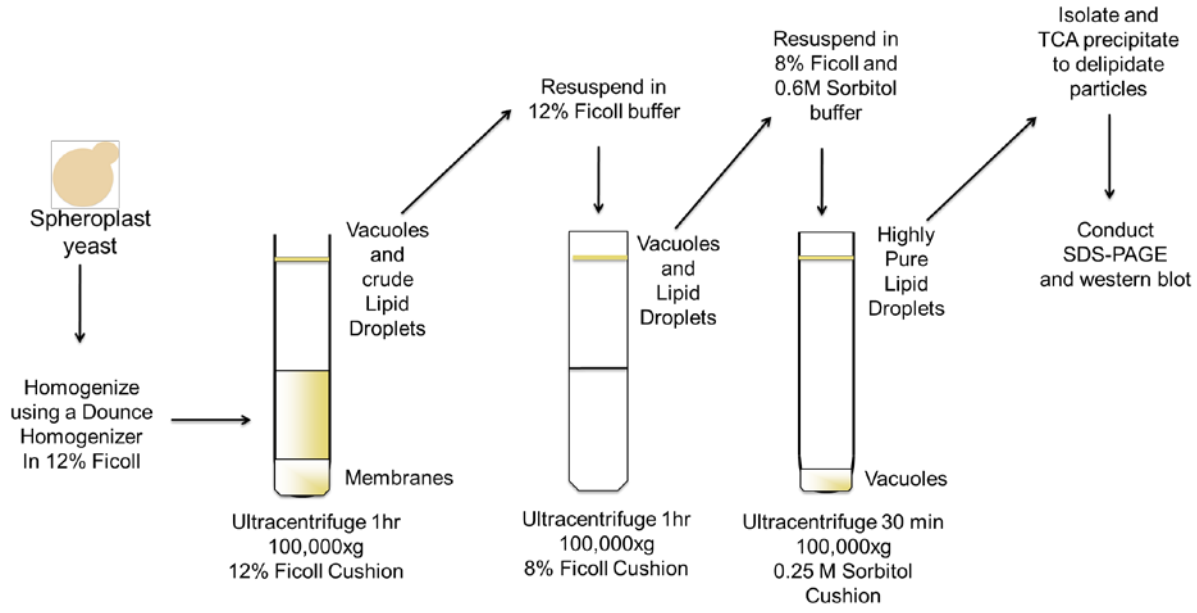


Figure 12. Isolation of Yeast Lipid Droplets.

To isolate lipid droplets from yeast, yeast cell walls were removed using lyticase for 15 minutes in a 30°C shaking water bath. Following collection, yeast cells were lysed in buffer containing 12% Ficoll using a Dounce homogenizer. Following 2-fold dilution in buffer containing 12% Ficoll, lysate was ultracentrifuged for 1 hour at 100,000g. Vacuoles and crude lipid droplets were isolated from the top of the gradient, diluted 2 fold, and mixed in a Dounce homogenizer. The solution was then overlaid with a solution containing 8% Ficoll and ultracentrifuged for 1 hour at 100,000g. Lipid droplets and vacuoles were isolated from the top of the gradient, diluted 2 fold in buffer containing 8% Ficoll and 0.6 M Sorbitol. This solution was next overlaid with buffer containing 0.25 M Sorbitol and ultracentrifuged for 30 minutes at 100,000g. Highly enriched lipid droplets were isolated from the top of the gradient, delipidated through TCA precipitation, and subjected SDS-PAGE followed by western blot analysis.

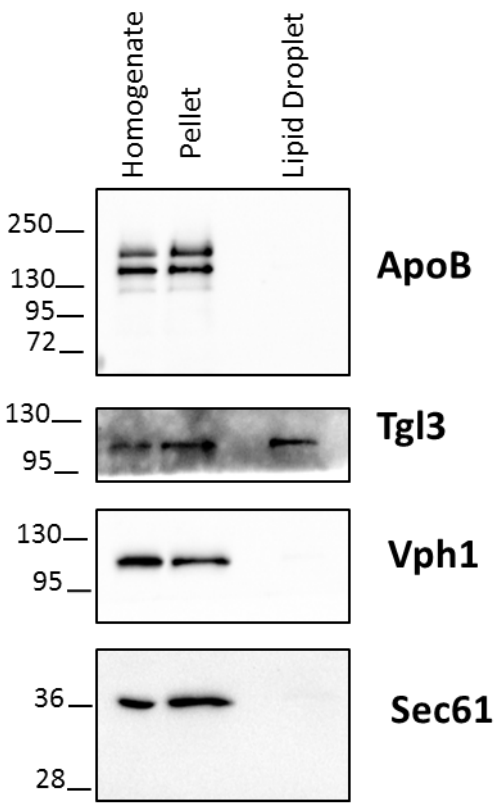


Figure 13. ApoB is Absent From Lipid Droplets.

Lipid droplets were isolated from a wildtype yeast strain expressing ApoB and containing an integrated copy of Tgl3-GFP. Fractions representing the crude homogenate, the first 5,500 rpm pellet fraction following Dounce homogenization, or the highly enriched lipid droplet fraction were obtained and examined by immunoblot analysis using anti-HA, anti-GFP, anti-Vph1, and anti-Sec61 antibodies. Tgl3 is a lipid droplet resident protein, Vph1 is a resident vacuole protein, and Sec61 is an ER resident protein.

highly enriched lipid droplets, indicating that the lipid droplet preparation was not contaminated with vacuoles. Furthermore, Sec61, an ER resident protein, was absent from lipid droplet fraction but was found in the homogenate and pellet fractions. I found that ApoB behaved like Sec61, residing only in the homogenate and pellet fractions but not the lipid droplet fraction. These results rule out the possibility that a significant amount of ApoB resides in lipid droplets in yeast, but the protein is instead primarily an ER resident.

2.2.4 The Small Heat Shock Proteins Do Not Affect ApoB Stability

Because ApoB does not appear to reside in lipid droplets, I next hypothesized that cytosolic factors, such as specific molecular chaperone "holdases", could interact with ApoB in the cytoplasm after retrotranslocation. Chaperone "holdases" are molecular chaperones that tightly bind to misfolded or unfolded polypeptides to prevent irreversible protein aggregation in a non-catalytic manner (Mattoo & Goloubinoff, 2014). One such family of these chaperones is the small heat shock proteins. The small heat shock proteins are ATP-independent and prevent protein aggregation via physically interacting with their substrates through a characteristic C-terminal α-crystallin domain (Burnie et al, 2006; Petko & Lindquist, 1986; Wotton et al, 1996). The small heat shock proteins are conserved from yeast to humans, which encode at least 10 family members (Bakthisaran et al, 2015; Kappe et al, 2003). In yeast, however, there are two small heat shock proteins, Hsp26 and Hsp42. Hsp26 is stress inducible (Susek & Lindquist, 1990; Tuite et al, 1990). In contrast, Hsp42 is constitutively expressed and is required to direct proteins to cytosolic aggregates (Haslbeck et al, 2004; Specht et al, 2011).

Previously, the small heat shock proteins were shown to facilitate the degradation of another ERAD substrate in yeast, the Cystic Fibrosis Transmembrane conductance Regulator

(CFTR) (Ahner et al, 2007). Deletion of Hsp26 slowed CFTR degradation while deletion of both small heat shock proteins almost completely stabilized CFTR. Consistent with this, the authors also observed that overexpression of a human small heat shock protein, αA-crystallin, enhanced degradation of CFTR in transfected HEK293 cells. Purified α-crystallin also slowed the aggregation of NBD1 in CFTR. Therefore, I hypothesized that the small heat shock proteins may also affect ApoB degradation, which is similarly aggregation prone. To investigate this hypothesis, I, with the help of an undergraduate, Ashley French, assayed ApoB stability in yeast strains that lack Hsp26 and/or Hsp42 (Figure 14). Cycloheximide chase assays were performed in wildtype, *hsp26Δ*, *hsp42Δ*, and *hsp26Δhsp42Δ* cells expressing ApoB under the control of the β-estradiol system. However, no effect on the ApoB degradation rate was observed in the absence of these chaperones, indication the small heat shock proteins do not affect ApoB degradation.

2.2.5 TRiC Does Not Affect ApoB Stability

The next chaperone "holdase" I investigated is the Tcp-1 Ring complex (TRiC). TRiC is a group II chaperonin and Hsp60 family member, which forms a large octameric complex to help refold proteins in an ATP-dependent manner. TRiC recognizes substrates through hydrophobic regions, electrostatic interactions, and/or polar motifs (Dunn et al, 2001; Kalisman et al, 2013; Spiess et al, 2004; Zhuravleva & Radford, 2014). The heteromeric complex forms a pore with a built-in lid and provides a favorable environment to facilitate protein folding. TRiC is required to help fold 5-10% of newly synthesized and aggregation-prone proteins, including huntingtin, actin, and tubulin, and helps fold β-strand-rich regions in proteins (Joachimiak et al, 2014; Nollen et al, 2004; Shahmoradian et al, 2013; Sontag et al, 2013; Ursic & Culbertson, 1991;

Ursic et al, 1994; Yam et al, 2008). As ApoB is also β-sheet rich, I hypothesized that TRiC may similarly play a role during ApoB folding or degradation.

I had previously investigated TRiC's effect on ApoB using the galactose inducible expression system and observed a small but reproducible effect on ApoB degradation rates at the final 90 minute timepoint, although this difference was not statistically significant (data not shown). I therefore investigated the effect of TRiC depletion on ApoB stability with my new β-estradiol expression system. I consequently conducted cycloheximide chase assays on wildtype and a temperature-sensitive mutation of TRiC (*tcp1* mutant strain) expressing ApoB (Figure 15). After shifting to the non-permissive temperature, no effect on ApoB degradation rates, indicating that TRiC is dispensable for the ERAD of ApoB. These results suggest that the modest effect of galactose on ApoB degradation might have arisen from a defect in growth/viability or the added stress of growth on galactose.

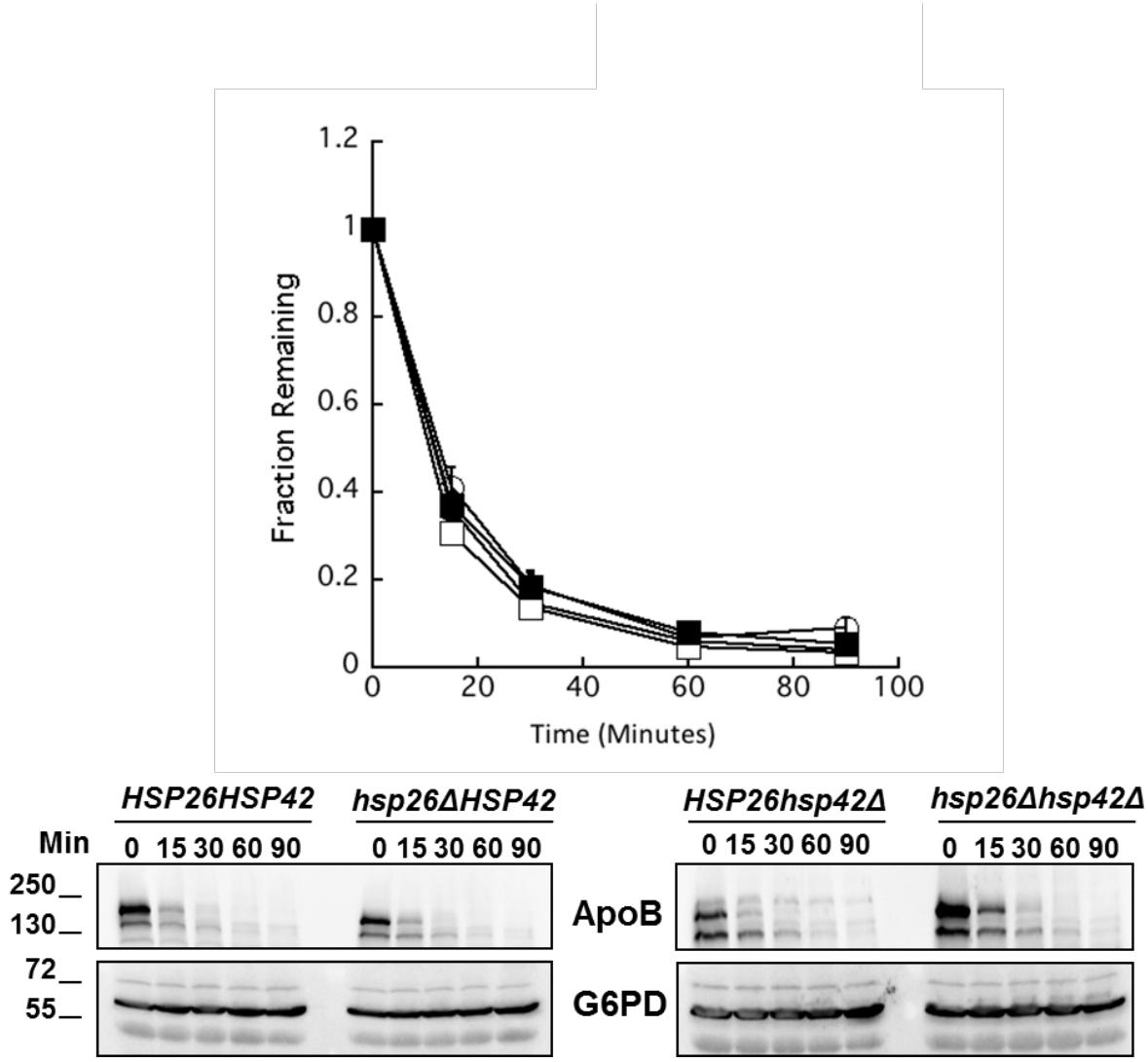


Figure 14. The Small Heat Shock Proteins Do Not Affect ApoB Stability.

A cycloheximide chase analysis was conducted in a wildtype yeast strain (N=7) (open circles), yeast lacking Hsp26 (N=11) (closed circles), Hsp42 (N=7) (open squares), or Hsp26 and Hsp42 (N=11) (closed squares) incubated at 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation.

2.2.6 Hsp104 is a Pro-degradation Factor for ApoB

Another chaperone holdase and disaggregase is Hsp104. Hsp104 is a hexameric AAA+ ATPase that helps refold aggregated proteins (Glover & Lindquist, 1998; Lee et al, 2004; Parsell et al, 1991; Sanchez et al, 1992; Zolkiewski et al, 2012). Hsp104 is essential for yeast survival under extreme stress conditions and for the propagation of yeast prions (Chernoff et al, 1995; Sanchez & Lindquist, 1990; Sanchez et al, 1992; Shorter & Lindquist, 2004). . Hsp104 is also thought to supply the mechanical force necessary to remove aggregated proteins from cellular aggregates. The protein substrate passes through the central pore of Hsp104 where it can interact with Hsp70 and Hsp40 to be refolded (Glover & Lindquist, 1998; Heuck et al, 2016; Lee et al, 2004; Yokom et al, 2016; Zolkiewski et al, 2012). This may help confer thermotolerance to the cell, as Hsp104 levels increase 8-10 fold between 30 mins and 2 hours of heat shock (Newnam et al, 2011). Since more Hsp104 is present, it may help dissolve aggregates formed during stress conditions. Curiously, Hsp104 has no direct sequence homolog in metazoans but is conserved across bacteria, archea, fungi, and plants. To compensate for this loss, Hsp110, Hsp70, and Hsp40 cooperate to prevent protein aggregation in metazoans (Glover & Lindquist, 1998; Mattoo et al, 2013; Parsell et al, 1991; Rampelt et al, 2012; Shorter, 2011; Shorter, 2017; Torrente & Shorter, 2013). More recent studies indicate that this activity is magnified when interacting Hsp40 partners with different chemical features are used in combination (Nillegoda et al, 2015).

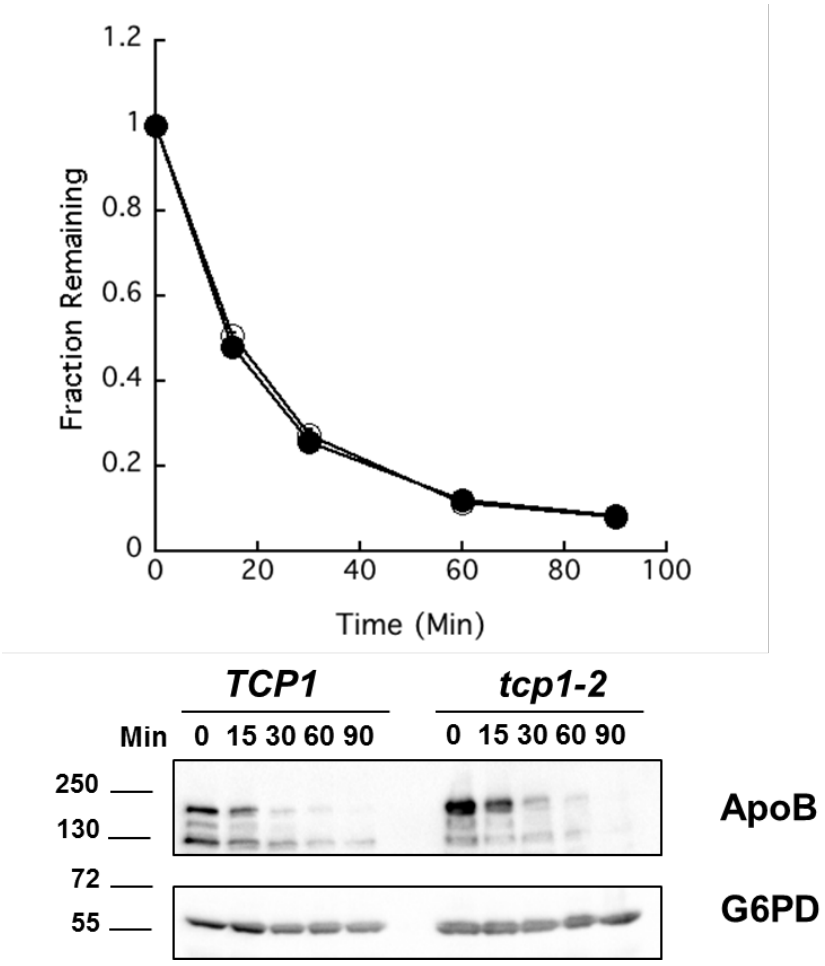


Figure 15. ApoB Degradation is Unaffected By the Absence of TRiC.

A cycloheximide chase analysis was conducted in wildtype (N=11) (closed circles) and *tcp1-2* (N=12) (open circles) yeast expressing ApoB incubated at 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation.

Hsp104 consists of 6 subunits, each containing an N-terminal domain (NTD), a middle (M) domain, two nucleotide binding domains (NBD1, NBD2), and a C-terminal domain (CTD). The NTD is a flexible region containing a long linker that has been implicated as a site for substrate interaction (Barnett et al, 2005; Doyle et al, 2012; Rosenzweig et al, 2015). Each NBD contains two AAA+ domains, which also interact with substrate to provide the energy for protein translocation, and comprise the ATP binding site. Additionally, the AAA+ domains consist of the conserved Walker A and B motifs, sensor-1 and sensor-2, and an arginine finger (Kedzierska et al, 2003; Lee et al, 2007; Rosenzweig et al, 2013; Zolkiewski et al, 2012). The NBD1 may also help to determine substrate specificity (Johnston et al, 2017). The M domain interacts with Hsp70, helps with communication between the NBDs, and is required for protein disaggregation (Cashikar et al, 2002; Desantis et al, 2014; Lee et al, 2013; Seyffer et al, 2012; Sielaff & Tsai, 2010). Recent cryo-EM structures of the M domain place it in close contact with NBD1. With individual Hsp104 subunits aligned next to each other, the M domain can essentially wrap continuously around the structure in order to regulate oligomeric function. Hsp70 can bind the M domain, causing a conformational shift and resulting in Hsp104's ability to interact with substrate (Heuck et al, 2016). The CTD is required for oligomerization of the subunits (Mackay et al, 2008).

Recent cryo-EM structures of Hsp104 from *Saccharomyces cerevisiae* and crystal structures from *Chaetomium thermophilian*, a filamentous yeast, have offered new insights into Hsp104's ability to disaggregate proteins (Heuck et al, 2016; Yokom et al, 2016). Instead of forming a closed cylinder, each subunit is tilted slightly and rotates approximately 53°, causing a corkscrew pattern to form and resulting the in final structure to rise nearly 10 Å from beginning to end. Nevertheless, the first and last subunit can interact, although this requires movement of

nearly 100°, forming a seam on the structure. Each NBD1 interacts with the M domain from the previous subunit to regulate movement. Following ATP hydrolysis, the substrate can be passed to NBD2 in the same subunit. After another round of hydrolysis, the substrate is passed to the adjacent subunit's NBD1. In this way, the substrate is passed through the channel, in a step like fashion.

To investigate if Hsp104 affects ApoB stability, I again worked with Ashley French, and we conducted cycloheximide chase assays on wildtype and *hsp104Δ* yeast expressing ApoB, which were temperature shifted to 37°C to induce stress, which is when Hsp104 activity is most essential (Figure 16A). Upon temperature shift, ApoB was significantly stabilized in the absence of Hsp104 throughout the chase period. To determine if Hsp104 affected ApoB stability under non-stress conditions, I therefore conducted cycloheximide chase assays In wildtype and *hsp104Δ* yeast expressing ApoB incubated at 30°C (Figure 16B). Under these conditions, Hsp104 had no effect on ApoB stability, indicating that Hsp104 is only important during stress.

As Hsp104 is also required for yeast prion propagation, I wanted to ensure that the effect Hsp104 exerted on ApoB was not due to the presence of prions, which might recognize ApoB aggregates (Chien & Weissman, 2001). I therefore cured putative prions in wildtype and *hsp104Δ* yeast by growing them on YPD media containing 3 mM guanidine hydrochloride. Millimolar amounts of guanidine hydrochloride prevent prions from forming and replicating new prions (Eaglestone et al, 2000; Jung et al, 2002; Tuite et al, 1981). Following the curing of the strains, I conducted cycloheximide chase assays on wildtype and *hsp104Δ* yeast expressing ApoB that were temperature shifted to 37°C (Figure 16C). Upon temperature shift, ApoB degradation was again significantly stabilized in the absence of Hsp104 throughout the chase period.

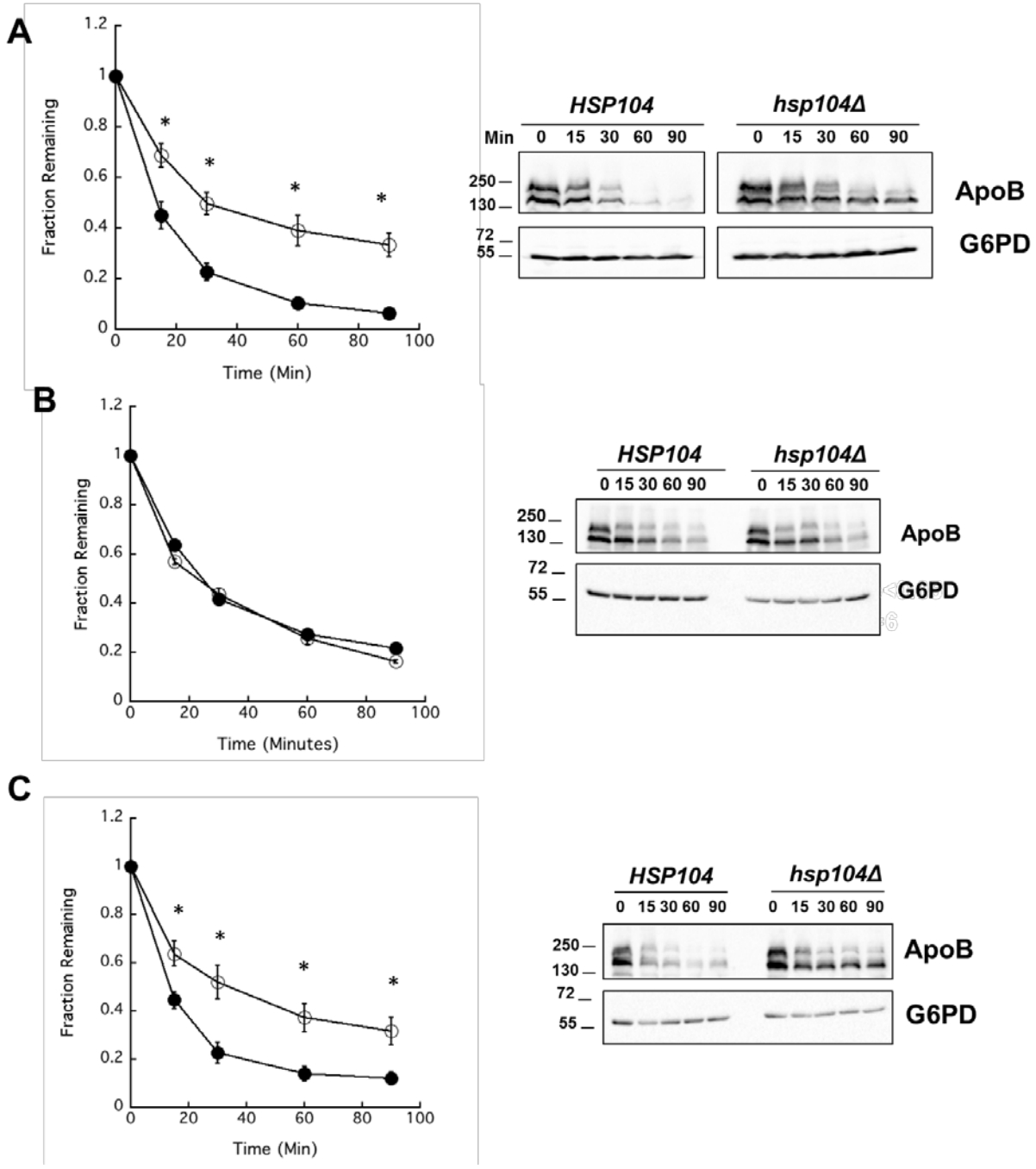


Figure 16. Hsp104 Stabilizes ApoB Degradation Under Stress Conditions.

(A) A cycloheximide chase analysis was conducted in wildtype (closed circles) (N=10) and *hsp104Δ* (open circles) (N=9) yeast expressing ApoB incubated at 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody

to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation. Asterisk denotes $p < 0.05$.

(B) A cycloheximide chase analysis was conducted in wildtype (closed circles) (N=11) and *hsp104Δ* (open circles) (N=15) yeast expressing ApoB incubated at 30°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation.

(C) Wildtype and *hsp104Δ* yeast were cured of prions by growth on YPD supplemented with 3 mM guanidine hydrochloride. A cycloheximide chase analysis was conducted in prion cured wildtype (closed circles) (N=11) and *hsp104Δ* (open circles) (N=15) yeast incubated at 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation. Asterisk denotes $p < 0.05$.

Since I had determined that ApoB species in the doublet exhibit different characteristics, I was curious if Hsp104 had differing effects on the glycosylated (ER soluble) species versus the non-glycosylated (aggregation-prone, cytoplasmic) species. The data in Figure 16A display the degradation rates for both bands. Therefore, I next calculated the degradation rates of the glycosylated or non-glycosylated versions alone and found that Hsp104 significantly affected the stability of both ApoB species (Figure 17). However, the non-glycosylated band was more dependent on Hsp104 for degradation than the glycosylated band, which is perhaps consistent with the more aggregation-prone nature of this species (Figure 17 right).

Because the loss of Hsp104 might affect secondary processes within the cell, I next sought to determine if Hsp104 was directly involved in degradation. I first investigated the direct role of Hsp104 on ApoB ERAD by conducting cycloheximide chase assays in wildtype or *hsp104Δ* cells that expressed ApoB and either expressed Hsp104 from a plasmid or that contained a vector control (Figure 18). After the cells were temperature shifted to 37°C, ApoB was again significantly stabilized in *hsp104Δ* cells containing a vector control compared to wildtype cells containing the same vector control. Importantly, ApoB degradation rates returned to the wildtype level upon expression of Hsp104 in *hsp104Δ* cells. Upon Hsp104 overexpression , ApoB was degraded to an even greater extent. These data strongly suggest that Hsp104 directly facilitates ApoB degradation.

To establish further that the effect of Hsp104 on ApoB stability is direct, I next tested if ApoB interacts with Hsp104 via coimmunoprecipitation experiments (Figure 19). Lysate from wildtype cells expressing ApoB was incubated in the presence or absence of a commercial anti-ApoB antibody and the associated proteins were isolated. As shown in Figure 19, I found that two chaperones previously shown to bind ApoB, Ssa1 (Hsp70) and Sse1 (Hsp110), were enriched

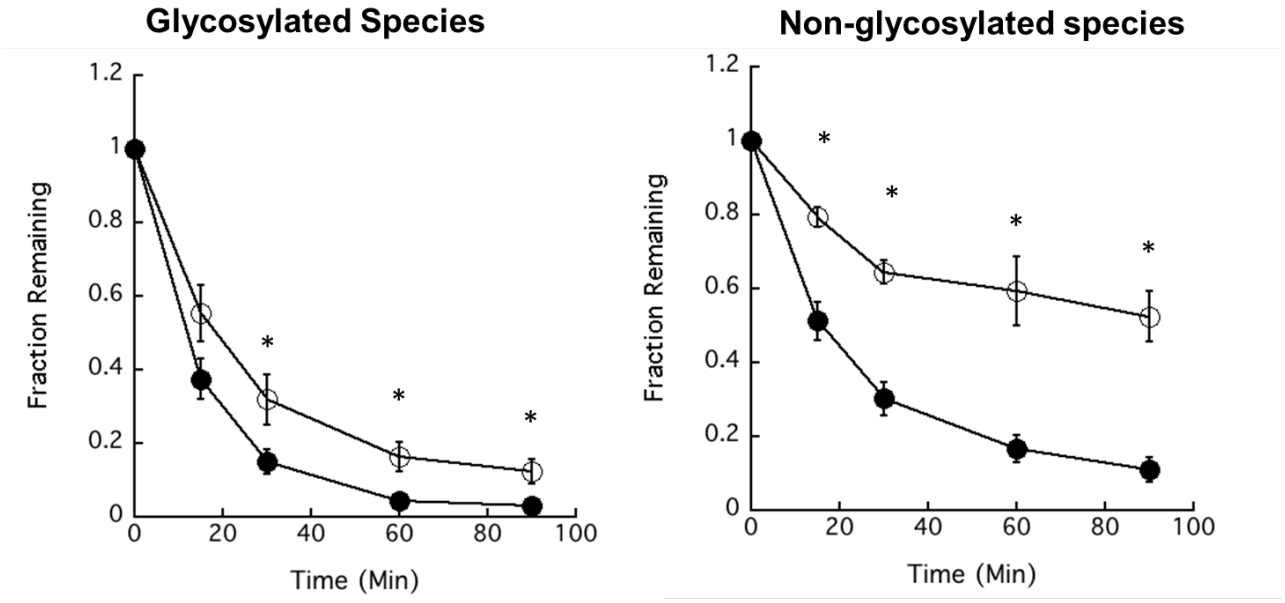
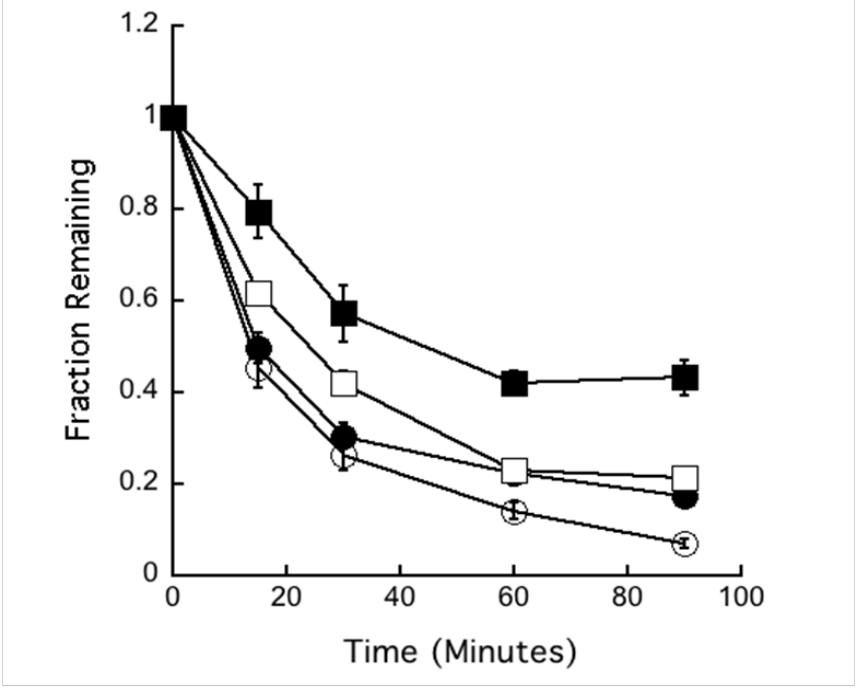


Figure 17. Hsp104 Affects Glycosylated and Non-Glycosylated ApoB Species.

Data from Figure 16A were re-calculated to show the effect of Hsp104 on individual ApoB species. Asterisk denotes $p < 0.05$.



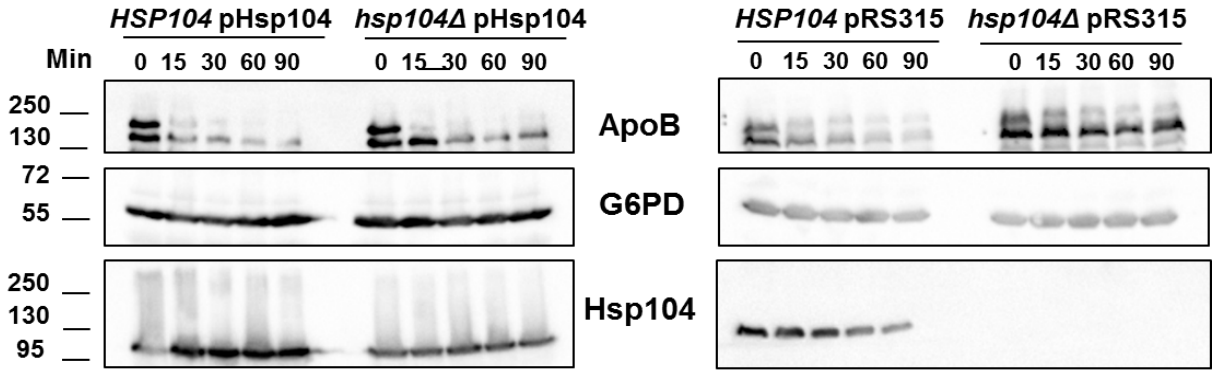


Figure 18. Hsp104 Facilitates ApoB Degradation.

A cycloheximide chase analysis was conducted in wildtype cells expressing ApoB and overexpressing Hsp104 (open circles) (N=12), *hsp104*Δ yeast expressing ApoB and Hsp104 (closed circles) (N=16), and wildtype cells containing a vector control (open squares) (N=10), and *hsp104Δ* cells containing a vector control (closed squares) (N=10) that also expressed ApoB. After a temperature shifted to 37°C, samples were taken at the indicated times and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation.

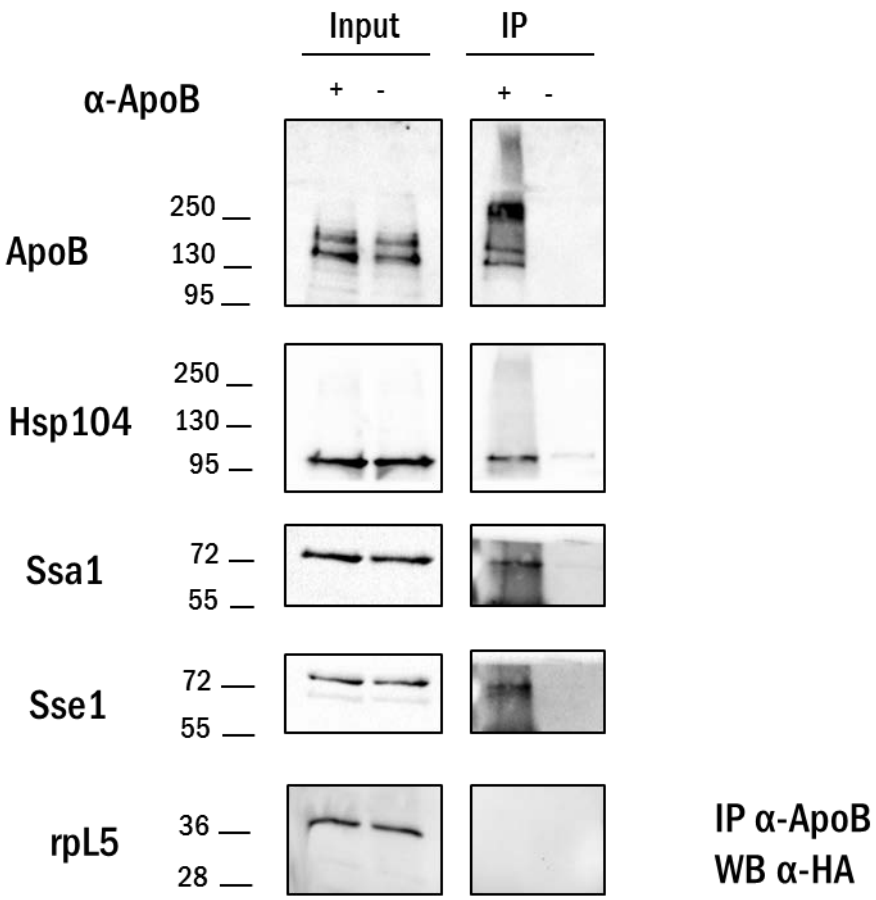


Figure 19. Hsp104 Coimmunoprecipitates with ApoB.

Lysates from wildtype yeast expressing ApoB were incubated in the presence or absence of an anti-ApoB antibody. Following incubation with protein G coupled Sepharose beads, samples were washed and processed for immunoblot analysis using anti-HA, anti-Hsp104, anti-Ssa1, anti-Sse1, and anti-rpL5 antibodies. Ssa1, a cytosolic Hsp70, and Sse1, an Hsp110 chaperone, have been previously shown to coimmunoprecipitate with ApoB. rpL5, a ribosomal protein, serves as a negative control.

in this pull-down assay when ApoB was present (Gusarova et al, 2001; Hrizo et al, 2007). Notably, there appears to be a larger molecular weight species present when ApoB was pulled down. I hypothesize that this is an aggregate which remains at the interface of the separating and stacking gels during SDS-PAGE. In contrast, rpL5, a ribosomal protein, was not observed after ApoB isolation. However, Hsp104 was also highly increased in the presence of ApoB, confirming that Hsp104 binds ApoB, perhaps in the context of a multi-chaperone complex.

2.2.7 The Rvb Proteins Do Not Compensate For The Loss of Hsp104

As Hsp104 has no obvious sequence homolog in humans, I was curious why this chaperone was required for ApoB degradation. I was intrigued when a recent paper reported that human RuvBL family members could compensate for loss of yeast Hsp104 (Zaarur et al, 2015). RuvBL1 and RuvBL2 are AAA+ DNA helicases but may also function independently of the helicase activity, including for example in TATA binding activity, assembly of RNA polymerase II, and as a chaperone (Gorynia et al, 2011; Jin et al, 2005; Kanemaki et al, 1997; Machado-Pinilla et al, 2012; Putnam et al, 2001; Qiu et al, 1998; Shen et al, 2000; Tsaneva et al, 1993; Yamada et al, 2001). RuvBL1 and RuvBL2 are conserved in yeast and are known as Rvb1 and Rvb2. These proteins also have DNA helicase activity and have been implicated in chromatin remodeling, snoRNP assembly, and DNA polymerase II assembly (Gribun et al, 2008; Jonsson et al, 2001; Kakihara & Houry, 2012; Lim et al, 2000; Shen et al, 2000). When yeast lacking Hsp104 and overexpressing Rvb1 or Rvb2 were heat shocked, the cells were protected from heat stress induced death, indicating that overexpression can at least partially compensate for the loss of Hsp104 (Zaarur et al, 2015).

Based on these data, I was curious if the Rvb proteins could rescue the ApoB degradation defect in *hsp104Δ* cells. To this end, I conducted cycloheximide chase assays in wildtype or *hsp104Δ* cells expressing ApoB and that either overexpressed Rvb2 or contained a vector control. After cells were temperature shifted to 37°C, overexpression of Rvb2 unexpectedly did not restore ApoB degradation to wildtype levels (Figure 20). These results suggest that the Rvb activity associated with the ability of the cells to compensate for survival after a severe heat shock is distinct from that required during ERAD.

2.2.8 Hsp104 Does Not Affect ApoB Aggregation Propensity

Based on the direct role of Hsp104 to facilitate the degradation of both an ER and cytoplasmic/ aggregated form of ApoB, I hypothesized that Hsp104 might maintain ApoB solubility prior to proteasomal degradation. To begin to test this hypothesis, I utilized a detergent solubility assay similar to that used by Zhao and colleagues (Zhao et al, 2013). This assay depends upon the observation that non-ionic detergents, such as dodecyl-maltoside (DDM), can solubilize membrane bound proteins but not aggregated proteins, while 1% SDS solubilizes proteins in both states. To this end, ER derived microsomes were incubated with multiple concentrations of various detergents, including DDM, at 4°C. Following centrifugation, supernatant (soluble) and pellet (insoluble) fractions were processed and subject to SDS-PAGE followed by western blot analysis. Detergents analyzed include DDM, Triton X-100, Digitonin, sodium deoxycholate, (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS), and Urea. Triton is a non-ionic detergent often used to extract proteins and permeabilize cell membranes. Digitonin is a non-ionic detergent often used to water solubilize lipids and membrane proteins. Sodium deoxycholate is a bile salt that is often used to lyse cells, solubilize cell membranes, and forms

mixed micelles with lipids and cholesterol. CHAPS is a zwitterionic detergent used to solubilize proteins and under non-denaturing conditions or purify membrane proteins. Urea denatures proteins by interfering with covalent bonds (Nicholls & Ferguson, 2013; Scientific; Scientific; Sigma-Aldrich). This wide range of detergents was chosen to test ApoB aggregates in a variety of conditions, as the ApoB-containing aggregates may behave differently in various conditions. Digitonin was especially interesting due to its association with cholesterol. Concentrations were chosen based upon each detergent's critical micelle concentration (CMC), which is the concentration when the solution experiences a dramatic change to form micelles (Tadros, 2013). The CMC can be altered based upon temperature, a protein's intrinsic molecular properties and concentration. The detergent concentration range was chosen by CMC/5, CMC, 5*CMC, 10*CMC, and 30*CMC for each specific detergent.

I first tested if there was a difference in ApoB solubility in the presence or absence of Hsp104 under non-stress conditions (Figure 21). Consistent with the cycloheximide chase assays conducted at 30°C, both ApoB species appeared in the supernatant with the highest concentration of all tested detergents in both wildtype and *hsp104Δ* yeast. Similar to the carbonate results, the higher molecular weight species shifted more readily to the supernatant fraction than the lower molecular weight species. Furthermore, there appeared to be no difference in ApoB solubility between the wildtype and *hsp104Δ* yeast. Furthermore, I calculated the amount of soluble upper band and insoluble lower band for each detergent, using the concentration at which a majority of ApoB has been release. In the wildtype strain, the glycosylated band is ~16% soluble in the presence of DDM, ~9% soluble in the presence of triton, ~46% soluble in the presence of digitonin, ~13% soluble in the presence of deoxycholate, and ~6% soluble in the presence of

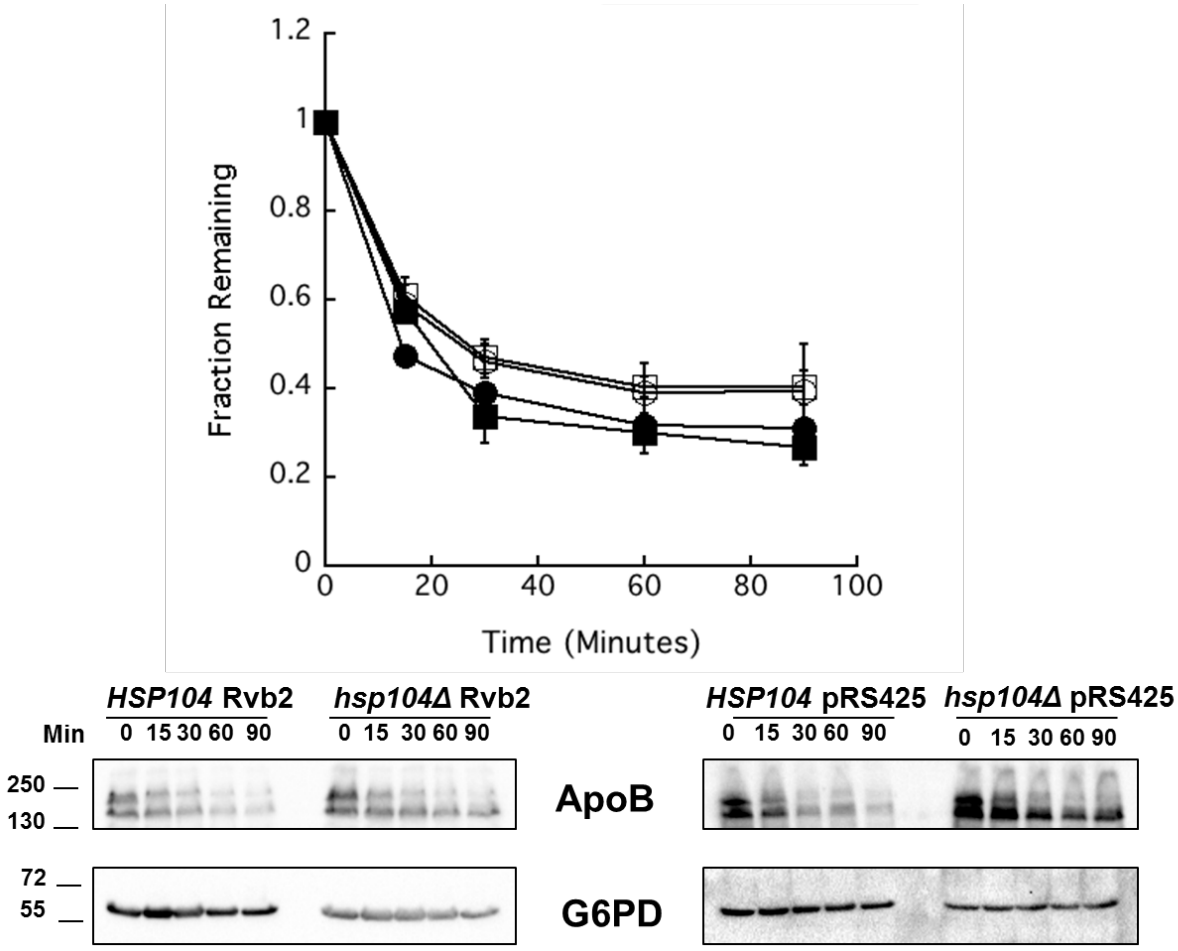


Figure 20. Rvb2 Does Not Compensate For Loss of Hsp104.

A cycloheximide chase analysis was conducted on wildtype (closed circles) (N=9) and *hsp104*Δcells (open circles) (N=10) expressing ApoB and overexpressing Rvb2, and on wildtype (closed squares) (N=8) and *hsp104Δ* (open squares) (N=6) cells expressing ApoB and containing an empty vector control that were temperature shifted to 37°C. At the indicated times, samples were taken and lysates were processed for immunoblot analysis using anti-HA antibody to detect ApoB. G6PD serves as a loading control. Examples of representative blots are shown below the data quantitation.

CHAPS. The unglycosylated band is ~51% insoluble in the presence of DDM, ~53% insoluble in the presence of triton, ~27% insoluble in the presence of digitonin, ~45% insoluble in the presence of deoxycholate, and ~50% insoluble in the presence of CHAPS. In the *hsp104Δ* strain, the glycosylated band is ~6% soluble in the presence of DDM, ~15% soluble in the presence of triton, ~33% soluble in the presence of digitonin, ~26% soluble in the presence of deoxycholate, and ~4% soluble in the presence of CHAPS. The unglycosylated band is ~74% insoluble in the presence of DDM, ~55% insoluble in the presence of triton, ~36% insoluble in the presence of digitonin, ~47% insoluble in the presence of deoxycholate, and ~51% insoluble in the presence of CHAPS. These results indicate that Hsp104 does not appear to affect ApoB aggregation propensity, at least under non-stress conditions.

I was next curious if Hsp104 helped to maintain ApoB solubility in cells grown under heat stress conditions. If this were true, then heat shocked strains lacking Hsp104 should contain higher levels of aggregated ApoB (and would be found in the pellet fraction) relative to wildtype cells. To test this hypothesis, ER-derived microsomes made from ApoB-expressing wildtype and *hsp104Δ* yeast temperature shifted to 37°C were subjected to detergent solubility assays, as above, with multiple detergents (Figure 22). For wildtype cells expressing ApoB (left), the detergent solubility assay looked similar to those from non-temperature shifted cells, as both ApoB species accumulated in the supernatant fraction in every condition tested when the highest concentration of detergent was used. Similarly, both species of ApoB shifted to the supernatant fraction in *hsp104Δ* yeast at the same concentrations of detergent as observed when microsomes from the wildtype cells were examined. In addition, I again calculated the amount of soluble upper band and insoluble lower band for each detergent. In the wildtype strain, the glycosylated band is ~5% soluble in the presence of DDM, ~10% soluble in the presence of triton, ~9%

soluble in the presence of digitonin, ~14% soluble in the presence of deoxycholate, ~5% soluble in the presence of CHAPS, and ~35% soluble in the presence of Urea. The unglycosylated band is ~55% insoluble in the presence of DDM, ~73% insoluble in the presence of triton, ~73% insoluble in the presence of digitonin, ~68% insoluble in the presence of deoxycholate, ~61% insoluble in the presence of CHAPS and ~2% insoluble in the presence of Urea. In the *hsp104Δ* strain, the glycosylated band is ~13% soluble in the presence of DDM, ~22% soluble in the presence of triton, ~12% soluble in the presence of digitonin, ~15% soluble in the presence of deoxycholate, 23% soluble in the presence of CHAPS, and ~26% soluble in the presence of Urea. The unglycosylated band is ~59% insoluble in the presence of DDM, ~66% insoluble in the presence of triton, ~70% insoluble in the presence of digitonin, ~64% insoluble in the presence of deoxycholate, ~53% insoluble in the presence of CHAPS and ~3% insoluble in the presence of Urea. These results suggest that Hsp104 does not play a role in maintaining ApoB solubility when it resides in the ER (higher molecular weight species) or is associated with the ER membrane (lower molecule weight species). Therefore, Hsp104 does not affect aggregation propensity of ApoB.

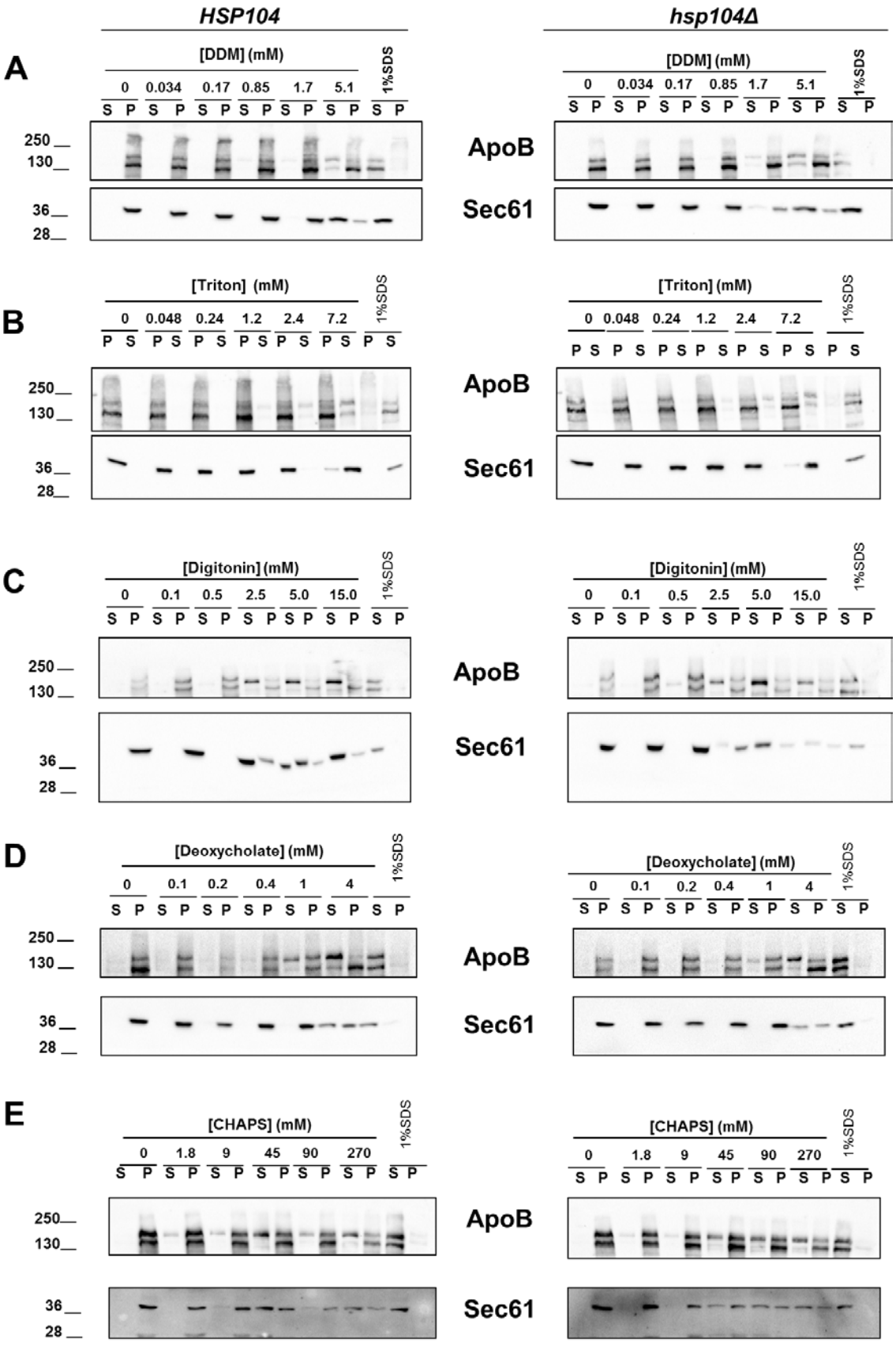
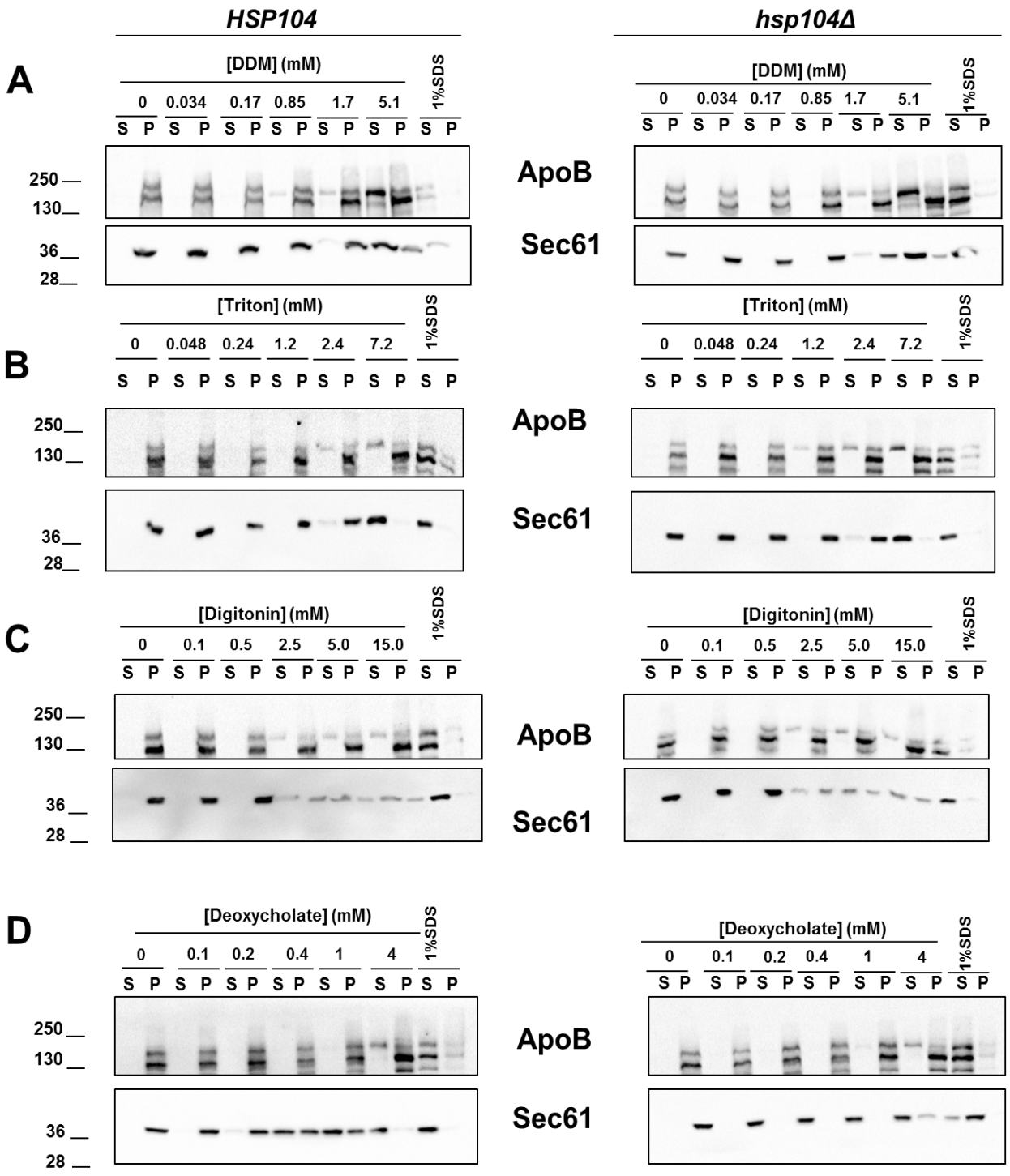


Figure 21. ApoB Aggregation Propensity is Unaffected by Hsp104 Under Non-stress Conditions.

ER derived microsomes were isolated from wildtype (left) and *hsp104Δ* (right) yeast expressing ApoB that were grown at 30°C. Microsomes were mixed with the indicated concentrations of (A) dodecyl maltoside (DDM), (B) Triton X-100, (C) Digitonin, (D) sodium deoxycholate, and (E) (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS) or 1% SDS and incubated on ice for 30 minutes. Following centrifugation, supernatant (soluble) and pellet (insoluble) fractions were processed for immunoblot analysis using anti-HA and anti-Sec61 antibodies. There appeared to be no difference in ApoB solubility between wildtype and *hsp104Δ* derived microsomes under any condition.


HSP104
hsp104Δ
A
[DDM] (mM)
0 0.034 0.17 0.85 1.7 5.1
1%SDS
S P
250
130
36
28
ApoB
Sec61
B
[Triton] (mM)
0 0.048 0.24 1.2 2.4 7.2
C
[Digitonin] (mM)
0 0.1 0.5 2.5 5.0 15.0
D
[Deoxycholate] (mM)
0 0.1 0.2 0.4 1 4

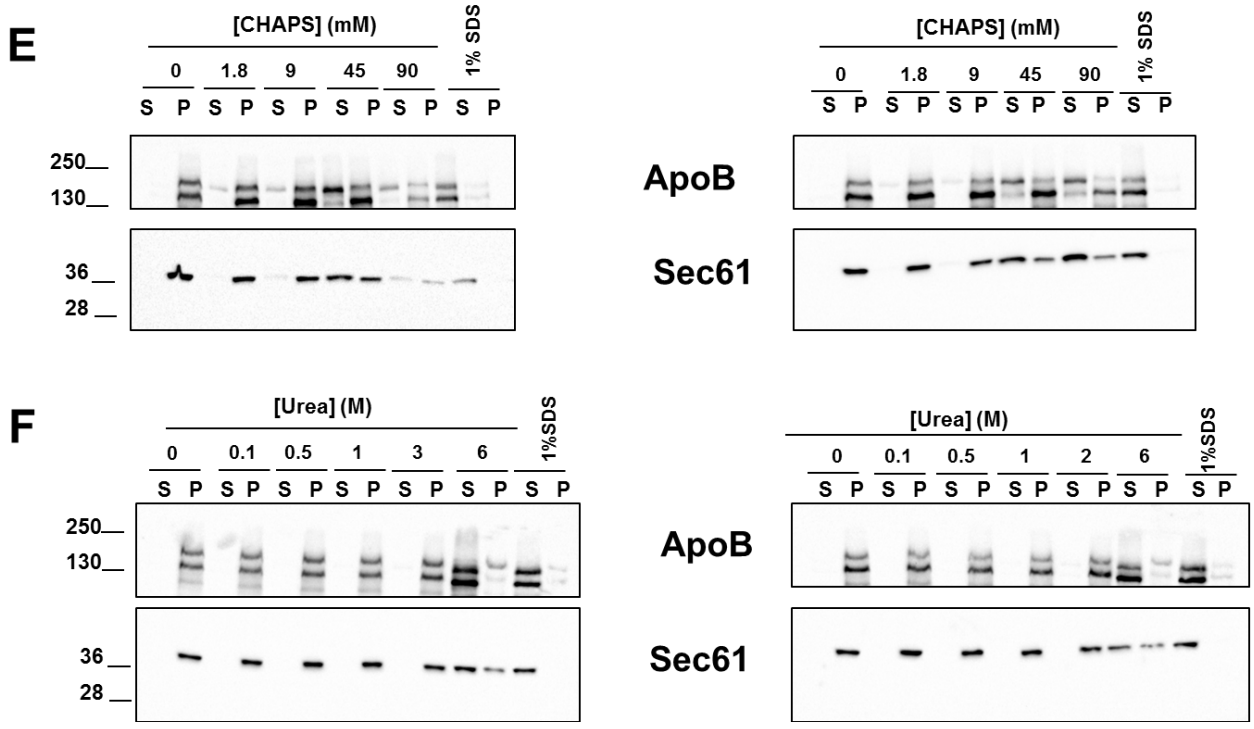


Figure 22. Hsp104 Does Not Affect ApoB Aggregation Propensity Under Stress Conditions.

ER derived microsomes were prepared from wildtype (left) and *hsp104Δ* (right) yeast expressing ApoB that had been temperature shifted to 37°C. Microsomes were mixed with the indicated concentrations of (A) dodecyl maltoside (DDM), (B) Triton X-100, (C) Digitonin, (D) sodium deoxycholate, (E) (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS), and (F) Urea or 1% SDS and were incubated on ice for 30 minutes. Following centrifugation, supernatant (soluble) and pellet (insoluble) fractions were processed for immunoblot analysis using anti-HA and anti-Sec61 antibodies.

2.2.9 Hsp104 Affects ApoB Retrotranslocation

Although Hsp104 does not appear to maintain the solubility of ApoB in microsomes, the fact that this AAA+ ATPase can extract aggregated proteins through the central cavity suggests that it might directly function with Cdc48 to retrotranslocate ApoB (Gates et al, 2017; Heuck et al, 2016; Yokom et al, 2016). I explored this question by using an *in vitro* retrotranslocation assay, with the help of Mike Preston, while he was a graduate student in our lab. This assay investigates the ability of any membrane protein expressed in yeast and that resides in the ER to be ubiquitinated and retrotranslocated (Buck et al, 2016; Nakatsukasa et al, 2008). In this assay, ER derived microsomes, yeast cytosol, an ATP regenerating system, and ^{125}I-ubiquitin are combined to allow for ubiquitination. Supernatant and pellet samples are separated by centrifugation and then the ERAD substrate in each fraction is subject to immunoprecipitation. By quantifying the amount of material in the supernatant and pellet fractions, one can determine how efficiently a ubiquitinated protein is retrotranslocated from the membrane based upon the presence of ^{125}I-ubiquitin that has been conjugated to the substrate.

Using this assay, I tested ApoB retrotranslocation. ER-derived microsomes from wildtype and *hsp104Δ* yeast that expressed ApoB and temperature shifted to 37°C were combined with yeast cytosol, an ATP regenerating system, and ^{125}I-ubiquitin. Following centrifugation immunoprecipitation, samples were washed, and subjected to SDS-PAGE followed by western blot or phosphorimager analysis. Following a temperature shift to 37°C, I found that ubiquitinated ApoB was retrotranslocated to a somewhat lower but reproducible

degree when Hsp104 was absent (Figure 23). This result suggests that Hsp104 may help facilitate ApoB degradation by directly aiding ApoB retrotranslocation.

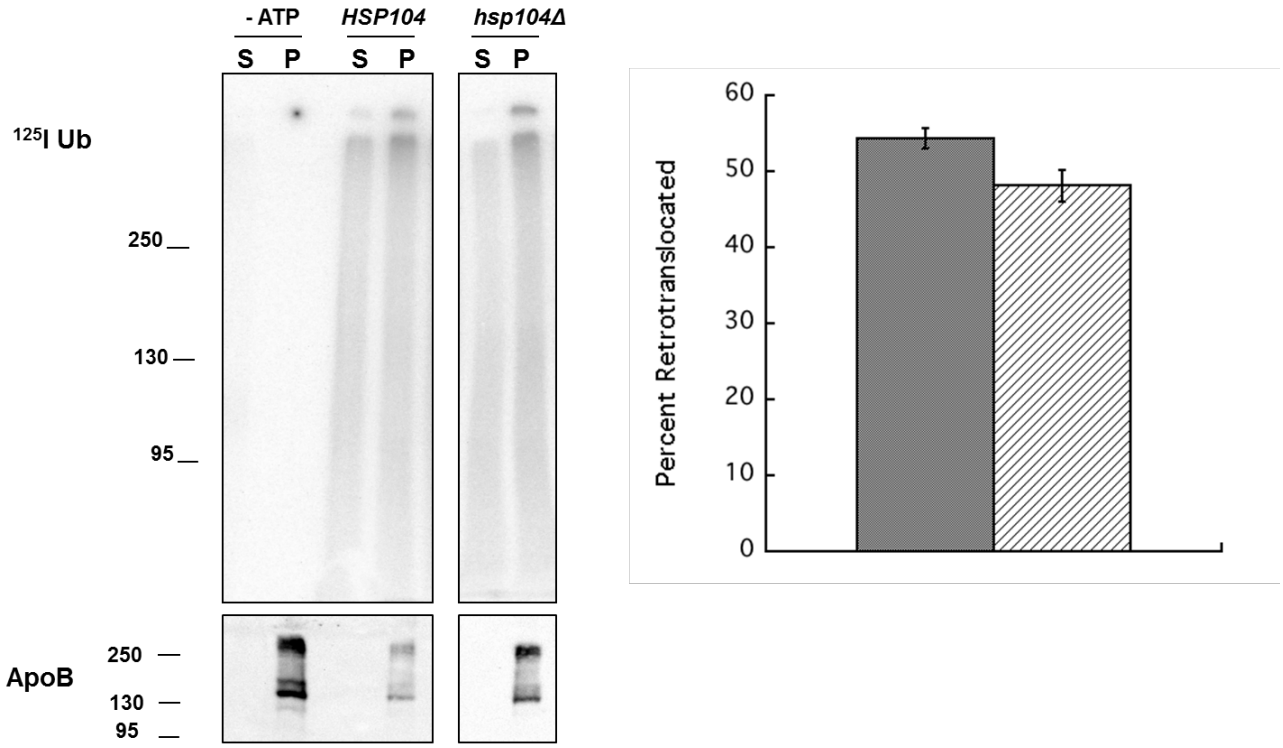


Figure 23.The Retrotranslocation of Ubiquitinated ApoB is Somewhat Lower in the Absence of Hsp104.

ER derived microsomes prepared from wildtype (N=10) (grey bar) and *hsp104Δ* (N=10) (striped bar) yeast expressing ApoB (temperature shifted to 37°C) were subjected to an *in vitro* retrotranslocation assay. Reactions containing microsomes, yeast cytosol, an ATP regenerating system and ^{125}I-ubiquitin were conducted at 37°C for 45 minutes. Control reactions lacked the ATP regenerating system. The reactions were quenched, centrifuged, and the supernatant and pellet fractions immunoprecipitated overnight using anti-ApoB antibody and protein G conjugated Sepharose beads. Following washing, samples were subjected to duplicate SDS-PAGE following by western blot or phosphorimage analysis. P value = 0.0197.

2.3 DISCUSSION

As described in this chapter, I developed a new expression system to investigate the fate of ApoB in the model yeast, *Saccharomyces cerevisiae*. This new system allows for the controlled induction of ApoB through the use of β-estradiol and a chimeric "GEV" transcription factor. Only small amounts of β-estradiol ($\leq$ 1 μM) were required to induce ApoB protein production, consistent with previous reports that GEV is saturated between 100 nM and 1 μM β-estradiol (McIsaac et al, 2011). ApoB appears as two bands via western blot, a larger, glycosylated molecular weight species and a smaller, non-glycosylated molecular weight species in both the galactose inducible (Grubb et al, 2012; Hrizo et al, 2007) and β-estradiol inducible expression systems (this study).

I hypothesize that the lower molecular weight species corresponds to an alternative start site being used to make the ApoB protein and that this protein lacks a signal sequence. Even though the full length protein appears to be synthesized and possesses features consistent with it being a translocated form of ApoB, DNA sequence analysis of the ApoB29 plasmid suggested the ApoB protein should not have been fully translated (Figure 10). To address this issue, I have attempted to make additional mutations in the ApoB expression plasmid. One mutation alters the predicted methionine for the alternative start site to alanine using the QuikChange XL II site directed mutagenesis kit (Agilent). Although I succeeded in introducing this mutation, I could only sequence the N-terminal 60% of the ApoB29 coding sequence. Furthermore, this plasmid was unable to sustain the growth of yeast on selective media (data not shown). Additional attempts to create another plasmid with the site directed mutagenesis kit led to the construction of plasmids that could not be recovered from either yeast or *E.coli*. Recently, I created an ApoB plasmid that lacks the signal sequence. Initial investigations with this construct confirm the role

of the proteasome and Hsp104 on the degradation of the lower molecular weight species of ApoB. Thus, the data in thesis on the lower molecular weight form have been validated.

Although the current ApoB plasmid may contain mutations (see below), I propose that this is still a valid model to investigate ApoB regulation and believe that *bona fide* ApoB is still being synthesized for a variety of reasons. First, I observe the correct predicted molecular weight for the ApoB29 protein. Next since ApoB contains a C-terminal HA tag and is detectable using anti-HA antibody, the HA tag must still be translated in frame. Furthermore, the protein can be immunoprecipitated using a commercial anti-ApoB antibody. Additionally, the higher molecular weight species is glycosylated and is carbonate extractable, indicating that it is, at least partially, able to enter the ER, as expected for ApoB. Finally, we have previously used this ApoB29 construct to identify factors in yeast that affect ApoB biogenesis and have confirmed these results in mammalian cells (Grubb et al, 2012; Hrizo et al, 2007). It seems unlikely that we would have obtained these results if the protein was completely irrelevant. It is also formally possible that the sequencing results are incorrect, as sequencing was only conducted once and data were obtained from only one DNA strand. As we have had trouble using this ApoB29 construct for cloning, it would not be surprising if sequencing issues arose from improper priming.

I used the new ApoB29 expression system to determine if the protein interacts with lipid droplets using a large scale lipid droplet isolation. Even though lipid droplets were isolated, ApoB did not reside in this fraction. Furthermore, cycloheximide chase assays conducted by a previous graduate student, Sarah Grubb, in lipid droplet deficient yeast using the galactose inducible system showed ApoB degradation was unaffected compared to the wildtype strain (Grubb, 2013). Taken together, ApoB does not interact with lipid droplets in yeast and its

degradation is independent of these species, which is consistent with reports that lipid droplets are not required for ERAD in yeast (Olzmann & Kopito, 2011; To et al, 2017).

Previously, ApoB crescents were observed in Huh7 cells (Ohsaki et al, 2006). This cell type may not be the most representative model for ApoB, as Huh7 cells do not normally secrete ApoB. Furthermore, the ApoB crescents may not specifically localize to lipid droplets but interact near the surface of the lipid droplets, as the outer leaflet of the ER is continuous with lipid droplets in yeast and in certain mammalian cells (Jacquier et al, 2011; Wilfling et al, 2013). In fact, using confocal microscopy ApoB was observed to be restricted to the ER membrane, which was in contact with the lipid droplet surface but not with the lipid droplet itself (Gao & Goodman, 2015; Suzuki et al, 2012). Additionally, Ohsaki and coworkers could eliminate this localization by inhibiting the MTP complex using drugs or siRNA, suggesting that only lipidated ApoB localized to these crescents (Ohsaki et al, 2008). As yeast lack an MTP complex, the ApoB protein is probably not lipidated, which may account for the lack of lipid droplet localization. Interestingly, a recent report implicated the E3 ubiquitin ligase, Doa10, as a mediator of ER protein selection into lipid droplets (Ruggiano et al, 2016). Doa10 targeted proteins that associate with the membrane via a "hairpin" and that localize to both the ER and lipid droplet membranes. The enzyme was responsible for degrading the ER pool of these proteins, thereby restricting these substrates to lipid droplets. Since ApoB is a Hrd1 substrate and is unaffected by deletion of Doa10, these data are consistent with ApoB being restricted from lipid droplets.

I hypothesized that Hsp104 affects ApoB degradation by preventing ApoB from aggregating once it is retrotranslocated but before it is targeted for proteasome-mediated degradation. I observed that Hsp104 facilitates ApoB degradation under heat stress but not

under non-stress conditions via cycloheximide chase assays. Degradation was accelerated upon over expression of Hsp104. These results were intriguing as ApoB29 is the first established ERAD substrate demonstrated to be an Hsp104 substrate. To show that this effect was direct, I determined that Hsp104 binds ApoB. I also observed Hsp70 in the coimmunoprecipitations. Since Hsp104 interacts with Hsp70 and Hsp40 once it has removed protein substrates from aggregates, Hsp70 also interact with ApoB and Hsp104 to prevent ApoB aggregation (Glover & Lindquist, 1998; Shorter, 2017).

Based on the established role of Hsp104 as a protein disaggregase, I first hypothesized that Hsp104 affects the aggregation propensity of ApoB (Zolkiewski et al, 2012). Since Hsp104 is a cytosolic protein, it most likely interacts preferentially with the cytosolic form of ApoB, i.e. the lower molecular weight form that fails to enter the ER. This was supported by quantifying the effect of Hsp104 on each band. As hypothesized, the non-glycosylated form had a much stronger dependence on Hsp104 for degradation than the glycosylated form. However, there was no apparent difference in ApoB aggregation in the presence or absence of Hsp104 under stress or non-stress conditions, as assessed using various detergents. Therefore, I next hypothesized that Hsp104 may affect ApoB's retrotranslocation efficiency. As hypothesized, in the absence of Hsp104 ApoB is retrotranslocated less efficiently to a small but reproducible degree. Although these data are preliminary, they lead to two potential models for how Hsp104 might affect ApoB degradation. In one view, Hsp104 may help keep ApoB in a retrotranslocation competent state and facilitate its retrotranslocation. ApoB forms large cytosolic loops that may interact with Hsp104. Thus Hsp104, in combination with Hsp70, may protect ApoB such that Cdc48 can more easily associate with it to facilitate retrotranslocation. The chaperone could also potentially hold ApoB in a position that makes it more Hrd1 accessible, which facilitates ubiquitination.

Alternatively, Hsp104 might work cooperatively with Cdc48 to directly drive retrotranslocation. Through its AAA+ domains, Hsp104 couples ATP hydrolysis to protein disaggregation by translocating protein substrates through the central cavity (Gates et al, 2017; Heuck et al, 2016; Yokom et al, 2016). Perhaps, Hsp104 can provide additional force with Cdc48 to move ApoB from the translocon. In turn, the disaggregase activity of Hsp104 might be more important to degrade the detergent insoluble untranslocated (i.e. lower molecular weight) form of ApoB. The action of Hsp104 as a cytoplasmic protein disaggregase is well established (Glover & Lindquist, 1998; Parsell et al, 1991; Shorter, 2011).

Surprisingly, the Hsp104 disaggregase is not conserved in metazoans. This poses the question as to what is the holdase for ApoB is in mammalian cells. I was intrigued when the conserved Rvb proteins were reported to functionally compensate for loss of Hsp104 in heat stressed cells (Zaarur et al, 2015). However, overexpression of Rvb2 did not affect ApoB protein degradation rates in yeast that lack Hsp104. This may be because the Rvb proteins are primarily associated with nuclear proteins and nuclear complexes, including those involved in snoRNP regulation, chromatin remodeling, RNA polymerase II assembly, and DNA damage responses (Gorynia et al, 2011; Gribun et al, 2008; Kakihara & Houry, 2012; Matias et al, 2015; Putnam et al, 2001). Furthermore, at a recent conference Dr. Walid Houry reported that the Rvb proteins form nuclear foci upon cellular stress (personal communication). As ApoB is a cytosolic protein, the Rvb proteins may not be accessible to ApoB to affect degradation, yet it is still able to rescue a nuclear defect in *hsp104Δ* cells. It has also been proposed that Hsp110, Hsp70, and Hsp40 cooperate to serve this holdase and disaggregase function in mammalian cells (Mattoo et al, 2013; Rampelt et al, 2012; Shorter, 2011; Torrente & Shorter, 2013). This complex preferentially refolded amorphous aggregates over amyloid fibrils, but folding was

slower in comparison to Hsp104-mediated protein refolding (also see Chapter 3). Current efforts in the laboratory are examining whether members of the mammalian Hsp70, Hsp40, and Hsp110 complex affect ApoB stability and act as disaggregases.

3.0 CONCLUSIONS

CAD is the leading cause of death worldwide and is characterized by the presence of atherosclerotic plaques, caused by an overabundance of circulating LDLs and cholesterol. The gold standard to combat CAD is the use of statins, which block the committed step during cholesterol synthesis. However due to side effects, nearly one third of all users stop the treatment. Alternative therapies recently have focused on regulators of cholesterol synthesis, delivery, or degradation. One of these regulators is ApoB, which is the essential structural component of LDL. Mipomersen is the first approved anti-sense oligonucleotide that targets ApoB to control cholesterol levels in humans. But based on limitations to the use of this drug (see Chapter 1), there must be other ways to regulate ApoB levels, in addition to targeting cholesterol or ApoB levels specifically. I identified one such factor, Hsp104, which promotes ApoB degradation. Hsp104 is a molecular chaperone holdase and a disaggregase that appears to modestly affect ApoB's retrotranslocation efficiency. Unfortunately, Hsp104 is not conserved in humans. This begs the question of what factor might perform an equivalent function for Hsp104 in humans. In this chapter, I suggest candidate factors that may serve as a holdase and disaggregase for ApoB in human cells.

3.1 HSP110 AND HSP40

For unknown reasons, metazoans do not have a homolog of the Hsp104 disaggregase. To compensate for the lack of this enzyme, it has been proposed that Hsp110, Hsp70, and Hsp40 act cooperatively to disaggregate proteins (Mattoo et al, 2013; Rampelt et al, 2012; Shorter, 2011; Shorter, 2017). Hsp70 is an ATPase associated with protein folding. Its ATPase activity is stimulated by Hsp40, and Hsp110 acts as a nucleotide exchange factor for Hsp70. However, it was observed that Hsp110 can also function as an ATP-dependent unfoldase and also has chaperone activity on its own, which is sufficient to prevent substrate misfolding and aggregation (Dragovic et al, 2006; Polier et al, 2008; Raviol et al, 2006; Ziegelhoffer et al, 1995). In yeast, one of the cytoplasmic Hsp70s is Ssa1, and its activity is stimulated by the Hsp40, Ydj1, as well as by Sse1, an Hsp110 family member. This conserved trio of proteins has the ability to disaggregate proteins in yeast, although the disaggregase activity was slow compared to Hsp104, at least under the conditions of this *in vitro* experiment (Shorter, 2011).

In cells, Hsp110 may serve as the holdase and further facilitate disaggregase activity. In yeast, Hsp110 helps to facilitate prion propagation and may localize to stress foci (Escusa-Toret et al, 2013; Spokoini et al, 2012). Mammalian Hsp110 has also been shown to assist in the removal of aggregated proteins (Eroglu et al, 2010; Olzscha et al, 2011; Yamashita et al, 2007). We previously investigated the role of Sse1 on ApoB degradation using our galactose inducible yeast expression system (Hrizo et al, 2007). In yeast, we found the ApoB degradation was faster in the absence of Sse1 and that ApoB coimmunoprecipitated with Sse1 and Ssa1. In mammalian cells, ApoB secretion was increased when Hsp110 was overexpressed. This result suggests that Sse1 interacts with ApoB to act as a holdase and in mammals the conserved protein promotes its maturation and secretion. However, the chaperone/holdase activity of Sse1 only requires the C-

terminal domain *in vitro* and it has been shown that the N-terminal ATP binding domain of Sse1 is required to rescue the growth of a temperature sensitive mutant form of Hsp40 when overexpressed (Goeckeler et al, 2002). This result may indicate that the chaperone activity of Sse1 alone is not sufficient to serve as a holdase.

Alternatively, Hsp40 could perform the holdase function for ApoB. This is an intriguing target as it has been shown that Hsp40 in mammals is a potent disaggregation machine (Nillegoda et al, 2015). Working in pairs, the Hsp40s (when combined with Sse1 and Ssa1) could disaggregate and refold a heat denatured luciferase protein with similar efficiency as the Hsp104 system.

Based on these and other data, we have recently begun to investigate if Hsp40 also affects ApoB degradation and aggregation. During her rotation in our lab when I served as her mentor, another graduate student, Deepa Kumari, observed that ApoB degradation was significantly slowed in a yeast strain containing a temperature sensitive mutant form of Ydj1. Most interestingly , we observed only the lower molecular weight species of ApoB in the temperature sensitive Ydj1strain, which we hypothesize is an untranslocated form of ApoB. Ydj1 has been previously shown to be required for the translocation of another protein into the yeast ER, prepro-alpha factor (Becker et al, 1996). We hypothesize that Ydj1 may play a dual role, assisting both in translocation of ApoB and in the subsequent degradation of the protein via ERAD. Since joining our lab, Deepa has begun pulse-chase experiments in rat hepatic McArdle RH7777 cells to see if the Ydj1 homolog, DNAJA1, affects ApoB stability, maturation, or both. Hsp40s could be regulators of ApoB function, if the chaperone indeed helps to promote ApoB translocation and degradation when lipids are depleted.

3.2 GET COMPLEX

Another possible candidate for an ApoB holdase function is the Guided Entry of Tail-anchored (GET) proteins complex, which help with the membrane insertion of tail-anchored proteins. Tail-anchored proteins are characterized by the presence of a C-terminal transmembrane domain which anchors the protein into the ER membrane after their complete synthesis (Chartron et al, 2012; Denic et al, 2013). These transmembrane domains must be shielded from the aqueous environment of the cell post-translationally and before insertion, which is performed by a complex consisting of Sgt2, Get4, and Get5 in yeast. Subsequently, the tail-anchored protein is shuttled to Get3 and then is inserted into the ER membrane by the integral membrane proteins Get1 and Get2. The Sgt2, Get4, Get5 complex could potentially act as a holdase for ApoB, serving a similar function as they do with tail-anchored proteins, except that in this case a hydrophobic protein is protected as it leaves the ER. In addition, the mammalian homologs of this complex have been shown to interact with retrotranslocated proteins during ERAD to help facilitate degradation (Hessa et al, 2011; Minami et al, 2010; Wang et al, 2011). Interestingly, the function of Sgt2 has been linked to Hsp104 in yeast. Specifically, the curing of yeast prions performed by Hsp104 could be reversed by overexpressing Ssa1, an Hsp70, and Sgt2 was the switch to increase targeting of Ssa1 to the prion (Kiktev et al, 2012).

With the help of an undergraduate in the Brodsky lab, Andrew Schulz, we have recently begun investigating if the Get Complex affects ApoB stability using our β-estradiol inducible expression system. Andrew examined if the absence of Sgt2 affects ApoB stability using cycloheximide chase assays. He observed that ApoB was degraded faster in the absence of Sgt2 compared to the rate in wildtype cells, indicating that Sgt2 plays a role in stabilizing ApoB. We are currently investigating the effect of Sgt2 deletion on another ERAD substrate, Ste6p*, to

determine if this effect is applicable to ERAD substrates besides ApoB. We are additionally creating a plasmid that will be used to overexpress Sgt2. I predict the introduction of this expression vector will slow ApoB degradation in yeast. In the future, we intend to investigate if the human homolog, SGTA, as well as other members of the GET complex affect ApoB degradation in mammalian cells through pulse chase experiments.

3.2.1 The Bag6 Complex

In humans, the Sgt2, Get3, Get4 complex consists of SGTA, UBL4A, and Trc35 but GET complex function also requires an additional cofactor, Bag6. Bag6 is a member of the Bag family of proteins, which are characterized by the presence of a C-terminal BAG domain which mediates interaction with Hsp70 (Casson et al, 2016; Kabbage & Dickman, 2008). Bag6 helps facilitate transfer of tail anchored proteins from SGTA to Trc40, the homolog of Get3. Bag6 was subsequently shown to help facilitate the degradation of ubiquitinated proteins and to have *in vitro* chaperone activity. Bag6 appears to recognize exposed hydrophobic regions in proteins (Hessa et al, 2011; Minami et al, 2010; Wang et al, 2011). Furthermore, Bag6 has been implicated in retrotranslocation of another misfolded, glycosylated ERAD substrate, TCRα (Claessen & Ploegh, 2011). As ApoB is also a ubiquitinated glycosylated protein with hydrophobic domains, perhaps Bag6 could serve a similar function for ApoB during the degradation of ApoB.

3.3 OTHER POTENTIAL TARGETS FOR TREATING CORONARY ARTERY DISEASE

As illustrated by drugs that target PSCK9 (see Chapter 1), current investigations into treating CAD without modulating ApoB can be extremely effective. Another way this may be accomplished is by targeting the assembly of the MTP complex. One drug, Lomitapide, prevents the action of the MTP complex (Cuchel et al, 2013). But since the MTP complex requires the assembly of the M subunit with a PDI, perhaps a drug that prevents the formation of the complex or the activity of PDI would also inhibit vLDL formation. Another potential target might be TANGO/TALI, which has been proposed to recruit lipids to ER exit sites to facilitate pre-vLDL formation (Santos et al, 2016). If these factors help to assemble pre-vLDLs, then perhaps in their absence (possibly by siRNA) circulating vLDLs and cholesterol would be reduced. Overall, there are a variety of factors that may serve as new targets for treatment of CAD.

3.4 CONCLUDING REMARKS

A great deal of effort has been put forth into understanding the underlying causes of cardiovascular diseases, generating a wealth of information about risk factors, pathology, factors that regulate the progression of CAD, and treatments, both approved for human use and in clinical trials. Understanding the basic biological causes of CAD has proven to be the one of the most exciting areas of research but also one of the most challenging due in part to cholesterol's important biological functions. As the field moves forward, it is becoming increasingly clear that targeting the factors that regulate cholesterol synthesis is vital for treatment of CAD. This

has led to the development of new treatments, such as PSCK9 antibody therapy, which have the potential to rival statins in their effectiveness for treating CAD. It is important to continue to pursue alternative treatments as individuals respond differently to various treatments. It is my hope that my investigations into the degradation pathway of ApoB will also contribute to therapeutics for CAD.

APPENDIX A

A.1 INVESTIGATION OF THE ROLES OF POTENTIAL TRANSLOCON REGULATORS ON APOLIPOPROTEIN B DEGRADATION

A previous student in the Brodsky lab, Dr. Sarah Grubb, was interested in factors that could potentially regulate the endoplasmic reticulum associated degradation (ERAD) of ApoB. One focus of her work was on factors that may affect the interaction between the Sec61 translocon and ApoB. ApoB is known to interact with Sec61 and then retrotranslocate to the cytoplasm for degradation (Cardozo et al, 2002; Chen et al, 1998; Fisher et al, 2008; Mitchell et al, 1998; Pariyarath et al, 2001). During my rotation in the Brodsky lab I was mentored by Dr. Grubb, and I investigated two potential translocon regulators, Ysy6 and *YKL207w* (Grubb, 2013). Ysy6 was first identified in an *E.coli* screen for mutations that suppress a secretion defect of SecY, the bacterial homolog of Sec61α, (Sakaguchi et al, 1991). Ysy6 was also identified as the yeast homolog of RAMP4 (ribosome associated membrane protein 4), which is proposed to help mediate protein translocation (Schroder et al, 1999). *YKL207w*, which has subsequently been renamed *ECM3*, is a member of the ER membrane complex (ERM), which facilitates protein folding, and results in upregulation of the unfolded protein response (Jonikas et al, 2009; Richard et al, 2013). To examine if these putative translocon regulators affected ApoB, I conducted cycloheximide chase assays in wildtype yeast and in yeast lacking Ysy6 and *YKL207w* at 30°C

using the galactose inducible expression system (Hrizo et al, 2007). Samples were TCA precipitated and to monitor ApoB levels western blot analysis was used. However, the degradation of ApoB was unaffected in the absence of either of these potential translocon regulators, indicating that these proteins do not affect ApoB ERAD (Grubb, 2013).

Dr. Grubb investigated another conserved family of proteins, the Yeast endoplasmic reticulum transmembrane (Yet) proteins, which she also hypothesized, may affect the ERAD of ApoB. The Yet proteins are a conserved family of integral membrane proteins thought to assist in membrane protein synthesis (Annaert et al, 1997; Ladasky et al, 2006; Paquet et al, 2004; Schamel et al, 2003; Wilson & Barlowe, 2010). She determined that ApoB degradation was slowed in *yet2Δ* and *yet3Δ* yeast while ApoB stability was unaffected in the *yet1Δ* strain (Grubb, 2013). In collaboration with the Fisher laboratory at the New York University School of Medicine, she went on to show that overexpression of Bap31, the mammalian homolog of Yet3, also affected ApoB degradation and secretion in mammalian cells. One of the remaining unanswered questions was how these proteins are regulating ApoB.

A.2 MATERIALS AND METHODS

A.2.1 Degradation Assays

Conducted as described in previous section 2.1.3

A.2.2 Denaturing Coimmunoprecipitation Assay

For ApoB29 protein production, plasmid pSLW1-B29 was used (Hrizo et al, 2007). For copper-inducible myc-tagged ubiquitin protein production, plasmid pKN31 was used (Nakatsukasa et al, 2008).

Cells were grown overnight in synthetic minimal media lacking uracil and histidine supplemented with 2% glucose at 30°C. Cells were diluted back in the same media and grown until log phase (OD_{600} = 0.4-0.6). ApoB29 production was induced using 300 nM β-estradiol for 2 hours at 30°C. A total of 150 ODs were harvested and resuspended in synthetic minimal media lacking uracil and histidine supplemented with 2% glucose and 300 nM β-estradiol to a concentration of 3 OD_{600}/mL. Next, the cells were incubated for 15 minutes at 30°C and ubiquitin was induced using 100 µM copper sulfate for 2 hours at 30°C. Cells were harvested and stored at -80°C.

Cells were resuspended in 500µL IP Extract Buffer (50 mM Tris, pH7.4, 0.5% SDS, 1 mM EDTA) supplemented with protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A), c0mplete EDTA free protease inhibitor cocktail (Roche), and 10 mM NEM. The cells were then lysed on a Vortex mixer 5 times for 30 seconds each round, with 30 second incubations on ice in between each agitation. The resulting cell lysate was centrifuged for 5 minutes at 13,000 rpm. The supernatant was saved and 10% was reserved for the "input". The remaining lysate was diluted to 1.5 mL using IP dilution buffer (60 mM Tris, pH 7.4, 190 mM NaCl, 2.25% Triton X-100, 6 mM EDTA) supplemented with protease inhibitors, protease inhibitor cocktail, and 10 mM NEM. Next, the samples were incubated with HA-conjugated beads or Sepharose 6B beads overnight (~20 hours) at 4°C with rotation. The next day, samples were centrifuged for 2 minutes at 5000 rpm at 4°C and washed 3 times in IP Wash buffer (50

mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, 5 mM EDTA) supplemented with protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A), c0mplete EDTA free protease inhibitor cocktail, and 10 mM NEM. The samples were then washed 3 times in Urea Wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% SDS, 5 mM EDTA, 2 M Urea) supplemented with protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A), c0mplete EDTA free protease inhibitor cocktail, and 10 mM NEM. Samples were washed twice in TEA (50 mM Tris, pH7.4, 150 mM NaCl, 5 mM EDTA) supplemented with protease inhibitors (1 mM PSMF, 1 µg/mL leupeptin, 0.5 µg/mL pepstatin A), c0mplete EDTA free protease inhibitor cocktail, and 10 mM NEM and resuspended in TCA sample buffer (80 mM Tris, pH8, 8 mM EDTA, 120 mM DTT, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue). The input material was TCA precipitated and all samples were heated at 37°C for 20 minutes followed by duplicate SDS polyacrylamide gel electrophoresis and western blots. One western blot was immunoblotted using HRP-conjugated anti-HA antibody (Roche Applied Sciences, 3F10) and the duplicate western blot was sandwiched between thick Wattman paper and boiled in a waterbath for 1 hour. The boiled western blot was blocked in fetal bovine serum containing triton and ubiquitin was detected with α-myc (Cell Signaling) or α-Ubiquitin (FL76) (Santa Cruz) antibodies and visualized using SuperSignal West Femto kit.

A.3 RESULTS AND DISCUSSION

I hypothesized that the Yet proteins may affect the ability of ubiquitin to be conjugated onto ApoB. Ubiquitin conjugation is a series of enzymatic reactions that attach a ubiquitin moiety

onto a substrate protein through an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ubiquitin ligase (see section 1.4) (Preston & Brodsky, 2017). I investigated this hypothesis through the use of denaturing immunoprecipitations (IP). By pulling down ApoB with anti-HA conjugated or Sepharose beads, I could investigate the potential change in ubiquitination level by western blot analysis when the *Yet* genes were deleted. I first optimized conditions using a mutated version of Sterol 6 (Ste6p*), which is an ERAD substrate, as a control for both IP conditions and ubiquitin detection, as Ste6p* has been previously shown to acquire detectable ubiquitin levels via this method (Nakatsukasa et al, 2008). Indeed, I was able to coimmunoprecipitate Ste6p* and ubiquitin was detected (Figure 24) as compared to the Sepharose bead control.

I next investigated whether ApoB was ubiquitinated using the conditions established with Ste6p*. Before conducting the IP, I screened colonies for expression levels of ApoB. Four colonies from BY4742 (wildtype), *yet1Δ*, *yet2Δ*, *yet3Δ*, and *yet1,2,3Δ* yeast expressing ApoB protein, induced using the β-estradiol system, (see section 2.2.1) were harvested. Following TCA precipitation and western blotting, colonies with near equal expression were chosen for subsequent use (data not shown). Next, I grew yeast containing copper inducible myc-tagged ubiquitin and either the empty vector or the ApoB expression vector in BY4742, *yet1Δ*, *yet2Δ*, *yet3Δ*, and *yet1,2,3Δ* yeast strains and subsequently induced the myc-tagged ubiquitin with 100 μM copper. Cells were lysed under denaturing conditions and IPs were conducted overnight with either HA-conjugated or Sepharose 6B beads. Samples were washed followed by SDS-PAGE and western blotting for both ApoB and ubiquitin (Figure 25). ApoB was enriched in the IP in the lanes in which ApoB was expressed and incubated with α-HA conjugated beads as

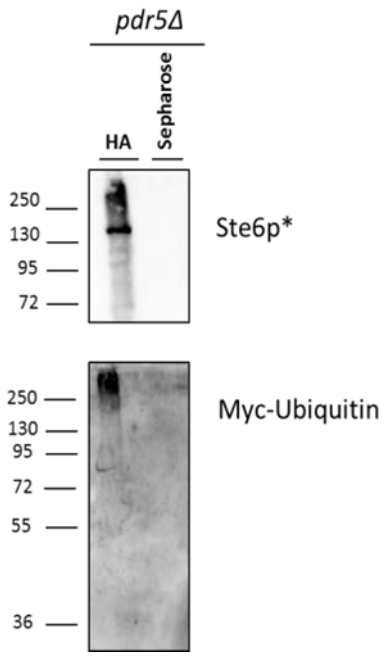


Figure 24. Detection of Ubiquitin Levels of Ste6p*.

Denaturing immunoprecipitations were conducted in *pdr5Δ* yeast expressing Ste6p* and overexpressing myc-tagged ubiquitin incubated with either HA-conjugated or Sepharose 6B beads. One hundred fifty OD_{600} equivalents of yeast expressing Ste6p* and myc-tagged ubiquitin were harvested and resuspended in IP Extract buffer (see section A1.2). Cells were lysed on a vortex mixer and lysate was immunoprecipitated overnight with either HA conjugated or Sepharose 6B beads. Following washing, samples were subjected to SDS-PAGE and western blotting using anti-HA or anti-Ubiquitin antibodies.

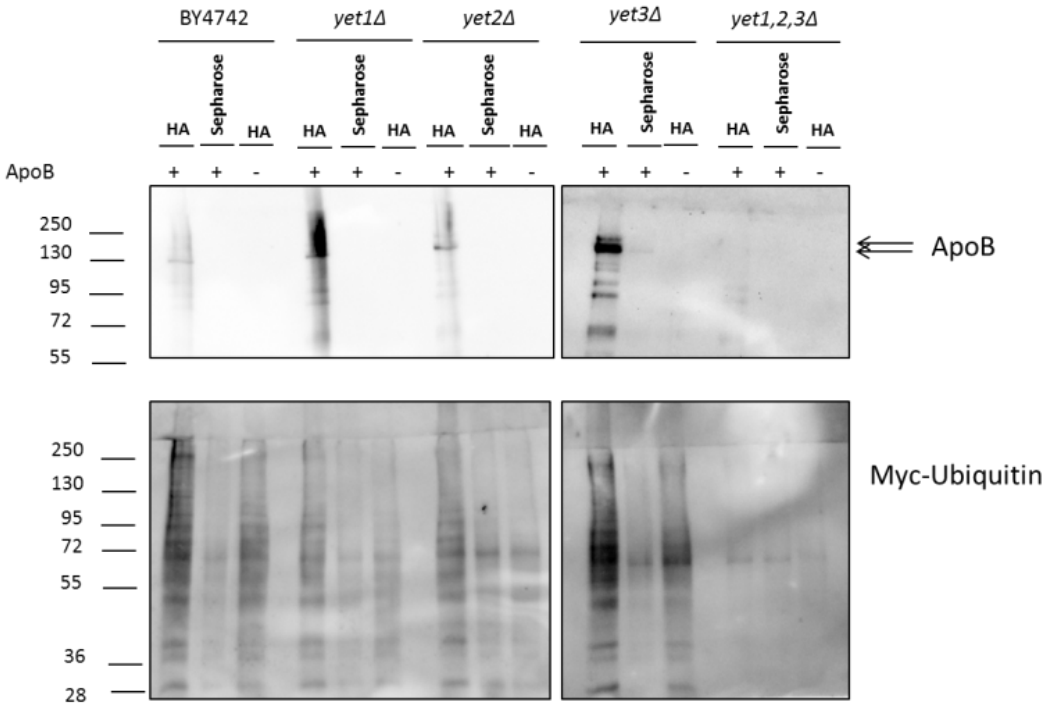


Figure 25. Preliminary Investigation of ApoB Ubiquitination Levels.

Denaturing immunoprecipitations were conducted in wildtype (BY4742), *yet1Δ*, *yet2Δ*, *yet3Δ*, and *yet1,2,3Δ* yeast expressing ApoB or containing a vector and myc-tagged ubiquitin expression vectors. IP conditions were conducted as in Figure 24. Following washing, samples were subjected to SDS-PAGE and western blotting using anti-HA and anti-ubiquitin antibodies.

compared to the Sepharose bead control or vector alone. However, variable amounts of ApoB were pulled down during the IP, even though these colonies were screened for equal expression prior to experimentation. Additionally, ubiquitination levels above the vector control were difficult to detect for ApoB.

I next wanted to investigate the stability of ApoB in the absence of the Yet proteins using my new β-estradiol inducible expression system (see section 2.2.1) as Dr. Grubb had identified these translocon regulators using our galactose inducible expression system. Since this is a less stressful induction system due to the lack of carbon source switching, I was curious as to how the Yet proteins affected ApoB. I predicted that the Yet proteins effect on ApoB stability would be similar regardless of whether the galactose inducible or β-estradiol inducible expression system was used, since I had not observed differences in stability between the expression systems in other experiments (see section 2.2.1). ApoB protein was induced in wildtype, *yet1Δ*, *yet2Δ*, *yet3Δ*, and *yet1,2,3Δ* yeast strains followed by cycloheximide chase assays conducted at 30°C. Samples were subsequently TCA precipitated and proteins were subjected to western blot analysis (Figure 26). Surprisingly, there appeared to be no difference in degradation rates for ApoB in the various strains.

If these experiments were pursued further, additional controls would be included. For the IPs, I have recently identified an anti-ApoB antibody (EMD Millipore) that appears to give a more reliable ApoB IP. The denaturing IPs could be repeated with this antibody. Perhaps this would produce a more robust and reproducible level of precipitation, which would allow me to determine potential differences in the ubiquitination state of ApoB. Additionally, the cycloheximide chase assays could be repeated at 37°C, which would be a stressful condition on the cells. In addition to the yeast result, Dr. Grubb also showed that Bap31 affected ApoB

degradation in mammalian cells. However, this leaves the puzzle of why the Yet proteins do not affect ApoB stability in the β-estradiol inducible system. Perhaps as this system is less stressful on the yeast cells compared to galactose, the Yet proteins are not as important for regulating ApoB. By conducting the cycloheximide chase assays under stressful conditions, such as a temperature shift, perhaps we could recapitulate the requirement for the Yet proteins for ApoB degradation.

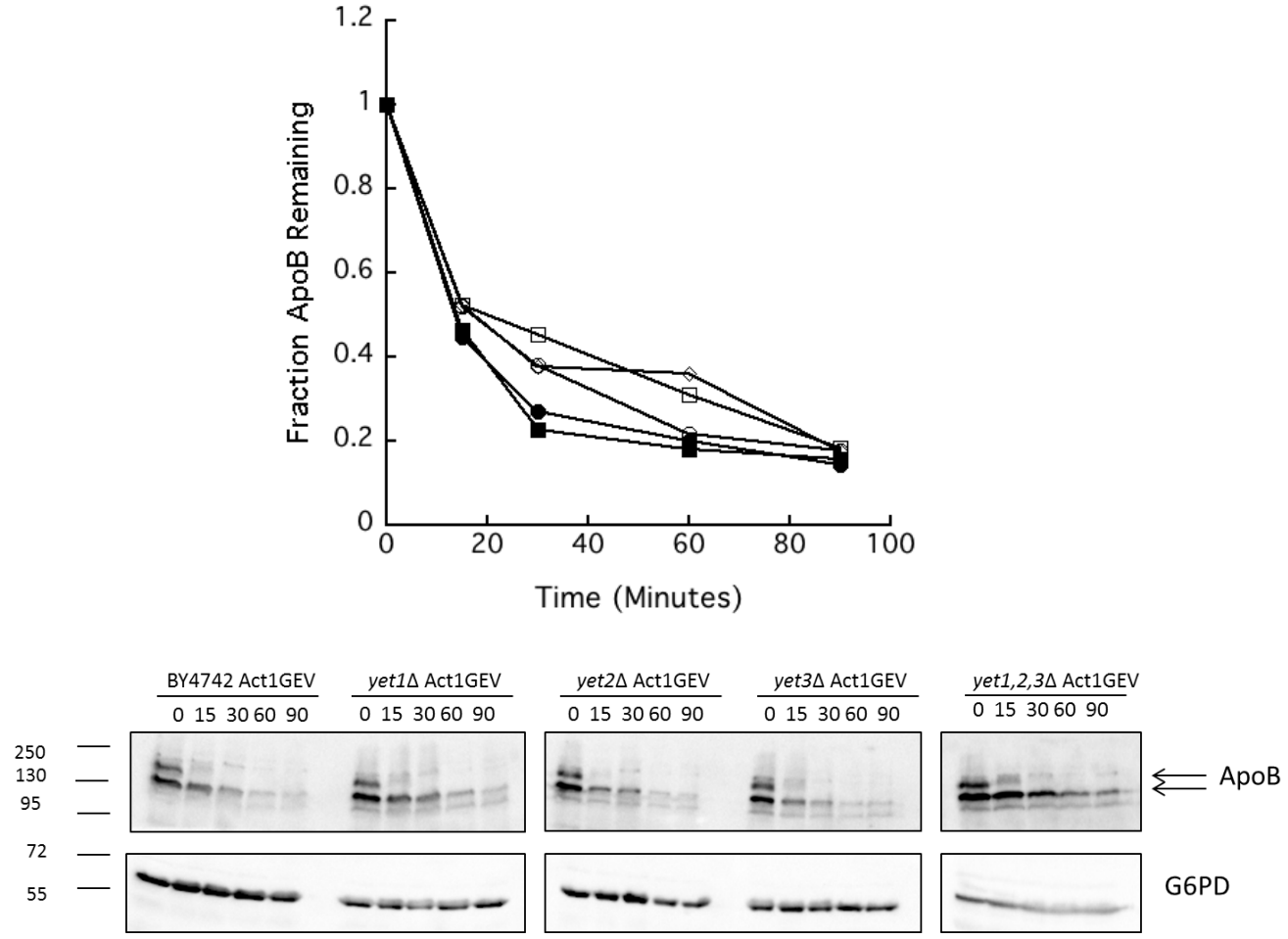


Figure 26. ApoB Appears Unaffected in Strains Lacking the Yet Proteins Where the β-estradiol Inducible Expression System Was Used.

Cycloheximide chase assays were conducted at 30°C in BY4742 (open circles) (N=4), *yet1Δ* (closed circles) (N=4), *yet2Δ* (open squares) (N=4), *yet3Δ* (closed squares) (N=3), and *yet1,2,3Δ* (diamonds) (N=2) yeast expressing ApoB. Samples were TCA precipitated and proteins were subjected to western blot analysis using anti-HA to detect ApoB. G6PD serves as a loading control.

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