PROGRESS TOWARD GENOMICALLY OPTIMIZED BEEF: CHOLESTEROL TRANSPORT PATHWAYS AND LIPID HOMEOSTASIS

By

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To the Faculty of Washington State University:

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Abstract

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Cholesterol is an essential substance involved with many functions such as maintaining cell membranes, manufacturing vitamin D on surface of the skin, producing hormones, and possibly helping cell connections in the brain. Homeostasis of cholesterol is centered on the metabolism of lipoproteins, which mediate transport of the lipid to and from tissues. In particular, lipoprotein pathways have become popular targets for genetic studies in humans, due to their causative relationship with cholesterol induced vascular diseases such as atherosclerosis. Since lipoproteins are central to lipid homeostasis in general, we hypothesize that they are promising candidate genes for mapping quantitative trait loci for fat deposition, eating quality traits, and fatty acid composition in beef cattle. In the present study, we targeted 13 candidate genes in the reverse cholesterol transport and low density lipoprotein receptor pathways with a goal to enhance beef palatability, while keeping the products as healthy as possible. The candidate genes are: ABCA1, APOA1, APOB, APOC2, APOE, LCAT, LDLR, LIPC, LIPG, LPL, PLTP, PON1, SR-BI. A total of 30 PCR amplicons were sequenced in 6 Wagyu x Limousin animals, generating 37 SNPs. A Sequenom assay system selected 13 mutations for genotyping on ~250

F2 progeny. Association analysis between these 13 SNPs and 19 phenotypes, including 5 carcass, 6 eating quality and 8 fatty acid composition traits revealed the following significant associations: marbling score (associated with 1 marker), subcutaneous fat deposition (6 markers), carcass weight (2 markers), rib-eye area (4 markers), kidney-pelvic-heart fat (1 marker), shear force (3 markers), muscle fiber tenderness (1 marker), overall tenderness (1 marker), flavor intensity (2 markers), cholesterol (1 marker), conjugated linoleic acid (2 markers), poly-unsaturated fatty acids (2 markers), saturated fatty acids (2 markers). Together, the results of this study strongly establish lipoprotein pathways as critical sources of phenotypic variation in beef cattle, while providing markers that can be utilized for animal breeding.

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Dedication

This thesis is dedicated to Douglas E. Farrell-Ranker.

Chapter One:

INTRODUCTION

Beef cattle production represents the largest segment of American agriculture, with over 94.5 million cattle and $66 billion in value added to the US economy in 2008 (National Cattleman's Beef Association, 2009). Beef producers are consistently looking to improve the palatability of their products, while maximizing output and reducing costs. According to the United States Department of Agriculture (USDA), beef carcass quality grading is primarily assessed based on physiological maturity of the animal, as well as the distribution of lipids, with intramuscular deposition of fat (marbling) leading to higher valued products. Conversely, as the levels of subcutaneous fat deposition increases, economic value of the beef declines. Strategic animal breeding can maximize or minimize individual health related characteristics of beef such as saturated fat, conjugated linoleic acid, and cholesterol as well as palatability characteristics such as juiciness and flavor. Unfortunately, these traits are very complex and polygenic, which makes the molecular processes that govern them difficult to understand and manipulate. However, motivation for beef producers to maximize product quality is strong, as beef consumers are certainly willing to pay for palatable, healthy beef.

Although fat growth and distribution plays a critical role in the economic value of beef, relatively little is known about the processes that regulate it. It is known however, that adipose tissue proliferation occurs as triglycerides are synthesized, which is dependent on three evens: i.) De novo synthesis of fatty acids in adipocytes, ii.) Uptake of fatty acids from plasma, and iii.) Fatty acids recruited via hydrolysis of lipoproteins (Kris-Etherton and Etherton, 1982). The latter mechanism of lipoprotein hydrolysis appears to regulate de novo fatty acid synthesis, as demonstrated in rats, which terminate synthesis in the presence of certain lipoproteins (Lakshmanan et al, 1977). Therefore, since meat quality traits are affected by adipogenesis,

which is highly related to lipoprotein homeostasis, our hypothesis that mutations in genes affecting lipoproteins will also regulate meat quality traits appears reasonable.

Genetic marker assisted selection greatly enhances animal breeding by establishing a specific link between polymorphism and a measurable difference in complex traits, thereby allowing for selection of preferred phenotypes based on genotype. Commercially available genetic panels containing SNPs known to associate with specific traits such as GeneSTAR (Pfizer Animal Health, New York, NY), and Igenity (Merial Limited, Duluth, GA) have been independently proven to supplement predictive abilities of beef phenotypes (Van Eenennaam et al. 2007). The goal of this research was to summarize current knowledge of lipoprotein homeostasis, and then use this knowledge to investigate lipoprotein pathways in order to produce genetic markers for use in beef cattle breeding. Our results have generated several significant associations between markers and important traits, and thus have developed potentially critical makers.

Chapter Two:

LITERATURE REVIEW

2.1 Introduction

The clinical manifestation of cholesterol buildup in arteries servicing the heart muscle causes more death and disability than all types of cancer combined (Lloyd-Jones, 2009). This is a remarkable outcome for a common, polycyclic lipid with the humble primary function of maintaining the permeability and fluidity of cell membrane. Nonetheless, cholesterol is required by all eukaryotic cells, which have specialized methods of recruiting and synthesizing the lipid only when it is needed. While effectively maintaining intracellular cholesterol homeostasis, these processes leave excess amounts circulating though the body, leading to atherosclerotic plaque development and subsequent coronary artery disease. Thus, levels of cholesterol and related lipids circulating in plasma are important predictive tools utilized clinically to gauge risk of a cardiac event. For example, a rise in total cholesterol in men from 200 to 240 mg DL^{-1} is associated with a three-fold increase in death from cardiac disease (Stamler, et al. 2000).

As a synopsis of the major events and proteins that manage cholesterol homeostasis, this review chapter contributes to the substantial attention that has recently been directed to this area. Despite the scrutiny, the majority of phenotypic variation in total cholesterol and related traits eludes explanation by current genetic knowledge (Pollex and Hegele, 2007). This is somewhat disappointing considering heritability estimates have established these traits as highly genetic (Chen et al. 1990, Goode et al. 2007). Thus, the continued search for candidate genes, mutations, and mechanisms is vital to our understanding of heart disease at the molecular level. Furthermore, as marker development continues to predict risk of vascular illness, this knowledge has the potential to revolutionize treatment of this leading human health issue.

2.2 Lipoproteins

While in circulation, cholesterol, being a lipid, requires a transport vesicle to shield it from the aqueous nature of plasma. Complex, micelle-like amalgamations of various proteins and lipids achieve cholesterol transport through the vascular system. These particles, known as lipoproteins, are heterogeneous in size, shape, composition, function, and perhaps most importantly, their contribution to vascular disease. High density lipoprotein (HDL) particles promote vascular health by extracting cholesterol from tissues (including atherosclerotic plaques) and delivering it back to the liver. Conversely, low-density lipoproteins (LDLs) are the classic antagonists of the circulatory system due to their propensity to bind to connective tissue in the intimal sub-layer of arteries (Mourão et al. 1984). These processes are at the motivation for the "good cholesterol," and "bad cholesterol" stigmas attached to HDL and LDL, respectively. Before any cholesterol reaches a particle of the HDL or LDL sub-fraction, it will usually have undergone a maturation process beginning with the hepatic or intestinal synthesis of very low density lipoprotein (VLDL). VLDLs, LDLs, and HDLs make up three of the six major sub-fractions of lipoproteins, which also include chylomicrons, chylomicron remnants (CRs), and intermediate density lipoproteins (IDLs). Together, lipoprotein particles are heterogeneous composites of the stored forms of fatty acids and cholesterol (triglycerides and cholesteryl esters, respectively), amphipathic phospholipids, and apolipoproteins.

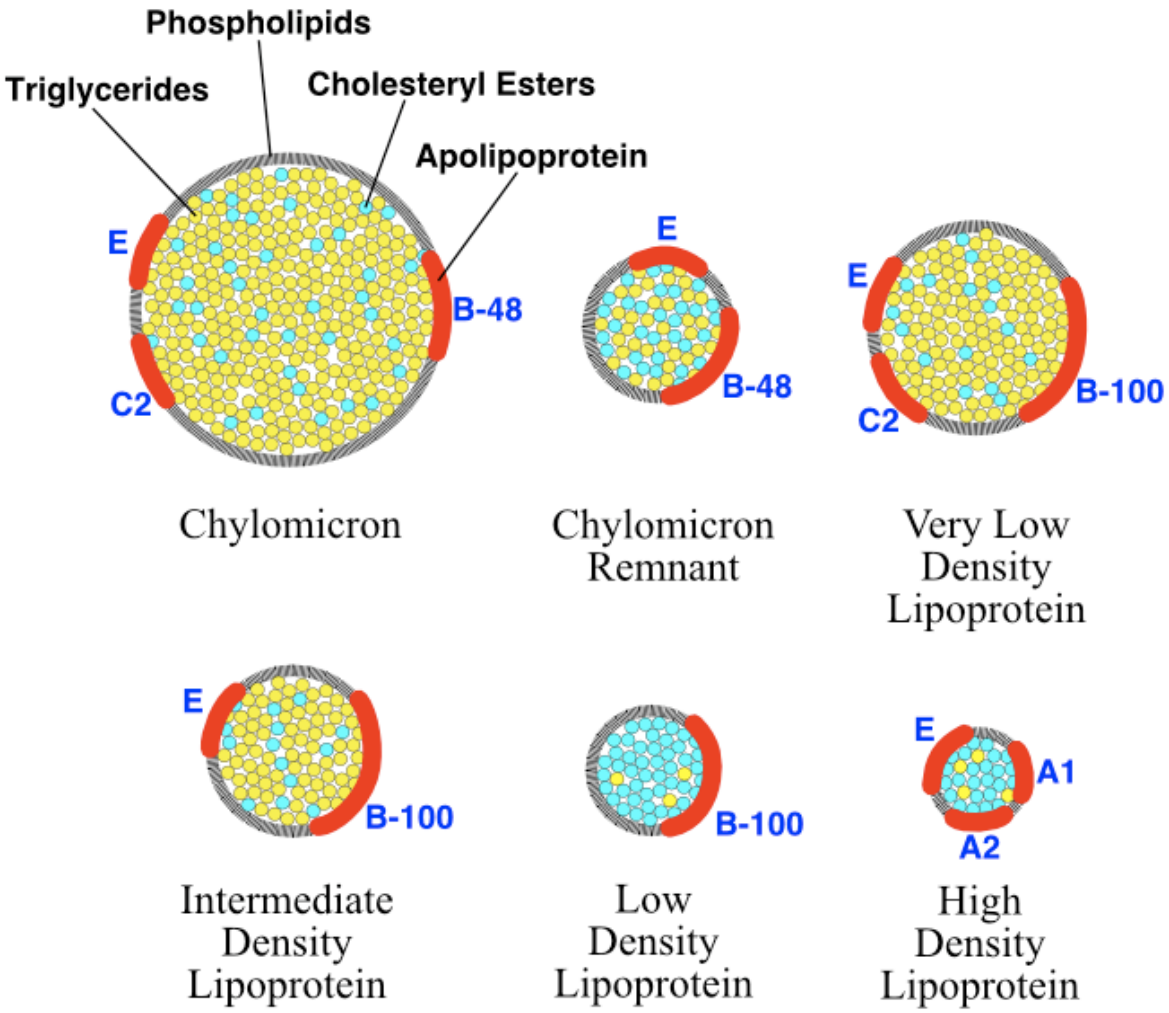

Figure 2.1: Approximate relative sizes and compositions of the six major classes of lipoproteins. The specific apolipoprotein components of each lipoprotein are also indicated.

2.3 High density lipoproteins and reverse cholesterol transport

High density lipoproteins prevent coronary artery disease by serving as transport particles for excess cholesterol to the liver, where it is converted into bile and excreted. In humans, HDL levels are an important measure of cardiac health due to their strong inverse relationship with coronary artery disease (Wilson et al. 1988). The principal HDL pathway, termed reverse cholesterol transport (RCT) is a major component of lipid homeostasis. Genetic variation with the RCT pathway contributes greatly to phenotypic variation in humans, potentially contributing as much as 50-70% of HDL variability (Cohen et al. 2004). The practical relevance of this statistic however, remains controversial, as it is possible that common mutations might contribute more minor effects to lipid phenotypes (Boekholdt et al. 2006).

2.3.1 Brief overview of reverse cholesterol transport

A schematic of RCT is shown as Figure 2.2. The process is initiated as apolipoprotein A1 (APOA1) protein is synthesized in the liver or the intestine and then secreted into circulation. Free APOA1 is immediately encompassed by phospholipids, forming discoidal pre-β1-HDL, which mature further into pre-β2-HDL upon accumulating cholesterol and additional phospholipids mostly from cell membranes (Francone et al. 1989). Circulating pre-β1 and β2 HDL particles have the critical task of extracting cholesterol molecules from various sources including the scavenger receptor BI protein (SCARB1) (Jian et al. 1999), the ATP binding cassette A1 (ABCA1) (Castro and Fielding, 1988), and through simple diffusion (Johnson et al. 1991). This movement of cholesterol from various tissues into HDL is known as cholesterol efflux. While continuing to circulate, lipid-rich pre-β-HDL particles become round in shape as

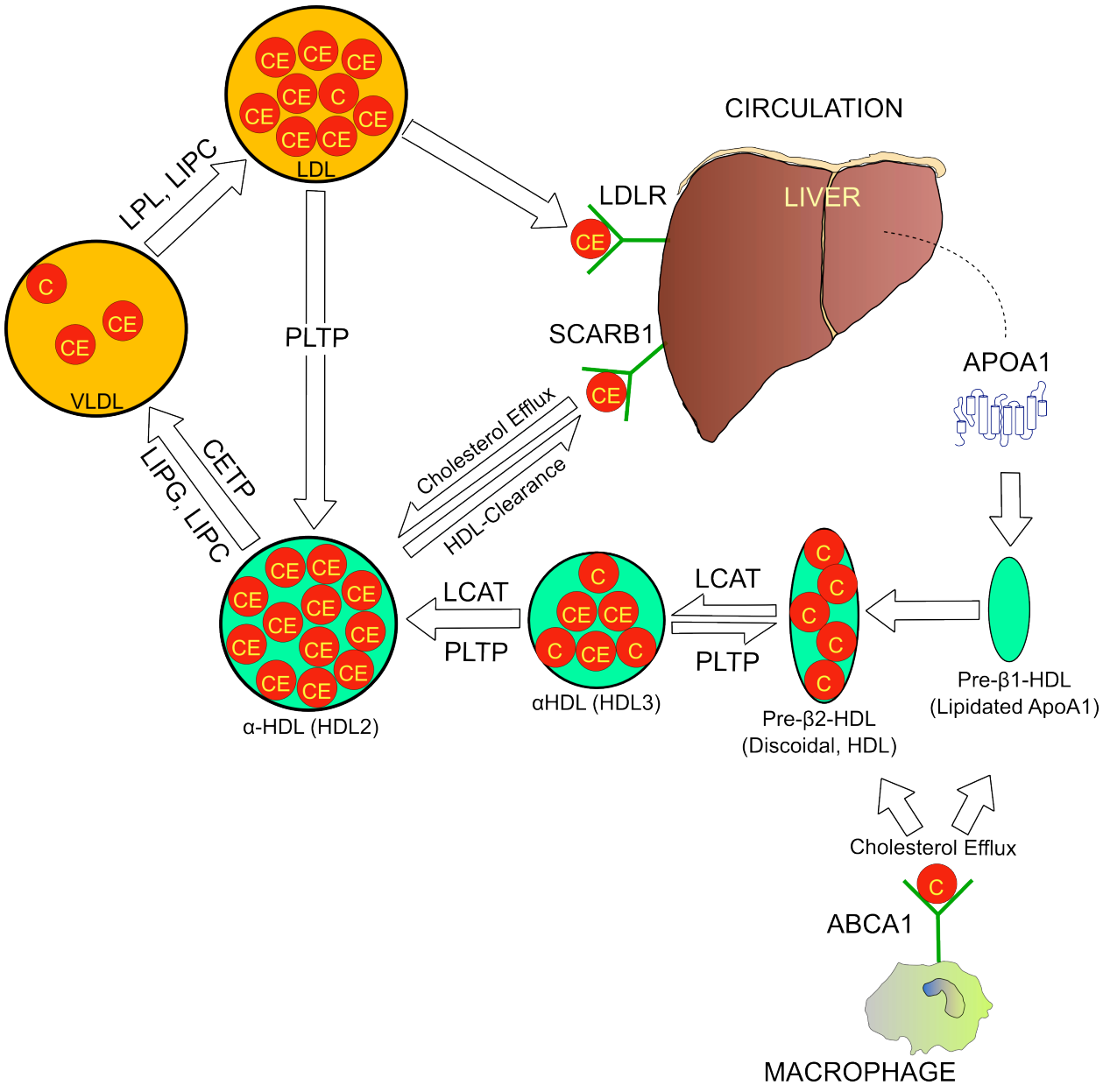

Figure 2.2: The transport of cholesterol through circulation. Cholesterol molecules are labeled "C", and cholesterol esters "CE". Gene symbols are abbreviated as follows: ABCA1 (ATP-binding cassette A1), ApoA1 (apolipoprotein A1), LCAT (lecithin:cholesterol acytransferase), PLTP (phospholipid transfer protein), CETP (cholesteryl ester transfer protein), LIPC (hepatic lipase), LIPG (endothelial lipase), LPL (lipoprotein lipase), LDLR (low density lipoprotein receptor), SCARB1 (scavenger receptor protein B1).

cholesterol inside the particles is esterified by the lecithin:cholesteryl acyltransferase (LCAT) protein, forming cholesterol esters (CE). The remodeled HDL particles are heavily influenced by several proteins including lipases, the phospholipid transfer protein (PLTP), and the cholesterol ester transfer protein (CETP). These complicated interactions establish a highly heterogeneous nature of HDL particles. Mature, CE-rich HDL particles (α-HDL) continue to roam the circulatory system, exchanging lipids with other lipoproteins and tissues. Eventually, most α-HDL particles return to the liver, binding SCARB1, which catalyzes delivery of cholesterol esters from HDL into hepatocytes of the liver (Acton et al. 1996). Once the cholesterol esters return to the liver, they are converted into bile salts, and eliminated through the gastrointestinal tract. Thus, the HDL pathway facilitates movement of cholesterol from various sources (including potentially harmful atherosclerotic plaques) to HDL particles, which deliver the excess cholesterol into the liver.

2.3.2 Structural components of HDL

The principal protein components of HDL are dual APOA1 peptides, which wrap around the particle in an anti-parallel, double-belt structure (Wu, et al. 2007b) (Figure 2.3A). Secondary structure of ApoA1 consists ten transmembrane amphipathic α-helices (Zannis et al. 2006) (Figure 2.3B). Functionally, ApoA1 forms the initial structure of discoidal HDL (Castro et al. 1988), and is recognized by LCAT (Fielding et al. 1972), ABCA1 (Wang et al. 2000), and SCARB1 (Rigotti et al. 1997a). Therefore, ApoA1 is not only the primary structural component; it serves as the recognition molecule for most of the proteins that interact with HDL. ApoA1 deficient mice do not form normal HDL particles and exhibit 70-80% reductions in plasma

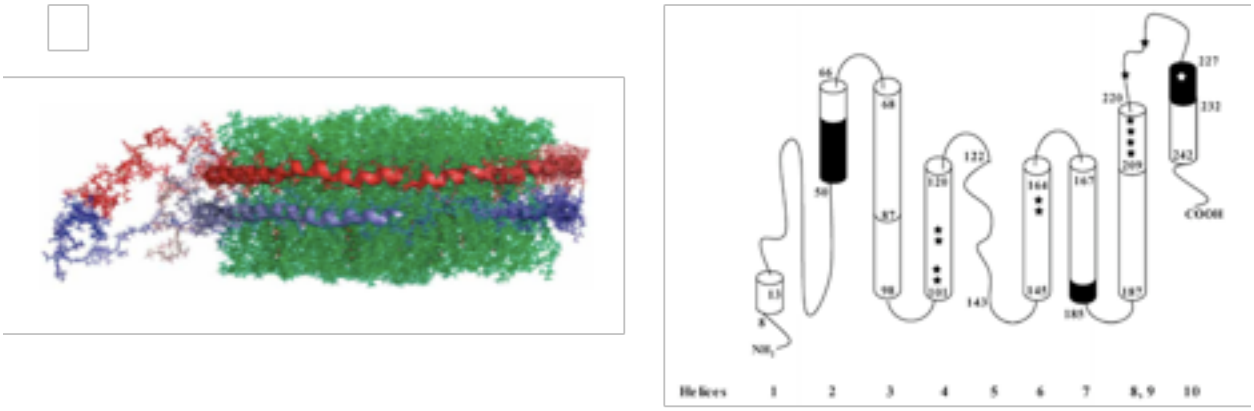

Figure 2.3A: Characterization of HDL particle showing twin ApoA1 peptides (Wu et al. 2007b), **2.3B:** ApoA1 protein structure (Zannis et al. 2006).

cholesterol and HDL (Williamson et al. 1992). Human studies have shown mutations in the ApoA1 gene to have the most profound effects on cholesterol phenotypes compared to other RCT proteins (Hovingh et al. 2005). However, some *ApoA1* mutations reduce levels of circulating HDL, but are reported to actually decrease risk of cardiac events. Notably, the *ApoA1 milano (R173C)* mutation has been implicated in improved circulatory health despite leading to reduced HDL and hypertriglyceridemia (Franceschini et al. 1980). A possible explanation for this anomaly was provided years later when it was discovered that mice carrying the *ApoA1 milano* mutation had more efficient cholesterol efflux capabilities then their wild-type littermates (Franceschini et al. 1999). This finding has recently been challenged, as Weibel and colleague's 2007 study compared efflux potentials of *ApoA1* wild-type and *milano* rats from 3 different cell types, and determined that efflux rates were not significantly different. Certainly, the elusive details of the potential atheroprotective effects resulting from ApoA1 mutations such as *milano* are worthy of intense scrutiny.

High density lipoproteins are intricate conglomerates of several different apolipoprotiens. Studies in humans and mice have identified ApoA2 (Ge et al. 2007), ApoA4 (Weinstock et al. 1997), ApoA5 (van der Vliet et al. 2002), ApoC1 (van Ree et al. 1995), ApoC2 (Huard et al. 2005), ApoC3 (Pollin et al. 2008), ApoD (Desai et al. 2002), ApoE (Mahley, 1988), and ApoF (Lagor et al. 2009) as associated with HDL particles. The genes encoding these apolipoproteins are distributed in clusters throughout the human genome, including chromosome 11q23, which harbors *ApoA1/A5/C3/C4*. This cluster has high genetic variability, which contributes to several obesity related phenotypes (Fullerton et al. 2005). Human chromosome 19 contains another cluster, which includes *ApoE/C1/C2*.

2.3.3 Cholesterol efflux into nascent HDL

The rate-limiting step in RCT is cholesterol efflux, a process mediated largely by the *ABCA1* gene product. ABCA1 promotes cholesterol efflux by catalyzing the transfer of cholesterol and phospholipids from potentially atherogenic cells in peripheral tissues to discoidal HDL (Wang et al. 2000) (Figure 2.2). ABCA1 induced cholesterol efflux is especially combatant to vascular disease when cholesterol is taken from macrophages inside artery walls because their cholesterol-induced differentiation into foam cells is at the foundation of atherogenesis (Schmitz et al. 1985a). The efflux process initiates as discoidal HDL binds to ABCA1 on the cell surface, is internalized, and transported into endocytic vesicles inside the cell (Schmitz et al. 1985b). While inside endosomes, ABCA1 and the Niemann-Pick C1 protein mediate the transfer of lipid pools into intracellular HDL (Choi et al. 2003). The complex then returns to the cell surface and dissociates, thus releasing the cholesterol-enriched pre-β2-HDL undegraded (Schmitz et al. 1985b).

Genetic studies have definitively established the crucial involvement of ABCA1 with HDL genesis. Humans with *ABCA1* inactivating mutations have Tangier disease, which is marked by orange, cholesterol ester laden tonsils, peripheral neuropathy, and predisposition to coronary artery disease caused by the virtual absence of HDL (Schmitz et al. 1985a, Bodzioch et al. 1999). Several recent studies have identified more common mutations to play important roles in HDL homeostasis. For example, in their study of 128 patients with the extremely low HDL levels, Cohen et al. (2004) identified an *ABCA1* mutation in 20 individuals. Furthermore, large-population studies have estimated that *ABCA1* mutations are responsible for 10% of cases of low HDL in the general population (Cohen et al. 2004, Frikke-Schmidt et al. 2004). Some common *ABCA1* mutations associated with HDL are shown in Table 2.1.

2.3.4 HDL maturation

During HDL maturation, discoidal Pre-β-HDL is converted into spherical α-HDL via LCAT mediated conversion of cholesterol into cholesteryl esters (Figure 2.2). The 416 amino acid LCAT protein is synthesized in the liver and secreted into circulation (Francone et al. 1989), where it exists both bound to lipoproteins and lipid-free (Mclean et al. 1986). On HDL particles, LCAT generates cholesterol esters by catalyzing the transfer of the 2-acyl group of lecithin to the hydroxyl group of cholesterol (Fielding et al. 1972) (Figure 2.4). LCAT also can esterify cholesterol in LDL, but HDL is the preferred lipoprotein substrate due to the presence of ApoA1, which is a cofactor (Norum et al. 1971). Cholesterol esters are more hydrophobic then free cholesterol, and accumulate in the center of HDL particles causing a change in the geometry from discoidal to spherical. LCAT activity is crucial to cholesterol efflux because it helps

maintain a concentration gradient favoring addition of free cholesterol to lipoproteins including HDL (Fielding, 1984).

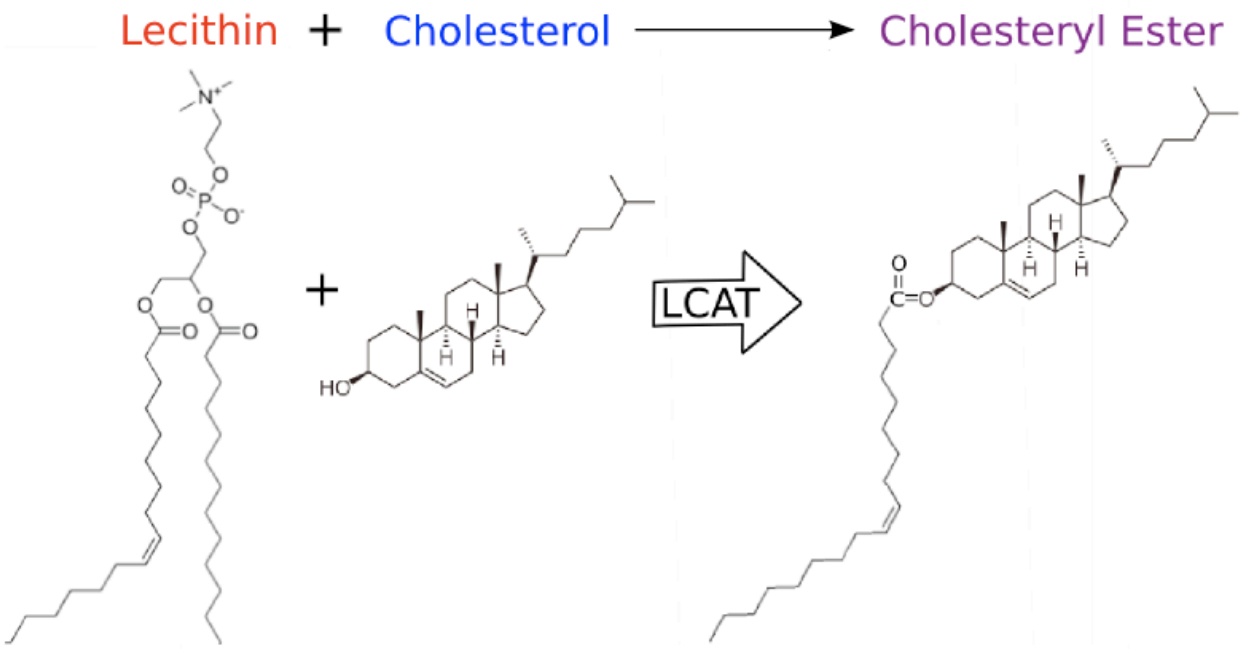

Figure 2.4: Molecular representation of the activity of the LCAT protein.

In humans, *LCAT* inactivating mutations cause familial LCAT deficiency (FLD), which is characterized by severe corneal opacification, low plasma HDL, sharply increased LDL triglycerides, and accumulation of discoidal HDL in plasma (Kuivenhoven et al. 1997). Additionally, some *LCAT* defects disrupt interaction with ApoA1, which leads to fish eye disease, decreased HDL (but not LDL), decreased cholesterol esters, and similar but milder phenotypes compared to FLD (Funke et al. 1991, Kuivenhoven et al. 1997). Table 2.1 summarizes common *LCAT* mutations found in human populations. Mutations in *LCAT* show association to HDL, however this is not always a consistent finding.

2.3.5 Lipase Activity

In order to understand HDL metabolism, it is important to be aware that lipoproteins of each class are heterogeneous particles that heavily interact, and often transition from one to the other. A common factor influencing lipoprotein interaction is activity of lipase proteins. Lipases are water-soluble enzymes that hydrolyze ester bonds of water-insoluble substrates such as triglycerides, phospholipids, and cholesteryl esters (Wong and Schotz, 2002). Endothelial lipase (LIPG), hepatic lipase (LIPC), and lipoprotein lipase (LPL) are three vascular lipase proteins that migrate to endothelial cells and anchor to the distal side via interaction with heparin sulfate proteoglycans (McCoy et al. 2002, Sendak and Bensadoun, 1998, Sendak et al. 2000). The exposed lipase proteins remodel circulating lipoproteins, generating important effects to lipoprotein metabolism and cholesterol homeostasis. Additionally, some lipase enzymes directly interact with lipoprotein receptors (such as the LDL receptor), thus enhancing metabolism of circulating lipoproteins.

The 71.4 kb *LIPG* gene contains 10 exons, and is located on human chromosome 18q21.1. The majority of the catalytic activity of LIPG is devoted to hydrolysis of phospholipids of VLDL, chylomicrons, and HDL (McCoy et al 2002). Interestingly, LIPG mediated phospholipid modulation in HDL inhibits cholesterol efflux from SCARB1, but enhances efflux from ABCA1, and HDL uptake in the liver (Yancey et al. 2004). In 2006, Badellino et al. established a correlation between LIPG and atherosclerosis, which seems to underscore the importance of SCARB1 cholesterol efflux and HDL longevity in ideal HDL function. Several commonly occurring mutations in the *LIPG* gene are associated with HDL in humans (Table 2.1).

The *LIPC* gene spans 135kb on human chromosome 15q21. It is synthesized primarily by hepatocytes, and then secreted and bound to the extracellular matrix of liver endothelial cells (Rea et al. 1993). LIPC has powerful VLDL and IDL triglyceride hydrolysis capabilities (Grosser et al. 1981), as well as the ability to catalyze conversion of αHDL subspecies HDL2 to denser HDL3 (Kuusi et al. 1980). The latter functionality has direct implications to RCT because HDL2 is more likely to interact with SCARB1 for cholesterol efflux or endocytosis (Catalano et al. 2008). Independently of RCT, LIPC appears to demonstrate pro-atherogenic effects by increasing artery wall retention of VLDL, chylomicrons, and LDL (Gonzalez-Navarro et al. 2002). Although mutations in human *LIPC* are associated with variations in HDL concentration (Table 2.1), the connection with coronary artery disease is controversial (Whiting et al. 2005, Eller et al 2005).

LPL is a critical enzyme involved in hydrolysis of triglyceride rich lipoprotein particles in muscle, adipose, and macrophages, a process which generates free fatty acids and glycerol for energy metabolism and storage (Goldberg, 1996). Expression of *LPL* has been implicated in atherosclerosis, citing the increased affinity for macrophage phagocytosis (and subsequent foam cell development) on LDL and chylomicron particles after LPL mediated remodeling (Zilversmitz 1973). Although LPL and HDL do not directly interact, association analysis of human mutations has repeatedly linked LPL genotype with HDL (Table 2.1).

2.3.6 Lipoprotein Remodeling in Circulation

Cholesterol homeostasis is greatly modulated by proteins that catalyze the exchange of cholesterol and other lipids between circulating lipoprotein classes. Mutations in lipid transfer

proteins are very important sources of lipoprotein phenotypic variation, as the genes are decidedly polymorphic. In plasma, there are two important lipid transfer proteins: cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP).

The CETP protein catalyzes the exchange of cholesteryl esters inside HDL for triglycerides of LDL and VLDL (Figure 2.2) (Pattnaik et al. 1978). Expression of CETP is fairly ubiquitous, however most is synthesized in the liver, and then excreted and bound to HDL in circulation (Quinet et al. 1991). Theoretically, CETP activity is attributed to coronary artery disease, as the movement of cholesterol from HDL to LDL is certainly pro-atherogenic. Surprisingly, studies have failed to clearly identify the relationship between CETP and atherosclerosis, suggesting additional function of CETP. In mice (which naturally lack *CETP*), expression of human *CETP* has been found to be both pro-atherogenic (Marotti et al. 1993), and anti-atherogenic (Hayek et al. 1995) depending on the metabolic state of the mouse model. In humans, markedly increased HDL has been a consistent finding in carriers of *CETP* nullifying mutations (Barzilai et al. 2003, Inazu et al. 1994). Some carriers of inactivated *CETP* have been linked to increased longevity (Barzilai et al. 2003), while other studies have reported no association (Ken-ichi et al. 1997), and still other mutations implicate increased risk of coronary artery disease (Zhong et al. 1996). The *CETP* gene, located on human chromosome 16q21 is highly polymorphic. One important polymorphism, *Taq1B*, accounted for 5.8% of variance of HDL in a Spanish population (Corella et al. 2000), and appears associated with coronary artery disease (Ordovas et al. 2000). Other important mutations discovered in humans are summarized in Table 2.1.

PLTP regulates the size and composition of HDL both by lipid exchange and particle remodeling. The gene is located on human chromosome 20q12-q13.1, and is expressed primary (but not solely) by adipocytes and hepatocytes (Dusserre et al. 2000). In circulation, normal PLTP activity appears protective against atherosclerosis due to its ability to both increase circulating levels of cholesterol efflux prone pre-β-HDL and to maintain levels of mature HDL. PLTP is able to catalyze the fusion of two HDL3 particles, forming one larger HDL2, while releasing lipid poor ApoA1, the precursor to pre-β-HDL (Jauhiainen et al. 1993). Enrichment of HDL with triglycerides enhances this process (Settasatian et al. 2001), which is termed HDL conversion. In addition to HDL conversion, PLTP facilitates the transfer of phospholipids and to a lesser extent, cholesterol from triglyceride rich lipoproteins such as VLDL and chylomicrons into HDL (Rao et al. 1997, Nishida et al. 1997). *PLTP* inactivated mice have 60-70% reductions in HDL, which suggests that phospholipid migration into HDL impedes catabolism of the particle (Qin et al. 2000). Interestingly, transgenic mice overexpressing *PLTP* have severely reduced HDL and inhibited RCT, despite increased levels of pre-β-HDL (Föger et al. 1997, Samyna et al. 2008). This activity has been linked to increased HDL catabolism (Föger et al. 1997), and excessive HDL conversion, a conclusion that challenges the theory that preβ-HDL particles are more important for reverse cholesterol efflux then α-HDL (Samyna et al. 2008). While these studies in animal models generate as many questions as answers, together they indicate that correctly balanced PLTP activity is essential in maintaining the normal plasma HDL concentration.

Oxidative modification of lipoproteins, especially LDL, strongly increases their accumulation in vascular tissues by stimulating migration of inflammatory cells that cause atherosclerosis (Steinberg et al. 1997). HDL is very adept at prohibiting LDL oxidation via the activity of the HDL-associated antioxidation enzyme paraoxonase 1 PON1 (Watson et al. 1995). While the mechanism is not clear, PON1 is thought to have peroxidase activity, directed specifically toward oxidized lipids of lipoproteins (Navab et al. 1996). Mice with inactivated PON1 have substantially decreased antioxidant capabilities and are more susceptible to atherosclerosis then WT littermates (Shih et al. 1998). Mutations in the PON1 gene have been associated with serum lipoprotein levels, and coronary artery disease (Table 2.1).

2.3.7 The Duality of the Scavenger Receptor Class B Type I Protein

Ultimately, the protective effects generated by RCT are accomplished through the removal of excess cholesterol (cholesterol efflux), and delivery of cholesterol rich lipoprotein particles to liver hepatocytes for lipid excretion (HDL clearance). The SCARB1 protein is very unique because it plays an integral role in both of these processes. The gene spans 86.3 kb of human chromosome 12q24.31, and is expressed primarily in adrenal tissues and the liver, where it is attached to epithelial surfaces (Acton et al. 1994).

SCARB1 is most notorious for catalyzing uptake of cholesterol from lipid-rich HDL particles into hepatocytes (Cohen et al. 2004), where it is converted into bile acids. The mechanism of HDL clearance is distinct from ABCA1 mediated cholesterol efflux and low density lipoprotein receptor endocytosis in that internalization of the entire particle does not occur, rather cholesterol esters are selectively taken into tissues (Cohen et al. 2004, Acton et al. 1996). In this way,

mature αHDL can quickly be recycled back into lipid-poor pre-β-HDL, and RCT can start again. Affinity of lipoprotein-SCARB1 interaction is complex and highly reliant on apolipoprotein composition, and particle geometry. SCARB1 binds both LDL (Acton et al. 1994), and HDL, however, preference is strongest for lipoproteins containing ApoA1, which it binds to tightly (Liadaki et al. 2000). In fact, three HDL associated apolipoproteins, ApoA1, ApoA2, and ApoC3, each interact with SCARB1, and increase affinity for cholesterol movement (Xu et al. 1997).

The availability of LDLR to commit lipoprotein endocytosis is regulated by Proprotein Convertase Subtilisin Kexin type 9 (PCSK9). In fact, this relationship is important enough to instigate a distinct classification of familial hypercholesterolemia (known as FH3), which is caused by mutations in *PCSK9* (Homer et al. 2008). Secreted PCSK9 competes with lipoprotein-associated apolipoproteins as a ligand for LDLR by binding to the receptor and causing endocytosis and degradation of the complex (Lagace et al. 2006). Additionally, PCSK9 appears to interrupt intracellular receptor recycling by binding to LDLR prior to its placement on the surface of hepatocytes (Homer et al. 2008). The molecular processes involved in PCSK9 induced degradation are not clear, although Geoghegan et al. have elucidated that PCSK9 exhibits acylenzyme activity on LDLR, in which residue Gln152 of the receptor is esterified by Ser386 of PCSK9 (Geoghegan et al. 2009). This study, and independent genetic studies have shown that the EGFP domain of LDLR is the active site of PCSK9 interaction (Zhang et al. 2007). The FH3 phenotype is rare, and is derived from missense mutations that increase functionality of PCSK9. However, this effect is not the only clinically important mutation in PCSK9, as loss-of-function mutations appear to generate positive effects on blood lipids. The

2005 Dallas Heart study identified two PCSK9 nonsense mutations among subjects with low plasma LDL levels: Y142X in exon 3, and C679X in exon 12 (Cohen et al. 2005). These mutations led to 28% decreased LDL levels, and significant reductions in risk of coronary artery disease. PCSK9 induced hypocholesterolemia has also been identified in Japanese (Miyake et al. 2008), and Italian (Fasano et al. 2007) patients.

As previously mentioned, SCARB1 plays a key role in cholesterol efflux by acting as a cholesterol donor to HDL. Unlike ABCA1 mediated cholesterol efflux, which targets pre-β-HDL, the ideal lipoprotein for SCARB1 mediated efflux is large, phospholipid-rich, spherical HDL2 (de la Llera-Moya et al. 1999, Catalano et al. 2008). Thus, SCARB1 is a bidirectional cholesterol transporter to and from mature HDL.

Studies in humans and animal models have established SCARB1 as vital to cholesterol homeostasis. Mice with inactivated SCARB1 have a twofold increase in plasma cholesterol, which is distributed in unusually large and homogenous HDL particles (Rigotti et al. 1997b). Furthermore, greatly reduced clearance of HDL from plasma was attributed to SCARB1 deficiency in a separate study (Out et al. 2004). These observations strongly suggest that SCARB1 is required to maintain normal cholesterol. Common mutations in human SCARB1 are summarized in Table 2.1.

2.3.8 Hepatic bile acid synthesis

Cholesterol delivered to the liver via HDL enters the bile acid synthesis pathway (also known as the cholesterol catabolic pathway), which begins with the enzymatic modulation of hepatic cholesterol to 7-α-hydroxycholesterol by cholesterol 7α-hydroxylase (CYP7A1) (Russell and Setchell, 1992). Transcriptional activity of CYP7A1 dictates the efficacy of the cholesterol catabolic pathway, and is critical to hepatic cholesterol homeostasis. One of many important transcriptional regulators of CYP7A1 is the Farnesoid X receptor (FXR). FXR acts as a bile acid sensor, suppressing CYP7A1 activity when hepatic concentrations of bile salts are high (Wang et al. 1999). FXR does not work on its target directly, but instead upon activation, it triggers a cascade involving small heterodimer partner 1 (SHP-1), which activates the direct CYPA1 inhibitor, liver receptor homolog 1 (LRH-1) (Goodwin et al. 2000). Not surprisingly, homozygous deletion of CYPA1 results in increased hepatic cholesterol content, deficient bile acid secretion, and hypertriglyceridemia (Pullinger et al. 2002). The remaining steps in the conversion of cholesterol into conjugated bile salts such as taurocholic and glycocholic acid involve at least 16 enzymes. After synthesis, these bile acids cycle from the liver, to the gall bladder, into the duodenum sub-layer of the intestine, back through enterocytes of the ileum, through the portal vein, and back into hepatocytes several times per day while carrying out their primary function of aiding lipid digestion (Russell et al. 2009).

Table 2.1: Common mutations (minor allele frequency >5%), and effects on HDL in genes participating in reverse cholesterol transport pathway

Gene	Rs#	Alias	Chrom.	Position	MA Freq.	Effect	Citation
LPL	rs268	Asn291Ser	8p22	19857809	5%	↓ HDL	Wittrup et al. (1999)
LPL	rs320	HindIII	8p22	19863357	30%	↓ HDL	Senti et al. (2001)
LPL	rs328	S447X	8p22	19864004	20%	↑ HDL	Wittrup et al. (1999)
LPL	rs326	rs326	8p22	19863719	44%	↑ HDL	Wittrup et al. (1999)
LIPC	rs1077834	T-710C	15q21-q23	56510771	22%	↑ HDL	Boes et al. (2009)
LIPC	rs1800588	C-514T	15q21-q23	56510967	25%	↑ HDL	Isaacs et al. (2004)
LIPC	rs2070895	G-250A	15q21-q23	56511231	22%	↑ HDL	Andersen et al. (2003)
LIPC	rs3829462	+1075C	15q21-q23	56640371	5%	↑ HDL	Fang and Liu (2002)
CETP	rs12149545	G-2708A	16q21	55550662	30%	↑ HDL	McCaskie et al. (2007)
CETP	rs1800775	C-629A	16q21	55552737	48%	↑ HDL	McCaskie et al. (2007)
CETP	rs708272	Taq1B	16q21	55553789	43%	↑ HDL	Ozsait et al. (2008)
CETP	rs34145065	+784CCC>A	16q21	55554147-8	39%	↑ HDL	Klerkx et al. (2003)
CETP	rs5880	A373P	16q21	55572592	5%	↓ HDL	Agerholm et al. (2000)
CETP	rs61212082	Ile405Val	16q21	55573593	30%	↑ HDL	Blankenberg et al. (2004)
LIPG	rs3813082	-384A>C	18q21.1	45342041	12%	↑ HDL	Hutter et al. (2006)
LIPG	rs2000813	584 C/T	18q21.1	45347862	24%	↑ HDL	Hutter et al. (2006)
LIPG	rs2276269	C+42T/ln5	18q21.1	45356000	44%	↓ HDL	Mank-Seymour et al. (2004)
LIPG	rs6507931	T+2864C/ln8	18q21.1	45367006	42%	↓ HDL	Mank-Seymour et al. (2004)
LIPG	rs3744841	2237G>A	18q21.1	45371372	36%	↓ HDL	Yamakawa et al. (2003)
LCAT	rs5922	608C/T	16q22.1	66533918	14%	↓ HDL	Zhu et al. (2006)
LCAT	--	P143L	16q22.1	66534134	6%	↓ HDL	Zhang et al. (2004)
LCAT	rs2292318	rs2292318	16q22.1	66543207	12%	↑ HDL	Pare et al. (2007)
ABCA1	rs1800976	G-273C	9q31.1	1.07E+08	40%	↑ HDL	Shioji et al. (2004)
ABCA1	rs1800978	G378C	9q31.1	1.07E+08	13%	↓ HDL	Porchay et al. (2006)
ABCA1	rs2853578	G596A	9q31.1	1.07E+08	28%	↑ HDL	Whiting et al. (2005)
ABCA1	rs2230808	R1587K	9q31.1	1.07E+08	24%	↓ HDL	Frikke-Schmidt et al. (2004)
ApoA1	rs5070	T84C	11q23-q24	1.16E+08	23%	↑ HDL	Shioji et al. (2004)
SR-BI	rs5888	C1050T	1q24	1.24E+08	49%	↑ HDL	Boekholdt et al. (2006)
SR-BI	--	HaeIII	1q24	--	33%	↑ HDL	Hong et al. (2002)
Pon1	rs662	Gln192Arg	7q21.3	94775382	29%	↓ HDL	Hegele et al. (1995)
Pon1	rs705379	C-107T	7q21.3	94791831	48%	↓ HDL	Blatter Garin et al. (2006)

ID = RefSNP ID#, MA Freq. = Minor allele frequency, Effect = phenotypic effect of minor allele, TC = total cholesterol

2.4 Cholesterol Distribution and Low Density Lipoproteins

The mechanisms that manage and utilize LDL are tightly controlled systems evolved to distribute cholesterol through the circulatory system and into cells that require extracellular cholesterol. Unfortunately, LDL-cholesterol does not always reach its most appropriate destination, but rather accumulates in artery walls causing atherosclerosis, the leading cause of death and disability in the developed world (Yusuf et al. 2001). For this reason, quantity of circulating LDL is a well-known risk factor for heart disease, and is the primary focus of most lipid lowering therapies (NCEP expert panel, 2001). The pathogenicity of LDL and likelihood of atherosclerotic development are heavily influenced by genetic composition of gene products involved with LDL metabolism. Patients with genetic defects that cause severely elevated LDL have familial hypercholesterolemia, which affects approximately 1 in 500 people (Koivisto et al. 1993). In normal individuals, approximately 50% of LDL variation is genetic. This section will address the mechanisms behind exogenous and endogenous cholesterol transport, with emphasis placed on the proteins that participate in this crucial pathway.

2.4.1 Brief Overview of Cholesterol Distribution Mechanisms

An overview of exogenous (dietary) and endogenous (de novo) cholesterol transport is shown in Figure 2.5. Cholesterol enters the body either from dietary sources, or from de novo synthesis in hepatocytes. Exogenous cholesterol is digested and absorbed into intestinal cells, then packaged into chylomicrons, which circulate the bloodstream. Eventually, the liver takes up chylomicron remnants, and dietary cholesterol joins newly synthesized cholesterol in hepatocytes. Free fatty acids, triglycerides, cholesterol esters, phospholipids, and apolipoprotein B-100 are transported to hepatocyte microsomes via the microsomal transfer protein (Gordon et al. 1995), where they

are processed into very low density lipoproteins (VLDL). VLDL particles circulate the bloodstream, and are eventually remodeled into intermediate density lipoprotein (IDL) by lipoprotein lipase (Goldberg, 1996), thus freeing fatty acids to be used in muscle or stored in adipose tissue. Approximately 50% of IDL is taken up by the liver (Mahley, 1988), and the remaining particles are again remodeled by lipases, (especially LIPC) into LDL (Fan et al. 1994). LDL particles are cholesteryl ester rich, and circulate the body in high concentrations compared to other lipoproteins of the LDLR pathway because plasma is deficient of cholesterol ester lipases. LDL binds with high affinity to LDLR, which is expressed at tightly regulated levels on the surface of all nucleated cells, but most notably hepatocytes. Transcription of the *LDLR* gene is regulated by levels of intercellular cholesterol. Additionally, LDL derived cholesterol accumulates in the arterial intima during atherosclerosis. While this process has given LDL cholesterol notoriety as harmful, the reality is that LDL facilitates the distribution of an essential structural element of all cells in a sophisticated and sensitive fashion.

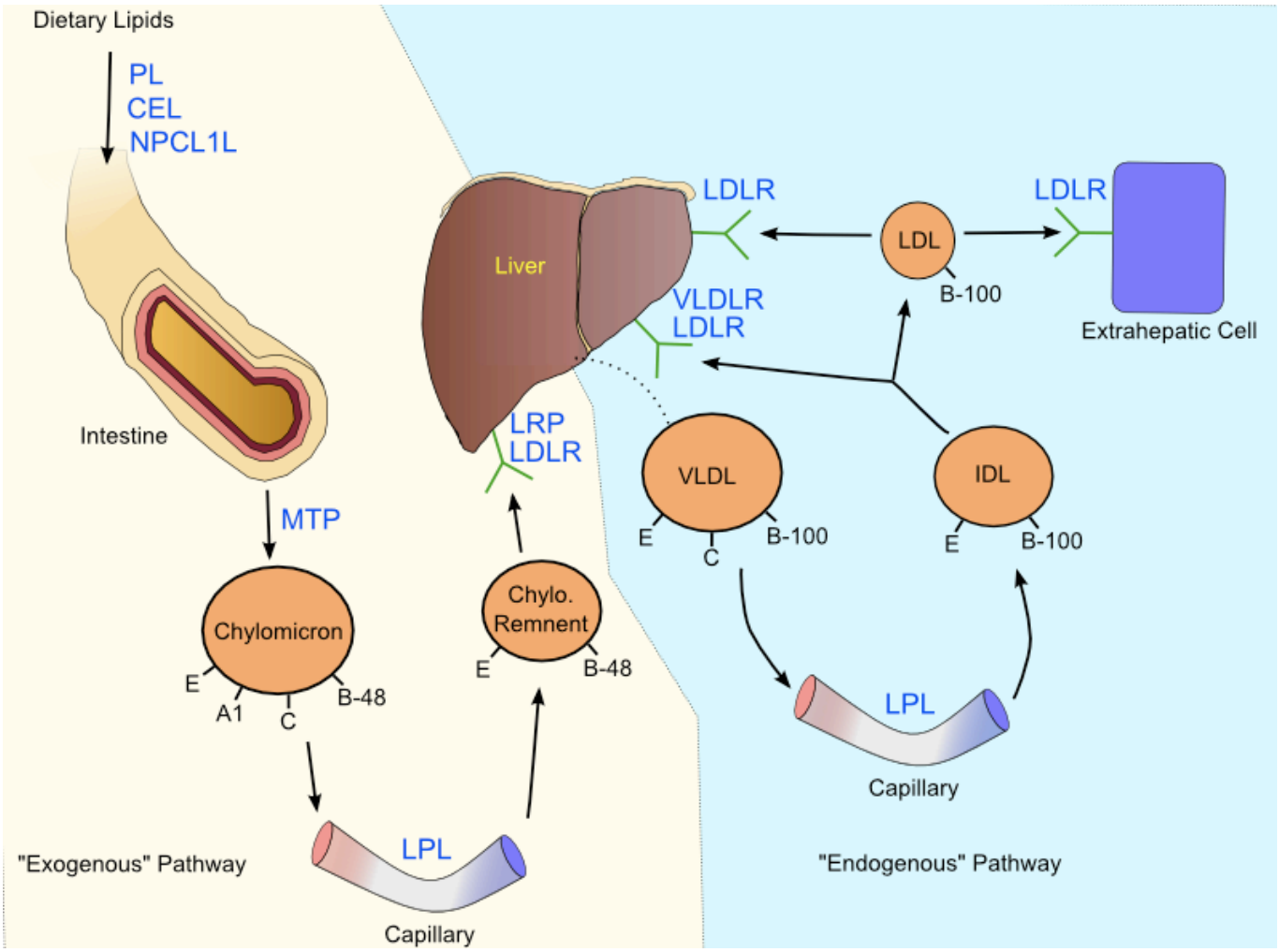

Figure 2.5: Transport of dietary exogenous cholesterol, and de novo endogenous cholesterol requires a diversity of lipoproteins and proteins shown above.

2.4.2 Exogenous lipid metabolism in the intestine

The amount of cholesterol absorbed from diet is a major contributor to levels of cholesterol in circulation. One study has estimated that in humans, the complete abolition of dietary cholesterol absorption would reduce plasma cholesterol by up to 62% (Gylling et al. 1995). About 50% of dietary cholesterol is absorbed through intestinal enterocytes, while the rest is excreted through feces (Ostland et al. 1999). This figure however, is extremely variable among individuals and this variation has been established as an inherited trait (Gylling and Miettinen, 2002). Thus, the mechanisms behind the entry of dietary cholesterol into the body are critical sources of variation in cholesterol homeostasis.

Early lipid digestion, from the oral cavity to the duodenum sub-layer of the intestine, produces crude emulsions consisting of free cholesterol, triglycerides, free fatty acids, and phospholipids. As these emulsions are delivered into the intestine, they are mixed with bile salt micelles, which are synthesized and secreted into the intestine from the liver (Yao et al. 2002). Total concentration of bile salt micelles is positively correlated with cholesterol absorption (Ponz et al. 1981), due to catalysis of lipid emulsification into smaller droplets, which interact more readily with lipase enzymes (Young and Hui, 1999). Bile salt emulsified triglycerides and cholesteryl esters are hydrolyzed by pancreatic lipase (PL) (Lowe, 1994), and carboxyl ester lipase (CEL) (Howles et al. 1996), respectively. Not surprisingly, *in vivo* knockout studies using murine models have confirmed the necessity of both *PL* (Young and Hui, 1999) and *CEL* (Howles et al. 1996) to normal cholesterol absorption, and chylomicron assembly.

Cholesterol absorption is achieved through passage across brush boarder membranes and into intestinal enterocytes in the jejunum. While details of the mechanism require further investigation, recent studies have identified key proteins to the process. One major breakthrough came with the identification of the substrate for the drug *ezetimibe* (brand: Zetia), which inhibits cholesterol entry into enterocytes without effecting de novo cholesterol biosynthesis (van Heck et al. 2003). *Ezetimibe* was approved by the United States Food and Drug Administration for treatment of hyperlipidemia in 2002, however, it took two additional years before Garcia-Calvo and colleagues (2005) discovered that the Niemann-Pick C1-Like 1 (NPC1L1) protein is the target of the drug. NPC1L1 is a putative transporter of cholesterol, and is expressed on the brush border membranes of enterocytes in a general pattern that parallels that of maximum cholesterol absorption (Altmann et al. 2004). Naturally occurring mutations in human *NPC1L1* are associated with reduced cholesterol absorption and circulating levels of LDL (Cohen et al. 2006). In addition to NPC1L1, the ATP binding cassette G5 and G8 proteins (ABCG5/8) appear to negatively regulate cholesterol transport into enterocytes. Mutations in the *ABCG5/G8* genes (they are neighbors on human chromosome 2p21) are associated with sitosterolemia, which is characterized by increased absorption of plant sterols. Sitosterolemic patients also have increased amounts of dietary cholesterol absorption and premature atherosclerosis (Berge et al. 2000). This data has been interpreted as evidence that ABCG5/G8 form a complex that promotes secretion of cholesterol back into the intestinal lumen, and that this activity targets plant sterols in normal individuals (Hui and Howles, 2005).

2.4.3 Movement of exogenous cholesterol from the intestines to the liver

Once inside enterocytes, dietary cholesterol is packaged into chylomicrons, and put into circulation. This process is initiated by the esterification of large amounts of free cholesterol by the a:cholesteryl transferase protein (Purdy and Field, 1984), and the synthesis of triglycerides from free fatty acids by mono- and di-acylglycerol acyltransferases (Hui and Howles, 2005). In the endoplasmic reticulum, cholesteryl esters, phospholipids, and triglycerides are amalgamated together with apolipoprotein B (ApoB)-48 by the microsomal triglyceride transfer protein (MTP) (Hui and Howles, 2005). The action of MTP is not localized to enterocytes, or even to chylomicron synthesis, but instead it is nearly ubiquitously expressed and a crucial element of VLDL synthesis as well. Inactivating mutations in MTP result in abetalipoproteinemia, in which chylomicrons and VLDL are not synthesized, ApoB containing particles in general are absent, and lipids accumulate in the intestine (Lackner et al. 1986). As nascent chylomicron synthesis nears completion, the particles are transported to the golgi apparatus where additional triglycerides are recruited, and then the particles are transported via vesicular structures to clatherin-coated pits where exocytosis occurs (Sabesin and Frase, 1977).

ApoB is the major protein component of all lipoproteins except HDL. During chylomicron synthesis in the intestine, ApoB mRNA is altered by the apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1) protein, so that the 6666th nucleotide is changed from a cytosine to a uracil (Tennyson et al. 1989). This unique process leads to a premature UAA stop codon, and production of a truncated ApoB peptide only 48% the length of full apoB (Tennyson et al. 1989). *APOBEC1* inactivated mice demonstrate a complete lack of ApoB-48, 178% increased ApoB-100, and decreased HDL cholesterol (Nakamuta et al. 1996). Mutations

in the *ApoB* gene itself are an important source of phenotypic variation in humans. In a recent review, 132 genetic variants in the ApoB gene are listed including one in the promoter region, one in the 5' untranslated region, 85 in the coding region (22 synonymous), 44 in the various introns, and one in the 3' UTR (Benn, 2009). Notably, genotypes of the very common *T2488T* and *E4154K* mutations have crucial implications to LDL homeostasis (Benn et al. 2005, Benn et al, 2007). Considering that ApoB is a direct measurement of potentially atherogenic particles, the levels of ApoB levels in circulation are considered a more appropriate trait to measure risk of cardiovascular disease than LDL (Talmud et al. 2002), however, LDL levels remain more commonly utilized clinically.

While the digestion and packaging of dietary lipids into chylomicrons takes about one hour, the halflife of lipids in chylomicrons is only 4.5 minutes (Grundy and Mok, 1976). Upon exiting enterocytes, the only protein component of chylomicrons is ApoB-48. After passage through the throacic duct and into the bloodstream, nascent chylomicrons accept ApoC2 and ApoE from HDL, a process that yields mature chylomicrons. As mature chylomicrons circulate, the newly acquired apoC2 on the particle surface activates LPL, which is bound to epithelial surfaces of capillaries in adipose and muscle tissue, where it is differentially expressed according to fed/fasting conditions (Goldberg, 1996). LPL catalyzes the hydrolysis of triglycerides in chylomicrons, a crucial process which distributes fatty acids to tissues, generates non-esterified fatty acids in plasma, and remodels chylomicrons into chylomicron remnants (CRs) (Goldberg, 1996). The absence of *LPL* in cases of familial LPL deficiency, which is characterized by hypertriglyceridemia, decreased HDL and LDL, and massive accumulation of chylomicrons in

plasma (Takagi et al. 2000). As chylomicrons shed triglycerides to epithelial cells, ApoC2 is lost (ApoE is retained), and as a result, the CR loses further lipase activation.

As CRs lose triglycerides, they become enriched in ApoE, and consequently are destined for the liver. ApoE peptides are essential for particle uptake into hepatocytes, as demonstrated by *ApoE* inactivated mice, which demonstrate negligible CR clearance (Zhang et al. 1992). The model for hepatic clearance involves ApoE interaction with hepatocyte cell surface molecules including heparan sulfate proteoglycans and LIPC, followed by an endocytosis step mediated by a complex of LDLR and the low density lipoprotein related 1 protein (LRP1) (Herz et al. 1995). Interestingly, researchers have learned that cholesterol content plays an important role in CR uptake. Chylomicron-like particles, created without cholesterol, undergo triglyceride hydrolysis by LPL but are not taken up by hepatocytes (Redgrave et al. 1987). This result has been interpreted as a failure of ApoE to attain the necessary confirmation to achieve receptor binding with LRP1 (Redgrave et al. 1987)). After delivery to the liver, CR lipids are hydrolyzed once again into free fatty acids and free cholesterol for eventual synthesis of VLDL.

2.4.4 VLDL synthesis and maturation into LDL

The hepatic assembly of VLDL begins inside the rough endoplasmic reticulum, at the site of *ApoB-100* translation. As the peptide is synthesized by membrane bound ribosomes, it is sent through a protein channel into the cytoplasm. Meanwhile, the aforementioned MTP binds the precursor peptide and recruits a small amount of triglycerides, phospholipids, and cholesteryl esters, allowing apoB-100 to fold around a small lipid core (Hebbachi and Gibbons, 2001). Next, the bulk of VLDL triglycerides are transferred into the precursor particle, and the now

larger precursor (sometimes referred to as VLDL2) is sorted to the golgi apparatus. Evidence for a two-step maturation system up to this point is given by experiments using Brefeldin A (BFA), which inhibits only the transition into VLDL2, and not the initial lipidation of ApoB-100 (Rustaeus et al. 1995). BFA selectively inhibits the ADP-ribosylation factor (ARF) protein, suggesting that ARF is a critical factor in VLDL assembly (Rustaeus, et al 1995). Once in the golgi, additional lipids are recruited to form the mature VLDL particle, but the mechanism behind this behavior is not yet clear. However, it is certain that the fatty acids used for the synthesis of VLDL are derived from triglycerides stored in cytosolic lipid droplets (Gibbons et al. 2000), so it is likely that the concentration of these lipids is the rate limiting property of VLDL biogenesis.

Just like chylomicrons, VLDLs exchange ApoC2 and ApoE with HDL in circulation, and distribute free fatty acids to muscle and adipose tissues expressing LPL. As remodeled VLDL particles lose triglycerides and ApoC2, they become IDLs, which are either removed by the liver or are subject to further lipase activity and develop into LDL. Endocytosis of IDL by the liver includes not only LDLR, but other receptors in the same class including the very low density lipoprotein receptor (Kobayashi et al. 1996), and LRP1 (Herz et al. 1995). Larger, triglyceride-rich IDL particles are generally more susceptible to liver re-uptake by these receptors compared to smaller particles, which are more likely to transition into LDL (Ginsberg, 1994). The triglyceride content (and subsequently, the size and likely metabolic fate) of IDL particles is heavily influenced by LIPC (Grosser et al. 1981), as well as the cholesterol ester transfer protein, (Pattnaik et al. 1978) and phospholipid transfer protein (Rao et al. 1997). LDL particles have a relatively long halflife of about 3 days (Langer et al. 1972), a property attributed to the lack of

receptor and lipase activating apolipoproteins, as well as the stability of cholesterol in the particles.

Variation among VLDL, IDL, LDL, and HDL levels in circulation are highly dependent on the genetic composition of ApoE. Mice lacking functional ApoE have 500% increased plasma cholesterol, rapidly develop severe atherosclerosis (Zhang et al, 1992), presumably from a complete loss of LDLR binding and chylomicron/LDL clearance (Mahley et al. 1988). Due to their tendency to develop atherosclerotic plaques, the ApoE knockout mouse model is a very commonly used model for human atherosclerosis. In humans, ApoE contains two very common SNPs at amino acid positions 112 and 158, which dictate the ApoE2, E3, and E4 genotype nomenclature (Kim et al. 1993). Evidence has established that the ApoE4 is associated with decreased HDL, increased LDL, and increased plasma cholesterol, while the E2 genotype has the opposite effect (Mahley and Rall, 2000, Wu et al. 2007a).

2.4.5 Intracellular cholesterol metabolism

The human LDLR stretches 44.4 kb across human chromosome 19p13.3, and is responsible for uptake of cholesterol carrying lipoproteins. The principal ligand for LDLR is ApoB-100 on LDL, however LDLR can catalyze endocytosis of lipoproteins containing multiple copies of ApoE such as VLDL, IDL, and HDL. The extracellular portion of LDLR consists of three protein modules including a domain with seven contiguous cysteine-rich repeats (referred to as LDLR type A, or LA domains), a 400-amino acid sequence that is strongly homologous to the epidermal growth factor precursor protein (referred to as the EGFP domain), and finally, a 58-residue sequence rich in serine and threonine (Figure 2.6) (Jeon and Blacklow, 2005). Genetic

studies have revealed that LA domains 3-7 are required for effective binding of LDL, while only LA5 is required for VLDL uptake (Russell et al. 1989). Studies utilizing antibody blocking and synthetic peptides have determined that amino acid residues ~3300-3600 of ApoB-100 are the active sites for LDL interaction with LDLR (Yang et al. 1986, Milne et al. 1989).

Figure 2.6 describes the intracellular process in LDL endocytosis. LDLR positioning and subsequent endocytosis of the receptor-ligand complex occurs at clathrin coated pits (Anderson et al. 1978). After endocytosis, acidic conditions of the endosome catalyze disassociation of LDL. Genetic studies have implicated the EGFP domain of LDLR as responsible for release of LDL, as deletion of this region produces a non-separable complex (Davis et al. 1987). After release of the lipoprotein, the LDLR is recycled back to the membrane in a process also controlled by the EGFP domain (Davis et al. 1987). Meanwhile, LDL particles that are released from the receptor fuse into lysosomes, and are degraded into lipid components and amino acids by enzymes of the vesicle. A large portion of lipids released are cholesteryl esters, which are hydrolyzed by lysosomal acid lipase (LIPA) into free cholesterol. Patients with complete and heterozygous *LIPA* deficiency have Wolman disease and cholesteryl ester storage disease, respectively (Hooper et al. 2008). The consequences of inactive *LIPA* are severe: liver failure, hypercholesterolemia, hypertriglyceridaemia, liver fibrosis, early atherosclerosis, and early death

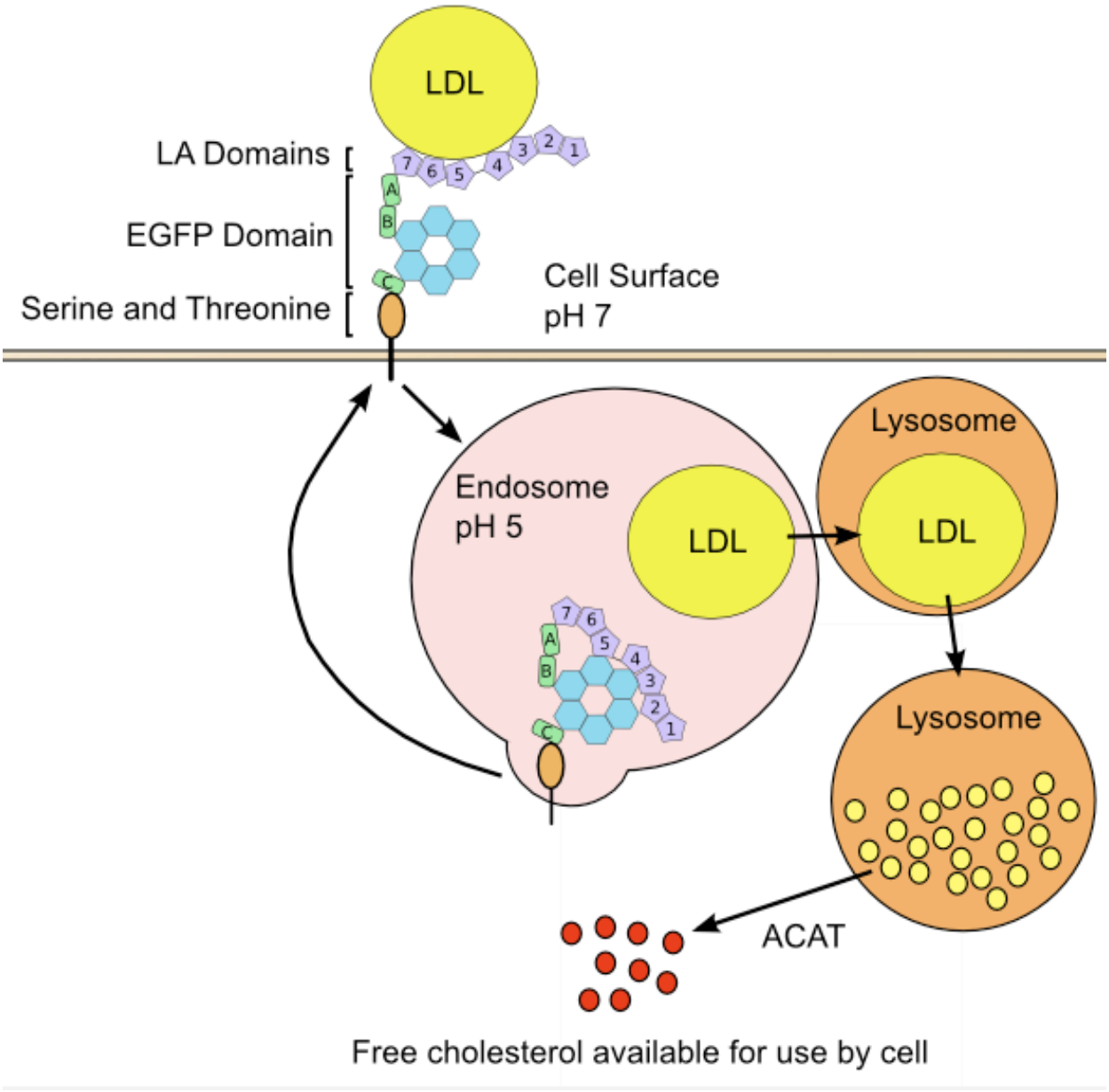

Figure 2.6: The LDLR structure contains three types of domain: LDLR-type A domains (LA), the epidermal growth factor precursor protein-like domain (EGPF), and a domain rich in serine and threonine residues. The endocytotic process is also shown, which yields free intracellular cholesterol.

(Hooper et al. 2008). Cholesterol that has been endocytosed and converted to free form is often incorporated into cell membranes, however depending on cell type, it has several other possible fates including efflux to cellular adaptors, conversion back into cholesterol esters, metabolism into bile acids, or synthesis of steroid molecules (Liscum and Underwood, 1995). Regardless of metabolic fate, levels of intracellular cholesterol are the controlling element behind overall cholesterol homeostasis of every cell via their impact on regulating LDLR protein and de novo cholesterol synthesis.

2.4.6 End product feedback regulation of LDLR and HMG Co-A Reductase

In addition to endocytosis of lipoproteins, mammalian cells increase cholesterol levels through de novo synthesis beginning from acetyl-CoA. The rate limiting reaction of the cholesterol biosynthesis pathway is production of mevalonate by HMG-CoA reductase (Goldstein and Brown, 1990). Importantly, expression levels of *HMG-CoA reductase* as well as *LDLR* are negatively controlled by intracellular cholesterol, which as a result, commands a powerful system of self regulation.

Transcription levels of both *LDLR* and *HMG-CoA* reductase genes are controlled by promoters with sterol regulatory elements (SREs), which are present on over 30 genes involved with lipid synthesis and uptake (Horton et al. 2003). Experiments using chimeric *LDLR* promoter and reporter gene constructs have localized the area of sterol responsiveness to 8bp of palindromic sequence, (5'-CACCCCAC-3') (Smith et al. 1990). The SRE in the *HMG-CoA reductase* promoter is homologous to *LDLR* SRE, with a substitution of a guanine for the central cytosine on the 3' side (Osborne et al. 1988). In both promoters, attachment of transcription factors called

SRE binding proteins (SREBPs) is required for efficient production of downstream transcription. There are three types of SREBP, (SREBP1a, SREBP1c, and SREBP2), which are encoded by two genes. Of the three isoforms, SREBP1a and SREBP2 are more implicated in cholesterol homeostasis then SREBP1c, which mainly alters expression of fatty acid synthesis genes (Horton et al. 2002). Despite discrepancies in their transcriptional targets, proteolytic activation of each *SREBP* isoform is regulated by cholesterol through a common mechanism. Complete disruption of *SREBP* activation in mice hepatocytes results in 75% decrease in sterol and fatty acid synthesis, 50% reduction in *LDLR* mRNA and LDL clearance, and a significant reduction in total plasma cholesterol (Yang et al. 2001).

The molecular basis for cholesterol sensitivity of SREBP has been elucidated greatly due to the efforts made by the Brown and Goldstein Lab of the University of Texas Southwestern Medical Center. Each newly translated *SREBP* is inserted into the ER membrane, where its C-terminal regulatory domain binds to the C-terminal of the SREBP cleavage activating protein (SCAP) (Horton et al. 2002). While associated with SREBP, SCAP binds to another ER bound protein known as insulin induced gene 1 (INSIG) (Yang et al. 2002). Interaction between SCAP and INSIG keeps the protein complex firmly in place in the ER. Importantly, this interaction between SCAP and INSIG is cholesterol-sensitive, and thus, acts as the cholesterol sensor of this system (Brown et al. 2002). In cholesterol abundant conditions, membrane-spanning domains 2, 3, and 6 of SCAP bind to cholesterol, causing a conformational change in the cytosolic domain between domains 7 and 8, resulting in strong affinity for INSIG (Brown et al. 2002). The cholesterol-absent confirmation of SCAP is not associated with INSIG, leaving the SREBP/SCAP complex free to leave the ER in COPII vesicles, which then migrate to the golgi

(Goldstein et al. 2006). Once there, the N-terminal transcription factor domain of SREBP is cleaved in a two-step process involving proteolytic enzymes site-1 and site-2 proteases (S1P and S2P, respectively) (Nohturfft et al. 2000). The nuclear form of SREBP migrates into the nucleus and activates genes such as *HMG CoA reductase* and *LDLR*, ultimately leading to increased cholesterol concentration in the cell.

Mutations in genes involved in the cholesterol biofeedback pathway produce powerful effects on cholesterol homeostasis, especially those in genes that encode cholesterol biosynthetic enzymes. Inactivating mutations in the cholesterol biosynthetic pathway cause accumulation of cholesterol precursors, which manifest other serious health defects besides dyslipidemia. Two examples of several such diseases include autosomal recessive Smith-Lemli-Opitz syndrome, which is caused by mutations in the *DHCR7* gene (Fitzky et al. 1998), and Conradi-Hünermann-Happle syndrome, which results from defective *EBP* (Braverman et al. 1999). Table 2.2 details common mutations that have been discovered in the genes regulating sterol biofeedback.

Table 2.2: Common mutations (minor allele frequency > 5%) in genes participating in sterol biofeedback pathway

Gene	ID	Alias	Chromosome	MA Freq.	Effects	Citation
SREBP1	--	-36DelG	17p11.2	42%	↑ TC, ↑ LDL	Védie et al. (2001)
SREBP1	rs11868035	17312450G>A	17p11.2	28%	↑ LDL	Liu et al. (2008)
SREBP1	rs2297508	54G/C	17p11.2	26%	↑ LDL	Liu et al. (2008)
SREBP2	--	1784G>C	22q13	20%	↑ TC, ↑ LDL	Duan et al. (2005)
SCAP	--	2386A>G	3p21.31	48%	↓ LDL	Fiegenbaum et al (2005)
Insig-2	rs7566605	rs7566605	2q14.1	32%	↓ TC	Oki et al. (2009)

IDRefSNP ID#, MA Freq. = Minor allele frequency, Effects = phenotypic effect of minor allele, TC = total cholesterol

2.5 Summary

Cells collect cholesterol molecules through de novo synthesis, or receptor mediated endocytosis of exogenous and endogenous cholesterol packaged in LDL. Extracellular cholesterol is initially packaged into triglyceride rich particles, which are hydrolyzed in circulation to move fatty acids into cells expressing LPL. As lipoproteins undergo triglyceride hydrolysis, they become increasingly dense and cholesterol-laden. Cholesteryl ester-rich lipoproteins interact with the LDLR, which is expressed selectively by the liver, and by cells low in intercellular cholesterol. Meanwhile, the liver is perpetually manufacturing HDL particles, which have the critical task of removing excess cholesterol from LDL particles, and the minute spaces that they might accumulate. Together, these systems interact, sharing various proteins and lipids in order to maintain the balance of the potentially deadly but undeniably vital cyclic lipid, cholesterol.

Interest in HDL is focused on its ability to reverse cholesterol accumulation in tissues. Conversely, LDL demands our attention so that we may comprehend the mechanisms that lead to heart disease. Unfortunately, the extremely polygenic and complex mechanisms that influence cholesterol homeostasis confound experimental design so that only narrow hypothesis may be investigated. However, the research community has established tremendous worth to deciphering these systems. Finally, the advent of new sequencing and analysis technologies assure that these pathways will continue to be investigated until preventative cardiac medicine is a reality.

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Chapter Three:

ASSOCIATION OF CHOLESTEROL TRANSPORT PATHWAYS WITH LIPID PHENOTYPES IN BOS TAURUS

Association of cholesterol transport pathways with lipid phenotypes in Bos taurus

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3.1 Abstract

The reverse cholesterol transport and low density lipoprotein receptor pathways are central to overall lipid homeostasis, leading to their promising roles in producing candidate genes for meat quality traits in Bos taurus. Thirteen genes related to lipoprotein homeostasis were investigated for association with 19 traits including marbling, subcutaneous fat depth, carcass weight, rib-eye area, kidney-pelvic-heart fat, cholesterol, conjugated linoleic acid, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, steatoyl-CoA desaturase activities (3), juiciness, muscle fiber tenderness, overall tenderness, connective tissue amount, flavor intensity, and shear force. 30 PCR amplicons were sequenced in 6 Wagyu x Limousin animals, generating 37 SNPs. A Sequenom assay system selected 13 mutations for genotyping on ~250 F2 progeny. Association between these markers and phenotypes was conducted under 2 statistical models. Resulting analysis yielded significant ($P<0.05$) association with one or more traits among mutations in the following genes: *ATP binding cassette A1* (*ABCA1), Apolipoproteins A1, B*, and *E, phospholipid transfer protein, paraoxinase 1,* and *scavenger receptor protein BI*. Notably, the *26132G>A* mutation in the *ABCA1* promoter appears to be strongly associated ($P<0.01$) with shear force, conjugated linoleic acid, and subcutaneous fat depth. Linkage disequilibrium analysis between this marker and *ABCA1* 70522C>G located in intron 15 reveals weak linkage ($R^2 = 0.18$). The latter SNP was associated with shear force, meat tenderness, saturated fatty acids, and subcutaneous fat depth. Taken together, these results and others of this study provide additional markers for animal breeding programs while emphasizing the central role that these pathways hold in beef cattle lipid homeostasis.

3.2 Introduction

Apolipoproteins provide the structural and catalytic basis of lipoproteins, and thus are strong candidate genes for obesity related phenotypes. Like other mammals, protein composition of cattle high density lipoproteins (HDL) and low density lipoproteins (LDL) is dominated by apolipoprotein (APO) A1 and APOB, respectively (Bauchart et al, 1989). APOA1 is an essential co-factor for several key components of reverse cholesterol transport including lecithin:cholesterol acyltransferase (LCAT) (Fielding et al. 1972), ATP binding cassette A1 (ABCA1) (Wang et al. 2008), and scavenger receptor BI (SCARB1) (Rigotti et al. 1997). Similarly, APOB is required for synthesis of chylomicrons and VLDL in the intestines and the liver (Hui and Howles, 2005, Hebbachi and Gibbons, 2001). Meanwhile, APOC2 is responsible for activation of lipases, on chylomicrons and very low density lipoproteins (Goldburg, 1996), a crucial aspect of fatty acid homeostasis. Finally, APOE, while surprisingly absent in cattle HDL (Brantmeier et al. 1996), is extremely important to LDL and chylomicron remnant clearance through the low density lipoprotein receptor (LDLR) (Zhang et al. 1992). Polymorphisms in *APOA1* (Hovingh et al. 2005, Sorci-Thomas et al. 2002), *APOB* (Benn, 2009), *APOC2* (Sepehrnia et al. 1989), and *APOE* (Mahley, 1988, Wu et al. 2007) have demonstrated association with lipid phenotypes in humans.

The principal HDL pathway, termed reverse cholesterol transport (RCT) is a major component of lipid homeostasis. Cholesterol efflux, the process in which cholesterol moves from the periphery into HDL, is largely mediated largely by interaction between immature HDL and ABCA1 expressed on cells in the vascular periphery (Wang et al. 2000). Mutations in human *ABCA1* are responsible for Tangier disease (Schmitz et al. 1985), familial high-density lipoprotein

deficiency (Huang et al. 2001), and more commonly, subtle alterations in overall lipid homeostasis (Brunham et al. 2006). As HDL particles become enriched in free cholesterol, the cholesterol inside the particles is esterified by the LCAT protein (Fielding et al. 1972), forming the stored form of the lipid, cholesterol esters. Additional cholesterol efflux, and perhaps more importantly, hepatic clearance of cholesterol-rich HDL, is mediated by the SCARB1 protein, which is considered the primary HDL receptor (Acton et al. 1996, de la Llera-Moya et al. 1999). HDL protects against atherosclerosis primarily via RCT, but also has powerful antioxidant properties directed specifically toward oxidized lipids inside lipoproteins. This effect appears to be caused by the paraoxidase I (PON1) gene product (Navab et al. 1996).

Endogenous and exogenous cholesterol molecules become components of LDL particles after being secreted into circulation as part of VLDL. VLDL particles undergo triglyceride hydrolysis, and eventually form first into IDL and then LDL. LDL particles have a relatively long halflife of about 3 days (Langer et al. 1972), a property attributed to the lack of activating apolipoproteins, the stability of cholesterol in the particles, and lack of cholesteryl ester hydrolysis enzymes. Removal of LDL from circulation is mediated by LDLR, which is under tight genetic control, regulated by sterol response elements (Horton et al. 2002). Over 700 mutations have been reported in human LDLR, many of which lead to familial hypercholesterolemia (Hutter et al. 2004).

In circulation, lipases and other proteins constantly remodel lipoproteins. Lipases are water-soluble enzymes that hydrolyze ester bonds of water-insoluble substrates such as lipoprotein bound phospholipids, triglycerides, and cholesteryl esters. In circulation, the activities of

lipoprotein lipase (LPL), endothelial lipase (LIPG), and hepatic lipase (LIPC) continuously remodel lipoproteins, which profoundly effects behavior of lipoproteins. The primary function of LPL is to hydrolyze triglyceride-rich lipoproteins, especially chylomicrons and VLDL, thereby generating free fatty acids and glycerol for energy metabolism and storage (Goldburg, 1996). These lipoproteins, along with HDL are also modified by LIPG, which primarily hydrolyzes phospholipids (McCoy et al. 2002). LIPC has powerful VLDL and IDL triglyceride hydrolysis capabilities (Grosser et al. 1981), as well as the ability to condense HDL into a subspecies that is more likely to interact with SCARB1 for cholesterol efflux or endocytosis (Catalano et al. 2008). Additionally, the phospholipid transfer protein (PLTP) catalyzes the fusion of two particles of the HDL3 subspecies, forming one larger HDL2, and releasing lipid poor ApoA1, the precursor to pre-β-HDL (Jauhiainen et al. 1993). Enrichment of HDL with triglycerides enhances this process (Settasatian et al. 2001), which is termed HDL conversion. In addition to HDL conversion, PLTP facilitates the transfer of phospholipids and to a lesser extent, cholesterol from triglyceride rich lipoproteins such as VLDL and chylomicrons into HDL (Rao et al. 1997, Nishida and Nishida, 1997).

All mammals including ruminant animals achieve intravascular lipid transport though production and metabolism of lipoproteins, which distribute cholesterol and fatty acids to peripheral tissues expressing the appropriate lipases/receptors. As these molecular processes are directly involved with lipid phenotypes, genetic markers among these genes can potentially contribute to animal breeding programs. For example, the levels of fat marbling appear to be inversely correlated to HDL levels and directly correlated to LDL (Noro and Kobayashi, 1995). Unfortunately, while association analysis of mutations among candidate genes for cholesterol homeostasis have

provided useful markers for obesity and plasma lipids in humans (reviewed in Boes et al. 2009, Soutar and Naoumova et al. 2007, Holleboom et al. 2008), little effort has been directed towards developing these candidate genes in production animals such as beef cattle. Furthermore, differences in dietary intake and digestive physiology between ruminants and non-ruminants produce questions regarding the importance of these pathways to cattle. To this end, the aim of the present study was to investigate the relationship between genes relevant to HDL and LDL homeostasis and meat quality traits in beef cattle.

3.3. Materials and methods

Animals. A Wagyu x Limousin reference population was jointly developed by Washington State University and the Fort Keogh Livestock and Range Research Laboratory, ARS, USDA, as previously described (Jiang et al. 2005). Japanese Wagyu cattle traditionally have much higher amounts of intramuscular fat deposition, as well as less saturated and more unsaturated fatty acids than the Limousin breed (Pitchford et al. 2002). Thus, our reference population provides unique gametic recombination emphasizing the genetic contributions to meat quality and obesity. The present study included 6 F1 bulls, 113 F1 dams, and ~240 F2 progeny from this population. DNA extraction and phenotypic data collection on F2 animals were conducted in the USDA laboratory.

Eating quality measurements. Marbling score (BMS), subcutaneous fat depth (SFD), carcass weight (CARC-WT), rib-eye area (REA), and percent kidney-pelvic-heart fat (KPH) were measured by a trained evaluator after 48h of chilling at 2ºC. Hot carcass weight was determined immediately after harvest before rinsing/washing and chilling. BMS was measured subjectively,

focusing on intramuscular fat stored in the *longissimus* muscle based on U.S. Department of Agriculture standards (http://www.ams.usda.gov). SFD was measured at the 12th to 13th rib interface perpendicular to the outside surface at a point three-fourths the length of the longissimus muscle from its chine bone end. KPH percentage was estimated relative to body weight. The area of the longissimus muscle measured in square inches at the 12th rib interface on the beef forequarter was recorded as REA.

Fatty acid composition analysis. Fatty acid composition was measured according to methodology previously described (Rule et al. 2002). In short, approximately 150mg samples of *longissimus dorsi* muscle tissue were completely saponified with 4.0 ml of 1.18 m in ethanol at 90 ºC. After about 45 minutes, 2.0 ml of water were added. Cholesterol (CHOL) was extracted with 2.0 ml of hexane, the resultant phase was then transferred to gas liquid chromatography vials and sealed. To prepare samples for fatty acid methyl ester (FAME) profile analysis, 1.0 ml of concentrated HCl was added to the original tubes. Fatty acids were then extracted in 2.0 ml of hexane, and the amount of conjugated linoleic acid (CLA) was measured using acid catalysis. FAME data was used to measure the following: saturated fatty acids (SFA) = myristic + pentadecanoic + palmitic + heptadecanoic + stearic, monounsaturated fatty acids (MUFA) = myristoleic + pentadecenoic + palmitoleic + heptadecenoic + oleic + vaccenic and polyunsaturated fatty acids (PUFA) = linoleic + linolenic. The relative amount of SFA, MUFA and PUFA was defined as SFA = (SFA/total fat in 100 g dry meat) x 100%, MUFA = (MUFA/total fat in 100 g dry meat) x 100% and PUFA = (PUFA/total fat in 100 g dry meat) x 100%, respectively. Three stearoyl-CoA desaturase activities were estimated as R1 = (14:1/14:0) x 100%, R2 = (16:1/16:0) x 100% and R3 = (18:1/18:0) x 100%.

Taste panel data. An eight-member sensory panel was conducted as previously described (Alexander et al. 2007). Panelists were selected and trained according to American Meat Science Association standards (American Meat Science Association, 1995). Panelists scored each (uniform 1.3 x 1.3 x 1.9 cm) sample on an 8-point scale for initial juiciness (JUICE), muscle fiber tenderness (MFTEND), connective tissue amount (CTISSUE), overall tenderness (OVTEND), and beef flavor intensity (FLAVOR).

Shear force. Peak shear force (SHEAR) was measured using an Instron testing machine filled with a Warner-Bratzler shear head as previously described (Alexander et al. 2007). Cores parallel to muscle orientation of cooked steaks were removed (6-10 each steak and 1.27 cm in diameter), cooled to room temperature, and sheared perpendicular to muscle fiber orientation.

Gene annotation and primer design. Manual annotation of each gene occurred in the following process: First, cDNA sequences of candidate genes were retrieved from the National Center for Biotechnology Information (NCBI) Entrez database. To produce complete length cDNA sequences, cDNA sequences were re-annotated using electronic rapid amplification of cDNA ends (e-RACE) (Jiang et al. 2008). This method ensures that the longest expressed sequences in the database are utilized to produce primary cDNA sequences of complete length. Next, the full-length cDNA sequence was used to search for genomic DNA contigs in the 7.15X bovine genome sequence database (see the Bovine Genome Resources at NCBI). For comparative annotation, human cDNA sequences were also retrieved from NCBI, and re-annotated in this way. Percent homology for protein sequences was calculated by the bst2seq function of BLASTP at NCBI.

Mutation discovery and genotyping. Primer design was completed using the Primer3 online oligonucleotide design tool (Rozen and Skaletsky, 2000). Based on genomic DNA sequences, 37 primer pairs were designed to amplify genetic targets located in 13 genes (Table 3.1). Approximately 50 ng of genomic DNA each from six Wagyu x Limousin F1 bulls was amplified in a final volume of 10 µl that contained 12.5 ng of each primer, 150 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl and 0.25 U of Amplitaq Gold polymerase (Applied Biosystems, Branchburg, NJ). Cycling conditions were as follows: 95ºC for 10 minutes, 8 cycles of 94ºC for 30 sec, 71ºC for 30 sec, and 72ºC for 30 sec, followed by 37 cycles of 94ºC for 30 sec, 59ºC for 30 sec, and 72ºC for 30 sec, and completed by an extension step at 72ºC for 10 min. PCR amplicons were sequenced on a capillary sequencer by High-Throughput Sequencing Solutions (Seattle, WA). Selected mutations were genotyped in 240 F2 animals using the Sequenom iPLEX assay service provided by Genomics Center at University of Minnesota.

Data Analysis. The HAPLOVIEW (Barret et al. 2005) program was utilized to determine degrees of Hardy-Weinberg equilibrium within each marker and linkage disequilibrium between markers. Phenotypic data for all measurements were adjusted for the effects of year, gender, age, and genotype for each marker under the GLM (general linear model) procedure of SAS v9.1 (SAS Institute Inc., Gary, NC). A protected t-test yielded pair-wise comparisons of least square means. Additionally, quantitative transmission disequilibrium test (QTDT) (Abecasis et al. 2000) was performed to further analyze the relationship between markers and adjusted phenotype data. P value <0.05 were considered statistically significant in each association test. Regulatory binding sites in promoter SNPs were determined using the Transcription Element Search System (TESS) software (Schug, 2008).

3.4. Results

***Comparative annotation*.** Using e-RACE, the 5' UTR regions of the following cattle genes were extended: *ABCA1* (ESTs *EE906192, EW684406, BF040532, DV776632, AM023804*), *ApoA1* (EST *EV787263*), *ApoE* (EST *CR852800*), *LIPC* (EST *EV611736*), *PLTP* (EST *EV797325*), *PON1* (EST *DT855088*), and SCARB1 (EST *EH146681*). The 3' UTR sequence of *LIPG* was extended via ESTs *BF230612*, *AV616502*, *DV907019*, *BE754248*, *CR455977*. Human 5' UTR sequences that were extended include: *ABCA1* (EST *BX955241*), *ApoA1* (EST *CD558050*), *ApoE* (EST *BM927791*), *LIPC* (EST *DA6270450*), and *PLTP* (EST *DA541411*). The cDNA sequence of the human *ApoB* gene was 82.5% homologous to two cattle cDNA sequences (*XM_001788185* and *XM_583270*), which decidedly encode the same gene.

The completely annotated sequences of *ABCA1, ApoA1, ApoC2, ApoE, LCAT, LDLR, LIPG, LPL, PTLP, PON1,* and *SCARB1* each had the same amount of exons in human and cattle orthologs. Upon BLAST searching the cattle *LIPC* mRNA (*NM_001035410*), the resulting three matches of genomic DNA (*AAFC03094839, AAFC03094837, AAFC03078250*) could account for only 43.1% of the mRNA contig. Therefore it appears that the current cattle build (4.0) lacks coding sequence of *LIPC*, preventing complete annotation of this gene. However, the WGS-traces database available on NCBI BLAST produced a fragmented set of genomic sequences to accommodate the cDNA (Trace IDs: *1663880125, 1081738980, 1082796348, 393920765, 1189812244, 514287263, 382466136, 514383498, 381230783*). The *ApoB* gene is the only one studied that appears to have different intron/exon architecture between human and cattle, as it contains 29 and 28 exons in the two animals, respectively (Table 3.2). Protein sequence homology between the human and cattle orthologs of thirteen genes is listed in Table 3.2. The

greatest homology appears between *ABCA1* (94%), while the *ApoC2* gene had the lowest, at 62%. Sizes (in bp) of cDNA, and UTR sequences of these genes are also listed in Table 3.2.

SNPs and Haplotypes. In total, 37 PCR amplicons of the F1 generation were sequenced in 13 genes, producing a total of 30 SNPs (Figure 3.1). Of these mutations, 15 were selected for genotyping of the F2 reference population using the Sequenon array platform, and 13 mutations generated quality data. Of these, 2 mutations were previously discovered: *ApoE:AAFC0303445:12439T>C* (Entrez SNP ID: *rs41884945*), and *ApoE:AAFC0303445: 11464T>C (rs41884933)*, which are both located inside the promoter region of *ApoE*. The remaining 11 SNPs appear to be novel mutations, and are located and identified as follows: *ABCA1* promoter region (*AAFC03037127:g.26132G>A*), *ABCA1* exon 14 (*AAFC03121742:c.70522G>C*), *APOA1* intron 3 (*AAFC03114751:g.79468G>A*), *APO*B intron 13 (*AAFC03076821:g.3553A>G), APOE 3'UTR (AAFC0303445:g.15442G>C* and *g.15696C>T*), *APOE* promoter (*AAFC0303445:g.11400G>A*), *PLTP* exon 16 (*AAFC03071797:c.3349C>T*), *PON1* intron 5 (*AAFC03037852:g.35193:A>G*), and *SCARB1* promoter *(AAFC03038307:g.17443C>A and g.17539:T>C*) (Figure 3.1). Among the 2 protein coding SNPs, *ABCA1 70522G>C* is a synonymous mutation, while the *PLTP 3349C>T* mutation is a missense SNP, leading to a change from GTC (codes for valine) to GCC (alanine). The minor allele frequencies of these mutations ranged from 0.141 for *ApoE:15442G>C* to 0.396 for *SCARB1:17539:T>C*. Each of the 13 mutations was found to be within Hardy-Weinberg equilibrium (P=0.05). Linkage disequilibrium was measured in 9 mutations using the HAPLOVIEW program. Output from HAPLOVIEW revealed strong linkage between three of the 5 *APOE* markers: *11464G>A, 15442G>C, and 15696C>T* (Figure 3.1). *11400G>A* was

weakly linked to these markers, while *15442G>C* appears to segregate independently of the other markers. *APOE* markers in linkage form 4 haplotypes: GCCCT, GTTCC, ATTGG, ATTGC (frequencies: 0.361, 0.310, 0.163, and 0.137, respectively), which accounts for 97.1% of allelic recombination. Additionally, strong linkage was detected between the 2 *SCARB1* markers, *17443C>A* and *17539:T>C* (Figure 3.1). Weak linkage ($R^2 = 0.18$) was found between the two markers in *ABCA1*, *26132G>C* and *70522G>C*.

Association analysis of mutations with obesity and meat quality traits. Table 3.3 reports the genotype frequencies, least square means, P-values, and QTL effects of statistically significant test results generated by QTDT and SAS. With the exception of the *APOE 15696C>T* mutation, each SNP was associated with one or more phenotype. P-values less than 0.05 were considered significant. Significance was reached between the following markers and carcass related phenotypes: BMS (1 marker: ABCA1 26132G>A), SFD (6 markers: ABCA1 26132G>A, ABCA1 70522G>C, APOE 11400G>A, APOE 15442C>G, SCARB1 17443 C>A, SCARB1 17539T>C), CW (2 markers: APOA1 79468G>A, SCARB1 17539C>A), REA (4 markers: ABCA1 26132G>A, APOA1 79468G>A, APOE 11464T>C, APOE 12439T>C), KPH (1 marker: PON1 35193C>T). The following eating quality phenotypes were significantly associated: SHEAR (3 markers: ABCA1 26132G>A, ABCA1 70522G>C, PLTP 3349C>T), MFTEND (1 marker: APOB 3553A>G), OVTEND (1 marker: ABCA1 70522G>C), FLAVOR (2 markers: APOE 11464T>C, APOE 12439T>C). The following phenotypes related to fatty acid composition were significantly associated: CHOL (1 marker: APOB 3553A>G), CLA (2 markers: ABCA1 70522G>C, PLTP 3349C>T), PUFA (2 markers: APOE 15442C>G, PLTP 3349C>T), SFA (2 markers: ABCA1 70522G>C, APOE 11400A>G).

Promoter SNPs and transcription factor binding sites. Transcription factor binding sites covering five promoter mutations are displayed in Figure 3.2. The *SCARB1 17539T>C* SNP does not appear to affect any transcription factor binding. Each of the other promoter mutations appears to lead to changes in transcription factor recognition. The G allele of *ABCA1 26132G>A* is associated with GCAGG and GAGGC binding sites, which are recognized by LVc and T-ag transcription factors, respectively. The A allele of this mutation is associated with two binding sites as well: GGAGA, which is a binding site for ADR1, and GACAG, which is recognized by NF-E. The A allele of *ApoE 11400G>A* contains a CCAAT sequence, which is recognized by transcription factors CRF, and NF-Y. These proteins do not appear to be associated with the G allele of this mutation. For *ApoE 11464T>C*, the T allele is not associated with any regulatory binding sites, while the C allele contains CCTGG and TGGA sequences, which are recognized by LPB-1 and NF-1. *ApoE 12439T>C* contains a recognition sequence GCCCC, which is recognized by T-Ag, and CCCC, which is bound by GAL4. The A allele of *SCARB1 17443C>A* contains ACTGACAA and TGAC recognition sequences, recognized by DEF and AP-1, respectively, while the C allele contains TGCCAA, GCCAAG, and GCCA, which are recognized by NF-1 and NF-1-like transcription factors.

3.5 Discussion

Table 3.2 lists the cattle/human comparative details of 13 genes included in the present study. As is typical in orthologs between these two species, intron/exon architecture is conserved. The *ApoB* gene is the only exception, as it contains 29 exons in humans, while the cattle annotation has 28. It is likely that this phenomenon is caused by the incomplete status of the current (4.0) cattle build. Cattle *ApoB* is listed as two genes in entrez database (Gene IDs 494004 and

507092), however, when taken together, the cDNA sequences of these genes clearly align to the human *ApoB* mRNA sequence (NM_000384). This exemplifies the need for additional inferences in order to correctly annotate cattle genes under the current bovine assembly. Another clear example occurs when annotating cattle *LIPC*. BLAST searches of cDNA against cattle genomic DNA matches the genomic sequence to only 43.1% of cDNA. However, when consulting the WGS-traces database, the complete coding sequence can be pieced together. This annotation is not yet complete however, as there are gaps in each intron, and additionally, the cDNA sequence can be extended after BLAST searching against the EST database. Cattle EST *EV611736* was matched to the 5' end of the cDNA transcript, and added 223 nucleotides to the UTR. When accurately annotating genes, it is very useful to extend cDNA ends using the EST database. This is also true in for human genes, in which the *LIPG* ortholog was extended 178 nucleotides using EST *DA627045*. These examples illustrate the difficulty in extracting the necessary information to compile an accurate and complete annotation using the current genetic resources.

Theoretically, lipoprotein pathways contain excellent candidate genes for obesity-related traits because of their direct involvement transporting cholesterol, triglycerides, and fatty acids to and from peripheral tissues. Other important lipid components are decidedly interconnected with levels of circulating lipoproteins, including saturated fat, which reduces expression of the *LDLR*, leading to increased levels of circulating LDL (Wollett et al. 1992, Mustad et al. 1997, and Boekholdt et al. 2006). However, the relevance of previous association studies to meat production can reasonably be questioned considering that the vast majority of the research to understand the genetics of lipoprotein and lipid homeostasis has been directed toward blood

lipids. Human ApoE for example, contains two very well known and common SNPs at amino acid positions 112 and 158, which are associated with decreased HDL, increased LDL, and increased plasma cholesterol in circulation (Mahley and Rall, 2000, Wu et al. 2007). The direct implications that these *ApoE* mutations have on intramuscular cholesterol are less apparent. Although skeletal muscle cells attain fatty acids via LPL mediated hydrolysis of circulating lipoproteins (Oscai et al. 1982), the lipid compositions of muscle cells and the blood might conceivably be reliant on different mechanisms. This idea is supported by the results of the present study. Of the 13 mutations genotyped and tested for association, only the *ApoB 3553A>G* SNP was associated with cholesterol levels, and this association was found in QTDT and absent in the GLM model. This finding is an interesting juxtaposition to the high level of significance found among other lipid traits and might be evidence that intracellular cholesterol is reliant on mechanisms independent of blood pathways.

In the present study, 5 SNPs were detected and genotyped in the *ApoE* gene. Of these, 3 were located in the promoter region, at positions 1392 bp (*11400G>A*), 1327 bp (*11464T>C*), and 353 bp (*12439T>C*) upstream of the transcription start site. Additional SNPs were analyzed in the 3‘UTR region of the gene (*15442G>C*) and slightly downstream of transcription termination site of ApoE (*15696C>T*). Association analysis between the promoter markers generated several significant tests including SFA and SFD for *11400G>A*, and additionally, FLAVOR and REA for both *11464T>C* and *12439T>C*. Linkage analysis of the 3 promoter mutations showed strong linkage between the latter two markers, *11464G>A* and *12439T>C*, and weaker linkage between the 5’ terminal marker with its downstream neighbors (Figure 3.1). Interestingly, SFD was also associated with the 3‘UTR marker, *15442G>C*. This association was detected in the

absence of strong linkage between *15442G>C* and *11400G>C*, suggesting that it is not necessary for two markers to have strong linkage disequilibrium to share association with a phenotype. TEFF analysis reveals allelic dependent transcription factor binding sites in each of the three *ApoE* promoter mutations. Notably, the minor allele of the *ApoE 11400G>A* mutation contains a CCAAT binding site (Figure 3.2), which is utilized by several well-conserved transcription factors including nuclear transcription factor Y family, (NFYA/B/C), the CCAAT/enhancer binding proteins (CEBP-A/B/D/E/Z), and the nuclear factor I/X (NFIX). Also of interest, the minor allele *of ApoE 11446T>C* produces a CpG island. CpG islands have traditionally been implicated in reduction of downstream gene expression, which is mediated by methylation of the cytosine. In both cases, it is plausible for expression of *ApoE* is to be altered by allelic composition of these SNPs, which would likely produce phenotypic results. Evidence supporting this conclusion comes from transgenic mice overexpressing *ApoE*, which demonstrate dramatically increased uptake of triglyceride-rich lipoproteins such as chylomicrons and VLDL (Shimano et al. 1992).

The statistically significant results of QTDT and GLM protected t-tests between 19 beef quality traits and 12 genetic markers are shown in Table 3.3. These association tests produced 27 significant associations. Least square means (LSMs) generated by the SAS program followed 3 different modes of quantitative trait loci (QTL) effects: dominant, additive, and overdominant. The *ABCA1 26132G>A* mutation demonstrated significant association with SHEAR, CLA, REA, BMS, and SFD. The minor A allele of this marker showed strong dominant effects in shear, BMS, and SFD. Overdominant effects were seen in CLA, in which the heterozygous genotype produced the lowest LSM, at 42.90. However, when considering that the LSM of the AA

genotype was 55.956, and the CC was 48.524, it appears possible that dominance effects of the A allele influenced CLA as well. Similar results were seen with the other *ABCA1* marker, *70522C>G,* which showed weak linkage to *26132G>A* (Figure 3.1). In this mutation, the common allele showed dominant effects over SHEAR, additive effects over PCTEND and SFD, and overdominant effects on SFA. These markers exemplify the inconsistent QTL modes that can occur inside the same gene, and even the same mutation.

Common mutations in the Bos taurus genome provide the basis of genetic panels utilized in animal breeding programs, which enhance the experience of consuming beef, while producing products that are as healthy as possible. For example, conjugated linoleic acid, which has been associated with anti-obesity (Delany et al. 1999) and anti-carcinogenic (Vanden Heuvel, 1999) properties, was strongly affected by genotypes of *ABCA 26132G>A* and *PLTP 3349C>T*. Additionally, subcutaneous fat deposition and marbling, which are directly related to consumer preference (Killinger et al. 2004) and USDA quality grading, demonstrate association to several of the genes studied including *ABCA1, ApoE, and SCARB1*. Overall, our study provides strong evidence for the involvement of RCT and LDL pathways toward improving animal breeding efficacy. Additionally, the SNPs included in the present study are strong candidates to join existing panels for marker assisted selection of quality phenotypes in beef cattle production.

The present study exemplifies some of the developments that are required before marker assisted selection assumes the revolutionary role in animal breeding that it seems to promise. First, it is inefficient to search for mutations using a PCR based approach. In order to capture the causative importance of a set of mutations, an exhaustive search of all mutations inside the candidate gene

is necessary. For example, the ABCA1 70522G>C mutation is a synonymous mutation in exon 14 associated with shear force, tenderness, saturated fatty acids, and subcutaneous fat deposition. It is extremely likely that the phenotypes associated with this marker (and other markers included in the present study) are due to genetic linkage with mutation(s) that directly cause the effects. To discover the causative mutation(s), we need more sequence data then scattered PCR amplicons can provide. With knowledge of the complete set of mutations in candidate genes, validation steps can be carried out efficiently, with strong hypotheses investigated.

Of additional interest, the practical obstacles regarding the incorporation of genetic data into animal breeding are formidable. As animal breeding traits are extremely polygenic, a very large subset of strongly associated markers will be required to effectively predict them. Technological advances in nucleic acid sequencing, bioinformatics analysis, and progeny screening will greatly assist our ability to produce candidate mutations, but this will take many more years. Also, the increasingly esoteric nature of scientific development in this field requires careful and skillful explanation to the layperson to avoid the distrust that is commonly attached to biotechnology. Finally, it should not be assumed that marker assisted selection will replace traditional trait-based selection methods. The ultimate goals of this research and marker assisted selection are to supplement animal breeding techniques, and not to replace them.

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Table 3.1: Sequences of 37 PCR primer pairs utilized in the present study.

Gene/Region	Primer Sequence	Product Size
ABCA1 Promoter (1)	F: ACTGTCCATCTTCTCCCCACTCTG	495
	R: TATCGGTCAAAGCCTGTTCTCTCC	
ABCA1 Promoter (2)	F: CAGGGTCCAGGTCACCAAGTTAT	576
	R: CAGTTCGCAAATTACGGCTAGAG	
ABCA1 5' UTR	F: TAAGTGGGAGAGTGGCTTGTTTG	592
	R: GACACAGATCCTGGCTTCAGAGAC	
ABCA1 Exon 10	F: AGAGGCGATGTCAGACAAGTTATGT	515
	R: GGTGTGTCTGGATGGCAAATAGA	
ABCA1 Exon 13	F: TGGTCAACTCCATGCCTATTAGTG	538
	R: AGGTTGGAGGGATGCTACTGTAAA	
ABCA1 Exon 14	F: CCTTTCCCTCCACAAAAATCAGTA	592
	R: CACCATGAATGTGCCAGTTACAC	
ABCA1 Exon 16	F: AGTTGTGCAAGGCAAGCTGATAG	600
	R: GAGGAGCCAGTGAAGCAGTCTTA	
ABCA1 Exon 39	F: TCACACTAGGGGAACCATTTTGA	519
	R: CCAAATCAGACACAGCGACACTT	
ABCA1 3' UTR (1)	F: GTTTCATTGTTGTCCCTTGCTTG	679
	R: GATGAACATGGCACAGGAAATCT	
ABCA1 3' UTR (2)	F: TTGAGCAGTTGGTTGATTTGGAG	583
	R: TGAGACATAGGCTACAAAGGCACTG	
APOA1 5' UTR	F: GCTTCCTGTTTGCCTGCTCTATT	451
	R: CAGGAAGAGCACAGCCAAGGT	
APOA1 Exon 3	F: GTCTGATCTGGGTCTCCCCTCT	586
	R: TGACCAGATGGATGGAGGAGTTA	
APOA1 3' UTR	F: GAGTTCCAGAAGAAGTGGCACGAG	597
	R: CCATACCCATTCTCCCCAAACAG	
APOB Promoter	F: AGTCAGATTGCAGGTGCCTTGTAG	520
	R: GGAGAAGTGGAGTAGTGGGTGGAC	
APOB Exons 13/14	F: ATGACACCTCTCCAGGGGATAAG	761
	R: TCATGGCTCCCTTGACTTGACTA	
APOB 3' UTR	F: CATTCCAAATACATTGTCGGTGCT	629
	R: CATTCCCCAGAGTTCAGAGACCTT	
APOC2 3' UTR	F: GATCCACGGCTTTGGGTTCTA	579
	R: CCTCGGTGAGGGTCAGTCTCTA	
APOE Promoter	F: ACTACATGCCTGAGTGTTCTCCCA	684
	R: GGTCCACACAACGAGGAAAAGAG	
APOE 5' UTR	F: CCTTACACTGTCCCCAGAACTCC	728

	R: GAACCCACCTTCCTGTCCAAAC	
APOE 3' UTR	F: CTGGGCTGGTGGAGAAGGTG	596
	R: TAGAGATGGAGGAAAACGGGAAAG	
LCAT 3' UTR	F: CCCAGTATCAACTACACAAGCCGT	501
	R: CTTTTCCTTCAACCCACAACACAC	
LDLR Promoter (1)	F: GCAGTGAAAACATGGTGTCCTAAC	597
	R: CCAAAACATCTCCGAAATCAACT	
LDLR Promoter (2)	F: TGGGGCTAAGTTTCCAACATCTCT	407
	R: GCAGCATTTCTATGTGAGGTTTCC	
LDLR Exons 13/14	F: CCTATGGCTGATGGGGATATGT	556
	R: AAAAGCCTTACAACAGCCTCCAG	
LIPC Promoter	F: ACAAGGCTCACCCTAACCATTCTA	598
	R: TGGGGTCTGTTTTCTGTTGAGTTTA	
LIPC 5' UTR	F: GCCCTGGAAGGCAAGAATACAT	503
	R: GACCTGTGATGCCGATCATAGAA	
LIPG Promoter	F: GCAGCAGGAAAGGGAGTAAAGAAG	639
	R: CTGAAGGTGGAGAAGGTGGAAGAT	
LIPG Exon 3	F: ACTGGGGGTCTTACAGTCTTGGA	521
	R: CAAAGAGGGCAAGGTTAAAGAGC	
LPL Promoter (1)	F: ACTGCTTGATCTGTTTGGCTACTTCT	565
	R: CCTTCTACTCATCCCTCCTTCTTCC	
LPL Promoter (2)	F: TTGTTTGGTGTTTGTGTAGGGGAC	633
	R: TCTTTCCTTCTCATCCTCACAACG	
PLTP Promoter	F: TCATTATTTCTCTTTCCCGCTTTC	511
	R: CCCATCTCTTCCTGTGTGATCCT	
PLTP 3' UTR	F: GCCTCTATTTCAGCACCCATCAC	739
	R: TCTAGTCCTTCCCAGCCTCTCCT	
Pon1 Promoter	F: CCTTTCTTAAACGGCACCTCAGTA	513
	R: CCCGAGGCTTTACAGAGAGAACTA	
Pon1 Exon 6	F: CGCACATAATTCTGGGCTGTAAC	550
	R: GCCTGGAAAACCTTCACAAAATAG	
SCARB1 Promoter	F: TGTCCTGTTACATCTTCCCACTCA	467
	R: TTACCAGAAGCAGAGACCCAACC	
SCARB1 5' UTR	F: GGCGAAATGAGAGGAGGAGGAG	662
	R: GAGGACCTGCTGCTTGATGATTG	
SCARB1 3' UTR	F: CTGACTTCTTGTGCCTGAACCTG	497
	R: CCAACCCAGCCTCCCATAAAC	

Table 3.2: Human/cattle comparative genomics data of 13 genes critical to lipoprotein homeostasis. Sizes of cDNA, and UTR regions are listed in basepairs. Protein sequences are listed in amino acid residues. Headings "H" and "C" represent human and cattle, respectively.

	EXONS		cDNA		5'UTR		3'UTR		Protein		
	H	C	H	C	H	C	H	C	H	C	Homology
ABCA1	50	50	10503	10682	404	643	3313	3253	2261	2260	94%
APOA1	4	4	1225	1013	366	151	55	61	267	265	78%
APOB	29	28	14121	13858	129	NA	301	NA	4563	3259	71%
APOC2	4	4	770	749	143	153	311	290	101	101	62%
APOE	4	4	1263	1216	166	124	142	140	317	316	90%
LCAT	6	6	1390	1385	47	42	20	18	440	440	72%
LDLR	18	18	5263	4371	169	94	2511	1727	860	845	81%
LIPC	9	9	1782	1803	235	254	45	13	499	500	77%
LIPG	10	10	4140	4155	252	279	2387	2374	500	500	85%
LPL	10	10	3747	3580	371	179	1948	1967	475	478	91%
PLTP	16	16	2051	2247	297	528	272	185	493	510	90%
PON1	9	9	2435	2253	97	51	1270	1134	355	355	82%
SCARB1	13	13	2741	2700	254	225	958	934	509	509	83%

Table 3.3: QTDT and GLM modeled association analysis of 12 SNPs with beef quality phenotypes in a Wagyu x Limousine F2 reference population. Only significant associations ($P < 0.05$) are listed.

ABCA1					
26132G>A	AA	AG	GG		
(N)	(20)	(83)	(97)	P(GLM)	P(QTDT)
SHEAR	4.360 ± 0.159a	3.909 ± 0.080b	3.823 ± 0.072b	0.0094	0.0752
CLA	55.956 ± 3.662a	42.940 ± 1.829b	48.524 ± 1.656a	0.0029	0.0057
REA	13.042 ± 0.324ab	12.931 ± 0.158a	13.465 ± 0.144b	0.0364	0.0311
BMS	6.725 ± 0.251a	5.972 ± 0.123b	5.924 ± 0.111b	0.0131	0.02
SFD	0.492 ± 0.039a	0.385 ± 0.019b	0.358 ± 0.017b	0.0061	0.0041
70522G>C	CC	CG	GG		
(N)	(35)	(108)	(89)	P(GLM)	P(QTDT)
SHEAR	0.492 ± 0.039a	3.916 ± 0.076b	3.777 ± 0.082b	0.0174	0.0316
OVTEND	7.006 ± 0.022a	7.435 ± 0.128ab	7.654 ± 0.139b	0.0437	0.0311
SFA	42.566 ± 0.346a	43.655 ± 0.203b	43.177 ± 0.217ab	0.0191	0.0134
SFD	0.445 ± 0.030a	0.401 ± 0.018a	0.334 ± 0.019b	0.0028	0.0012
APOA1					
79468G>A	AA	AG	GG		
(N)	(24)	(84)	(85)	P(GLM)	P(QTDT)
CW	661.864 ± 13.556a	686.851 ± 7.441a	709.636 ± 7.384b	0.0044	0.0111
REA	12.745 ± 0.289a	13.084 ± 0.158ab	13.495 ± 0.157b	0.039	0.0404
APOB					
3553A>G	AA	AG	GG		
(N)	(72)	(99)	(32)	P(GLM)	P(QTDT)
MFTEND	5.724 ± 0.092a	5.702 ± 0.079a	6.092 ± 0.138b	0.0393	0.0441
CHOL	222.853 ± 2.731a	230.836 ± 2.349b	220.836 ± 4.096ab	0.049	0.1978
APOE					
1**1400G>A**	AA	AG	GG		
(N)	(13)	(103)	(87)	P(GLM)	P(QTDT)
SFA	42.922 ± 0.529a	42.975 ± 0.189a	43.701 ± 0.210b	0.0273	0.0157
SFD	0.358 ± 0.046a	0.352 ± 0.017a	0.421 ± 0.018b	0.0172	0.008
11464T>C	CC	CT	TT		
(N)	(22)	(93)	(88)	P(GLM)	P(QTDT)
FLAVOR	5.538 ± 0.074a	5.711 ± 0.036b	5.599 ± 0.037a	0.0315	0.0408
REA	12.920 ± 0.307a	13.017 ± 0.149a	13.492 ± 0.155b	0.048	0.0491
12439T>C	CC	CT	TT		
(N)	(31)	(82)	(87)	P(GLM)	P(QTDT)
FLAVOR	5.570 ± 0.062ab	5.713 ± 0.038a	5.598 ± 0.038b	0.0466	0.057
REA	13.044 ± 0.258ab	12.949 ± 0.159a	13.485 ± 0.157b	0.0435	0.0471
15442C>G	CC	CG	GG		
(N)	(147)	(52)	(5)	P(GLM)	P(QTDT)
PUFA	4.438 ± 0.096a	4.924 ± 0.153b	4.128 ± 0.499ab	0.0191	0.0148
SFD	0.411 ± 0.014a	0.313 ± 0.023b	0.303 ± 0.074ab	0.001	0.0002

PLTP					
3349C>T	CC	CT	TT		
(N)	(110)	(84)	(9)	P(GLM)	P(QTDT)
SHEAR	4.020 ± 0.071a	3.762 ± 0.078b	4.022 ± 0.237ab	0.0428	0.125
CLA	47.333 ± 1.631a	45.228 ± 1.802a	61.284 ± 5.486b	0.022	0.0164
PUFA	4.502 ± 0.110ab	4.723 ± 0.121a	3.820 ± 0.369b	0.048	0.0478

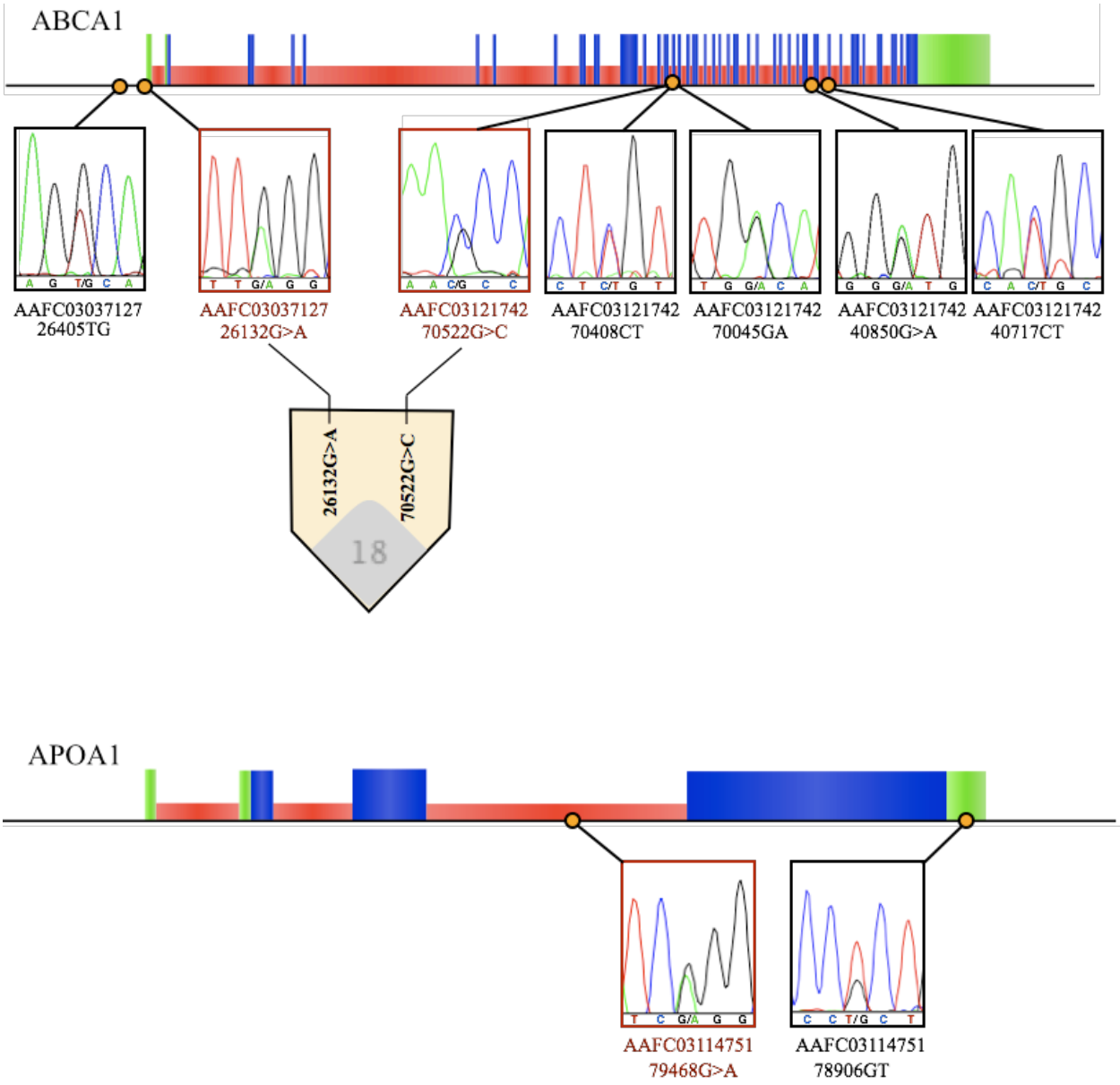
PON1					
35193A>G	AA	AG	GG		
(N)	(115)	(77)	(9)	P(GLM)	P(QTDT)
KPH	2.693 ± 0.033a	2.762 ± 0.039a	2.425 ± 0.115b	0.0186	0.016

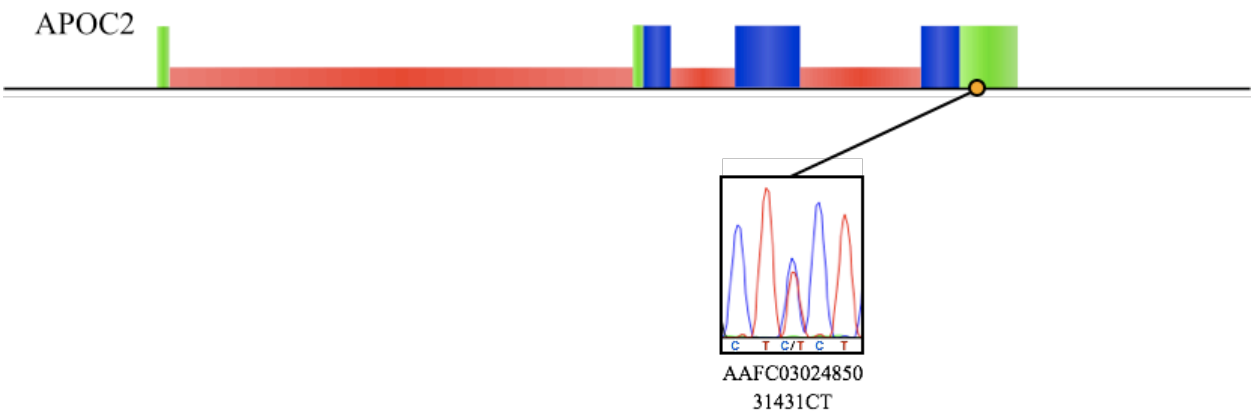
SCARB1					
17443C>A	AA	AC	CC		
(N)	(6)	(90)	(74)	P(GLM)	P(QTDT)
SFD	0.331 ± 0.028a	0.373 ± 0.018ab	0.421 ± 0.020b	0.0261	0.0059
17539T>C	CC	CT	TT		
(N)	(34)	(93)	(75)	P(GLM)	P(QTDT)
SFD	0.325 ± 0.029a	0.372 ± 0.018ab	0.423 ± 0.020b	0.015	0.0035
CW	669.968 + 11.606a	697.674 + 7.184b	697.120 + 8.090ab	0.1057	0.0407

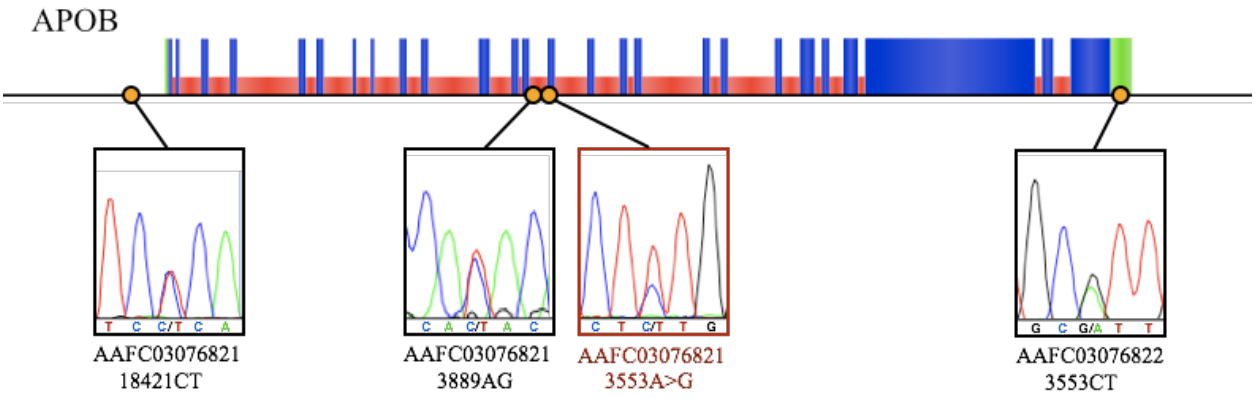
Genes: ABCA1 (ATP binding cassette A1), ApoA1 (apolipoprotein A1), ApoB (apolipoprotein B), ApoE (apolipoprotein E), PLTP (phospholipid transfer protein), PON1 (paraoxonase 1), SCARB1 (scavenger receptor B1).

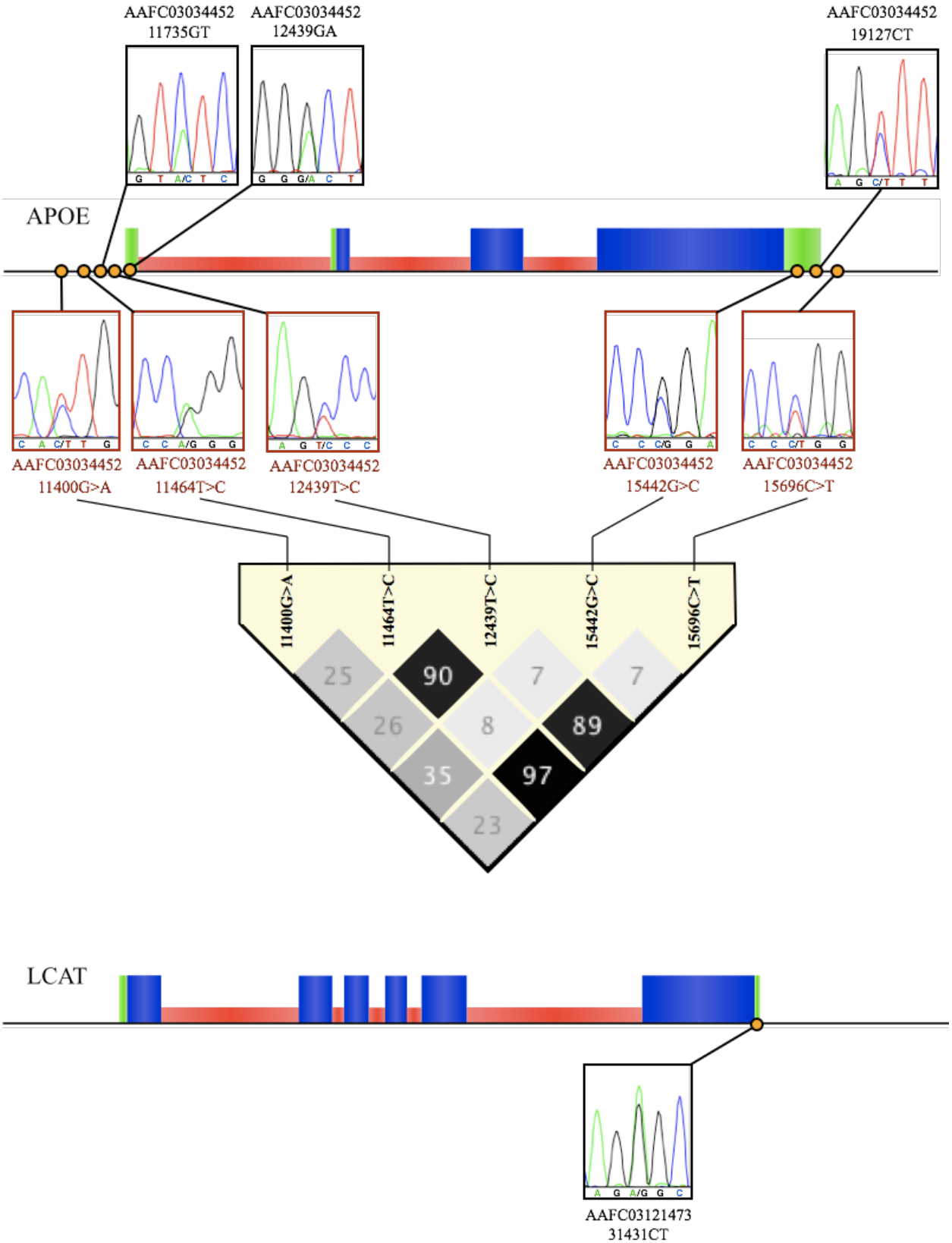
Traits: SHEAR (shear force), CLA (conjugated linoleic acid) REA (rib-eye area) BMS (beef marbling score) SFD (subcutaneous fat depth) SFA (saturated fatty acids) OVTEND (overall tenderness) CW (carcass weight) MFTEND (muscle fiber tenderness) CHOL (cholesterol) FLAVOR (flavor intensity) PUFA (poly-unsaturated fatty acids) KPH (kidney-pelvic-heart fat)

Figure 3.1: Genetic architecture of candidate genes, SNP locations, sequence traces, and linkage disequilibrium analysis. The green, blue, and red colors of genetic structures denote UTRs, exons, and introns, respectively. Only those mutations shown below that are outlined in red were genotyped and tested for association. Linkage data is shown based on R^2 measurements.




APOC2
C T C/T C T
AAFC03024850
31431CT


APOB
T C C/T C A
AAFC03076821
18421CT
C A C/T A C
AAFC03076821
3889AG
C T C/T T G
AAFC03076821
3553A>G
G C G/A T T
AAFC03076822
3553CT


AAFC03034452
11735GT
AAFC03034452
12439GA
AAFC03034452
19127CT
APOE
AAFC03034452
11400G>A
AAFC03034452
11464T>C
AAFC03034452
12439T>C
AAFC03034452
15442G>C
AAFC03034452
15696C>T
11400G>A
11464T>C
12439T>C
15442G>C
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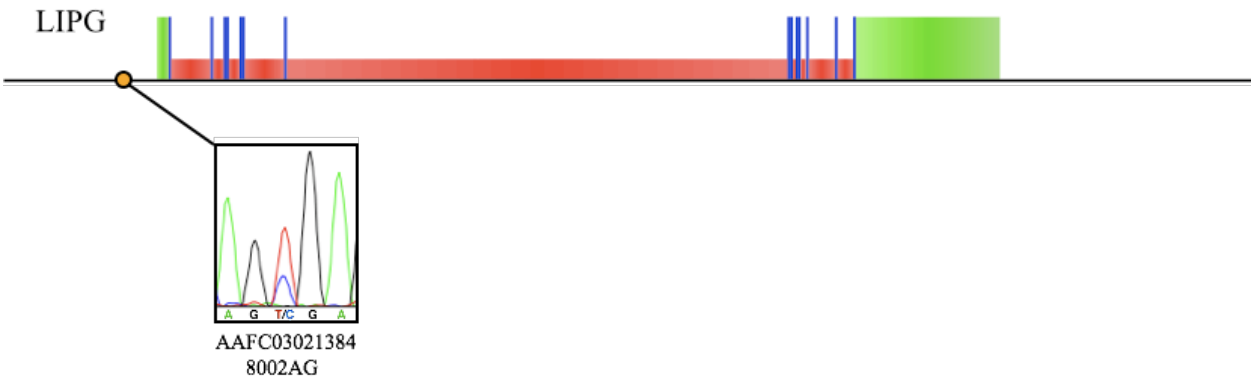
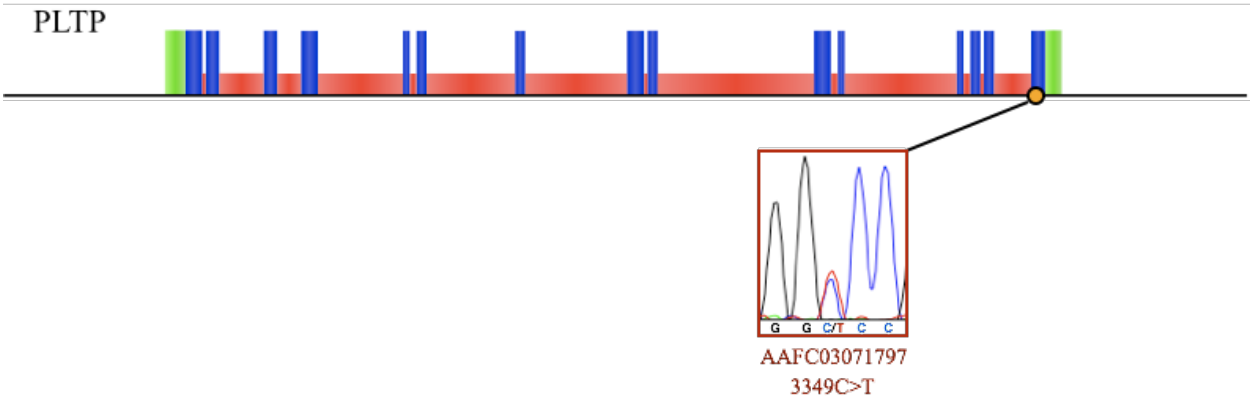
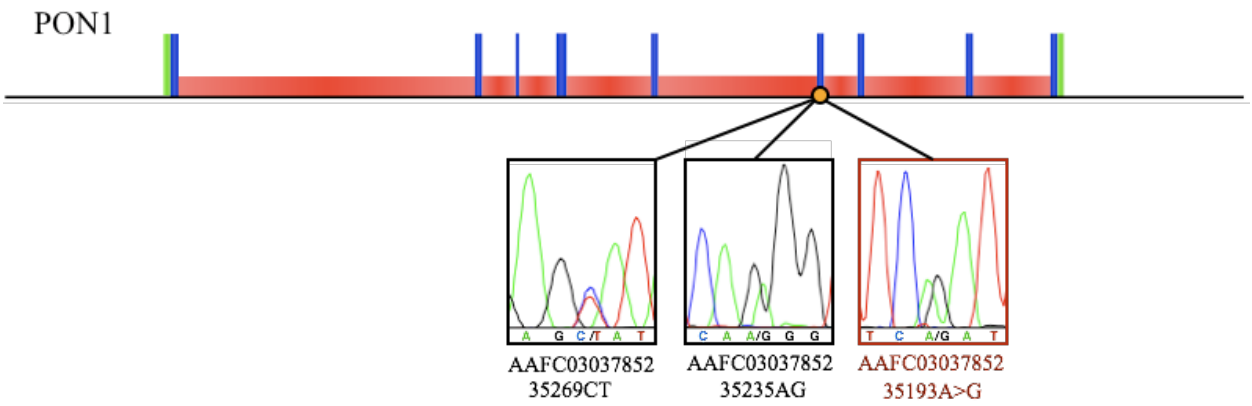
89
35
97
23
LCAT
AAFC03121473
31431CT


LIPG
A G T/C G A
AAFC03021384
8002AG


PLTP
G G C/T C C
AAFC03071797
3349C>T


PON1
A G C/T A T
AAFC03037852
35269CT
C A A/G G G
AAFC03037852
35235AG
T C A/G A T
AAFC03037852
35193A>G

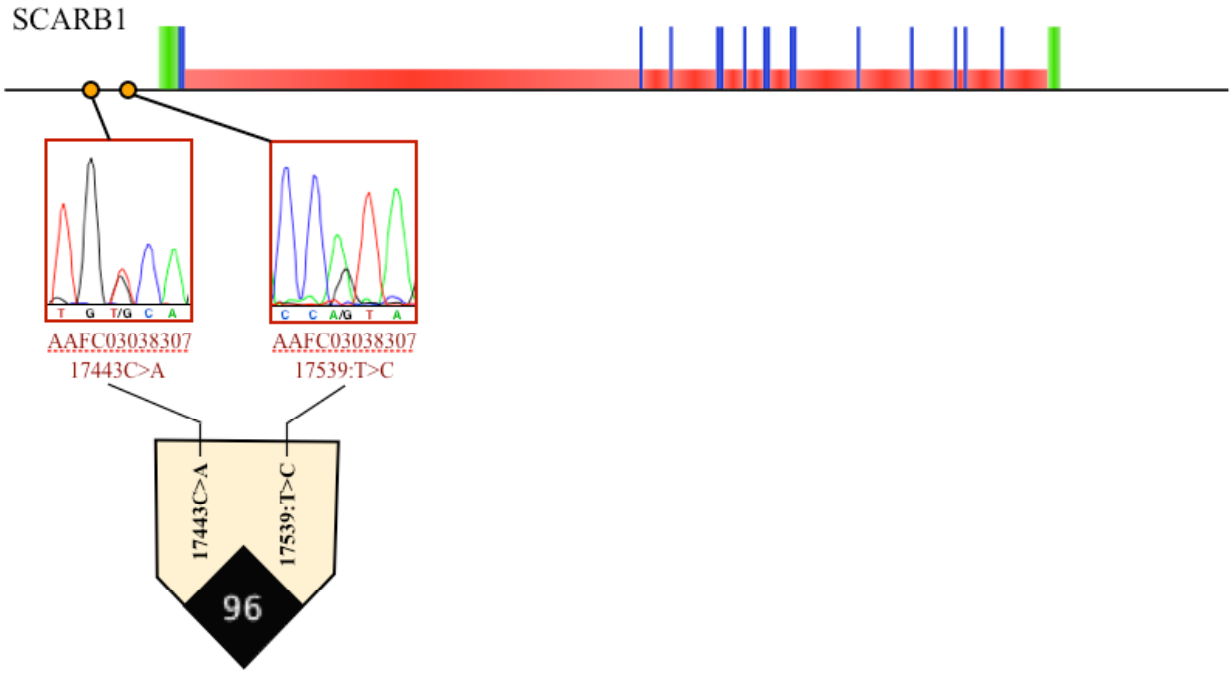

SCARB1
T G T/G C A
C C A/G T A
AAFC03038307
17443C>A
AAFC03038307
17539:T>C
17443C>A
17539:T>C
96

Figure 3.2: Nucleotide sequences and transcription factor binding sites of proximal promoter regions of genes containing SNPs.