THE ABDOMINAL FAT CONTRIBUTION TO ADIPOSITY IN CHICKENS DIVERGENTLY SELECTED FOR FATNESS OR GROWTH: CROSS-MODEL ELUCIDATION AND VALIDATION OF GENE EXPRESSION

by

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ABSTRACT

The domestic chicken (*Gallus domesticus*) is an important global source of high-quality dietary protein and a widely used biological model. Decades of intensive genetic selection have established the remarkable growth rate of the commercial broiler today; however, increased growth rate has been accompanied by the magnification of several unfavorable traits. This thesis addresses abdominal fatness, one of the traits that are incidentally amplified by selection for increased growth. Excessive fatness, coupled with several unique avian features of metabolism (i.e., fasting hyperglycemia and insulin insensitivity), parallels conditions observed in humans with metabolic diseases. Thus, understanding the genetic influence on excessive fatness in chickens will not only serve to improve the quality of production from an agricultural standpoint, but will also advance the knowledge of metabolic disorders in humans.

Four experimental lines of meat-type chickens that were divergently selected for either a large difference in abdominal (visceral) fatness or in growth rate were used to characterize the role of adipose tissue (classically thought to have a minimal lipogenic contribution) in regulating adiposity. At the age of selection (9 weeks), the fat line (FL) and lean line (LL) chickens exhibit a 2.5-fold difference in abdominal adipose weight, while their body weight and feed intake are similar. The high growth (HG) and low

growth (LG) chickens were divergently selected for either high (HG) or low (LG) body weight at 8 and 32 weeks of age resulting in a 2.7-fold increase in bodyweight and an 8-fold increase in abdominal fatness (as a percentage of BW) in HG chickens on average from 1 through 11 weeks. The adipose transcriptomes of these four genotypes (FL compared to LL and HG compared to LG) were analyzed at 1 through 11 weeks of age using the Del-Mar 14K Chicken Integrated Systems microarray, and at a single age (7 weeks) by RNA sequencing.

Microarray analysis of abdominal fat in FL and LL chickens revealed 131 differentially expressed (DE) genes (FDR≤0.05) as the main effect of genotype, 254 DE genes as an interaction of age and genotype and 3,194 DE genes (FDR≤0.01) as the main effect of age. The most notable discoveries in the abdominal fat transcriptome during juvenile development were higher expression of many genes involved in hemostasis in the LL and up-regulation of numerous adipogenic and lipogenic genes in FL chickens. Many of these DE genes belong to pathways controlling the synthesis, metabolism and transport of lipids or endocrine signaling pathways activated by adipokines, retinoids and thyroid hormones. The importance of these processes in regulating adiposity in abdominal fat of FL and LL chickens was reinforced by the deep RNA sequencing analysis at 7 weeks. Remarkably, the highest expressed genes at this age included those involved in the metabolism of lipid and carbohydrates which are functionally associated with endocrine system and metabolic disorders. There were 1,687 DE genes between fat and lean chickens at 7 weeks including transcription factors and metabolic enzymes which have direct influences on

lipogenesis and adipogenesis. The findings of the microarray analysis were further verified by the abundance of DE hemostatic factors uncovered by RNA sequencing analysis. This deep sequencing analysis also revealed a number of ectopically expressed genes suggesting that visceral fat functions autonomously as well as an endocrine organ in the regulation of lipid metabolism and perhaps feed intake.

Microarray analysis of HG and LG chickens at 1 through 11 weeks of age revealed DE genes (FDR≤0.05) as the main effect of genotype (321 genes), the interaction of age and genotype (718 genes), and the main effect of age (2,918 genes). RNA sequencing at 7 weeks uncovered 280 DE genes (FDR≤0.1). Similar to the FL, HG chickens over-express many genes involved in adipogenesis and lipogenesis (including biosynthesis of fatty acids, cholesterol and triglycerides) which could at least partially account for their increase in abdominal fatness. Conversely, LG chickens up-regulate several energy producing processes (i.e., peroxisomal β-oxidation, mitochondrial β-oxidation, ketogenesis and oxidative phosphorylation) early on in juvenile development which are likely responsible for their extreme leanness. Hemostasis also appears to have a critical role in the maintenance of the lean phenotype at the age of maximal difference in adiposity in these chickens (7 weeks). These findings validate abdominal fat as a major contributor to adiposity in response to either divergent selection on abdominal fatness in the FL and LL or body weight in the HG and LG chickens.

Chapter 1

BACKGROUND

The domestic chicken (*Gallus domesticus*) serves a dual purpose as a world-wide source of high-quality dietary protein and an important biological model. It was the first avian species and domestic animal selected for complete genome sequencing and assembly [1]. Subsequently, the chicken emerged as a premier model in animal agriculture [2-4] and developmental biology [5]. The chicken has been used to understand basic mechanisms controlling embryonic development, immune system function, nutrient utilization, hormone sensitivity, and adiposity. Although now recognized as a model organism for biomedical research [6], the chicken has not been extensively used for the study of human diseases, especially metabolic disorders (i.e., insulin resistance, diabetes, obesity and metabolic syndrome).

1.1 The Domestic Chicken as a Model of Obesity

1.1.1 Lipid breakdown and synthesis are conserved in chickens

The fundamental metabolic processes that regulate lipid breakdown and synthesis are conserved between avian and mammalian species. The mechanisms

required for the breakdown of carbohydrate and fatty acids for generation of energy during times of increased need are summarized in Figure 1.1 (reviewed in [7]).

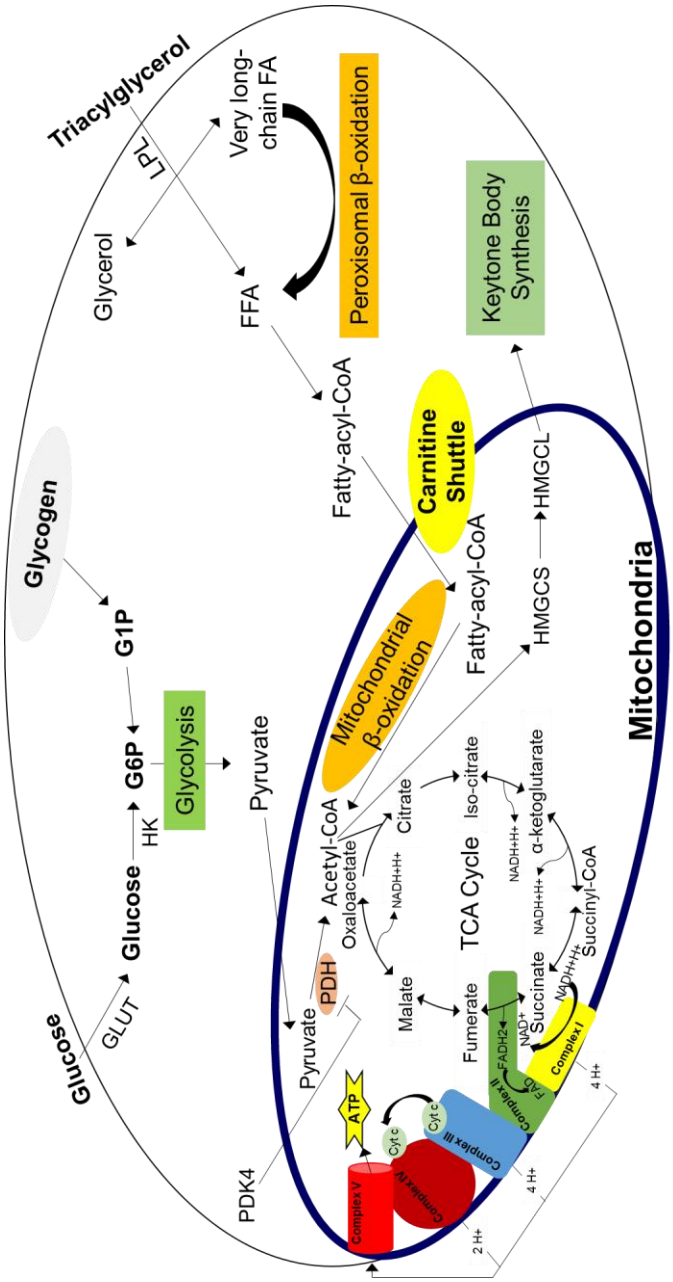

Figure 1.1 Summary of major energy producing mechanisms.

Briefly, in the metabolism of carbohydrates, glucose-6-phosphate produced from glucose (by hexokinase or glucokinase) or glycogen (including the glucose-1-phosphate intermediate) enters the glycolytic pathway for production of cytosolic pyruvate. Cytosolic pyruvate is then transported into the mitochondria via active transport where it is converted to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex (also producing the electron donor NADH).

Acetyl-CoA is also the end product of the breakdown of fatty acids which includes: transport of triglycerides into the cell, triglyceride cleavage to form free fatty acids, fatty acid activation, and β-oxidation. Lipoprotein lipase (LPL) has the critical roles of regulating triglyceride uptake into the cell and hydrolyzing triglycerides to free fatty acids (removal of the glycerol backbone). The fatty acids entering the cell are of different lengths. Fatty acids larger than C-22 (22 carbons) undergo peroxisomal β-oxidation (similar to mitochondrial β-oxidation described below) to enable transport into the mitochondria. These shortened fatty acid chains (along with those that did not undergo peroxisomal β-oxidation) are then activated for degradation by acyl-CoA ligase (thiokinase), which joins the fatty acid with coenzyme A, producing a fatty-acyl CoA. Fatty-acyl CoAs are then transported into the mitochondria for further degradation. This process is dependent on the carnitine shuttle which uses several enzymes [carnitine O-acetyltransferase (CRAT), carnitine palmitoyltransferase I (CPT1) and carnitine O-palmitoyltransferase 2 (CPT2)] to transport fatty acids as fatty

acyl-carnitine (produced by CPT1), which is converted back to fatty acyl-CoA (by CPT2) inside the mitochondria.

Once in the mitochondria fatty-acyl-CoA undergo an iterative series of four reactions (collectively termed mitochondrial β-oxidation) forming an acetyl-CoA with each iteration. A fatty-acyl-CoA of n carbons (given the number of C is even) will generate n/2 acetyl-CoAs. In the first step, acyl-CoA dehydrogenase (ACAD gene family) removes two hydrogen between carbon 2 and 3 which produces trans enoyl-CoA (and the electron donor FADH2). Enoyl-CoA hydratase (part of the tri-functional protein, HADHA/HADHB) then catalyzes the hydrolysis of trans enoyl-CoA to 3-L-hydroxyacyl-CoA. Hydroxyacyl CoA dehydrogenase (the second enzyme of the tri-function protein) removes two hydrogen forming 3-ketoacyl-CoA (and electron donor NADH). Finally, the terminal acetyl-CoA group is cleaved by beta-ketothiolase (thiolase; the third enzyme of the tri-functional protein) generating an acyl-CoA two carbons shorter and one acetyl-CoA.

The resulting acetyl-CoA will be coupled with oxaloacetate to form citrate in the first reaction of the citric acid (TCA) cycle. Citrate will then undergo three subsequent reactions for conversion to succinyl-CoA followed by four additional steps to convert succinyl-CoA back to oxaloacetate to start the cycle over. Steps 3, 4 and 8 each generate the electron donor NADH and step 6 produces FADH2. Alternatively, if the production of acetyl-CoA is too great, TCA cycle will be overwhelmed and the excess acetyl-CoA will be used in the generation of ketone bodies by the mitochondrial enzymes, thiolase, HMG-CoA synthase (HMGCS) and HMG-CoA

lyase (HMGCL). Of the three endogenous ketone bodies formed, acetoacetic acid and beta-hydroxybutyric acid are the most important as they are used as an energy source for the heart and/or brain when glucose is not available. The third endogenous ketone body, acetone, is released as waste.

The electron donors produced during the conversion of pyruvate to acetyl-CoA, the conversion of fatty acyl-CoA to acetyl-CoA and in TCA cycle enter the electron transport chain which includes five complexes located in the mitochondrial membrane. Complexes I-IV (NADH dehydrogenase, fumerate reductase, cytochrome c oxidoreductase and cytochrome c oxidase, respectively) use these electron donors in order to build a potential energy which Complex V (ATP-Synthase) uses to produce ATP.

When switching from high to lower energy requirements (e.g. exercise to rest), ATP concentrations will rise within the cell initiating the lipogenic mechanism [Figure 1.2 (reviewed in [7]). The increased concentration of ATP will effectively inhibit several steps of TCA cycle (i.e., alpha ketoglutarate dehydrogenase and isocitrate dehydrogenase) resulting in: 1.) the decreased production of electron donors for entry into oxidative phosphorylation, and 2.) the increased mitochondrial concentration of citrate. Citrate will then be transferred out of the mitochondria into the cytosol by the tricarboxylate transport system. ATP citrate lyase (ACLY) catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA, a reaction requiring ATP. This acetyl-CoA is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACACA), the first committed step of fatty acid synthesis. Malonyl-CoA is converted to palmitic

acid by fatty acid synthase (FASN), a multifunctional protein with seven distinct catalytic activities. Palmitic acid is a saturated fatty acid which can be used directly in the formation of triglycerides or it can be further elongated (initiated by the ELOVL proteins family) and/or desaturated by fatty acid desaturases (i.e., DEGS1, DEGS2, FADS1, FADS2, FADS6, SCD, SCD4, SCD5, etc.) producing essential fatty acids for storage or many other functions throughout the organism.

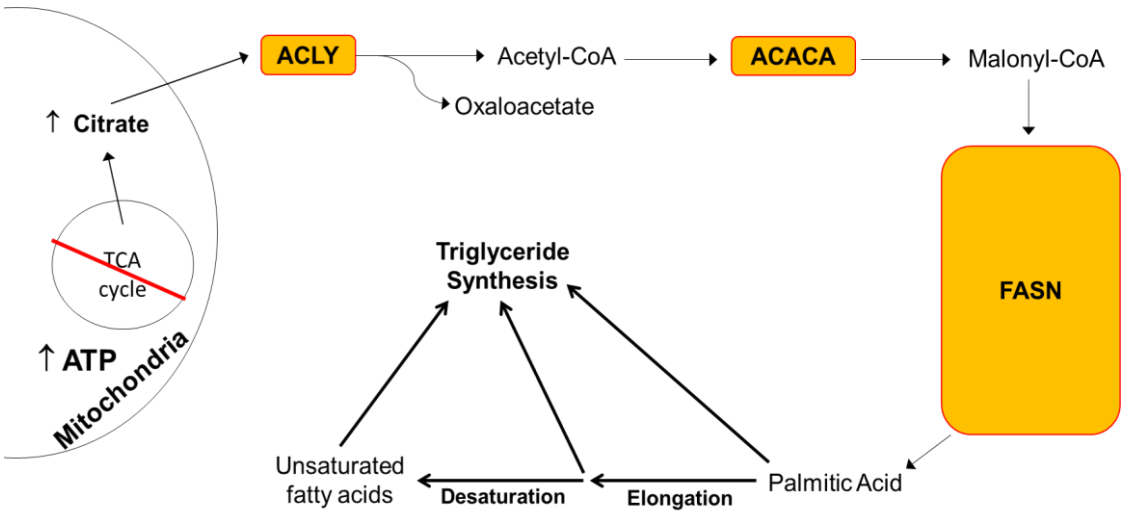

Figure 1.2 Summary of fatty acid and triglyceride synthesis.

1.1.2 Intriguing features of avian metabolism bolster the chicken as a model for metabolic disorders

While the anabolic and catabolic mechanisms controlling lipogenesis and lipid breakdown are conserved across species, the hormonal regulation of these processes is altered in chickens. These unique features of avian metabolism make the chicken an

interesting model for understanding the interactions between genetic and endocrine factors that contribute to the development of obesity and related metabolic disorders. In particular, chickens normally exhibit "hyperglycemia" [8, 9], insulin resistance [9-12], and hepatic *de novo* synthesis of lipids [13], while like humans [14], abdominal (visceral) fatness is a polygenic trait [15-20]. Despite their relative insensitivity to insulin, acute immuno-neutralization of insulin in the chicken provokes differential expression of more than a thousand genes in both liver and in skeletal muscle [21]. In contrast, only 69 genes were differentially expressed in abdominal fat of chickens following insulin immuno-neutralization, albeit short-term fasting produced a much larger change (1,780 genes) in transcription of abdominal fat genes [22]. This recent work also shows a rather large decrease in expression of lipogenic genes in abdominal fat of fasted chickens. A detailed examination of the insulin signaling cascade in adipose tissue of the chicken shows a distinct unresponsiveness to insulin [23]. Furthermore, numerous adipokines and metabolic enzymes which have been identified as important regulators of obesity in mammals have not been mapped to the current draft of the chicken reference genome (Gallus gallus v4.0). Some adipokines not mapped to the chicken genome include omentin (*ITLN1)*, leptin (*LEP*), plasminogen activator inhibitor 1 (*PAI-1*), resistin (*RETN)*, and tumor necrosis factor alpha (*TNFA*). The presence of chicken *LEP* is still an unresolved controversy [24-28]. Extensive expressed sequence tag (EST) [29] and whole genome sequencing projects have failed to identify a *bona fide LEP* gene. However, the leptin receptor (*LEPR*) gene is expressed in several chicken tissues [29-33]; and is capable of activating the JAK-

STAT pathway *in vitro* [34, 35]. Similarly, several components of TNF signaling are over expressed in the hypothalamus of lean chickens [33]. Despite the absence of several mammalian adipokines and metabolic enzymes (i.e., *LIPE*), adipogenesis and lipid metabolism in the chicken are regulated by mechanisms that are robustly similar to those described in mammals. This suggests that there are alternative methods for activating these signaling pathways in chickens, which may also be present in mammals, thus, intensifying the need to understand these processes. Collectively, these observations support the chicken as a unique model for the study of the genetic and biological mechanisms controlling obesity and the metabolic disease.

1.2 Our Models for Studying Fatness and Leanness

1.2.1 The fat line and lean line chickens

The fat line (FL) and lean line (LL) chickens are two experimental lines of meat-type chickens that were divergently selected over seven generations for either high (FL) or low (LL) abdominal (visceral) fatness [36, 37]. These chickens exhibit a 2.5-fold difference in abdominal fat weight at 9 weeks (wk) of age, albeit their body weight and feed intake are similar [38]. An image of the FL and LL chickens is presented in Figure 1.3 (note that there is little to no visual difference between the FL (right) and LL (left) individuals). The divergence of adiposity between the FL and LL chickens occurs at 3 wk of age [39]; hyperplasia of adipocytes was found as early as 2 wk of age in the FL [40], which was followed by marked hypertrophy of adipocytes

by 9 wk of age [39]. The FL chickens appear to favor partitioning of energy and nutrients into abdominal fat, whereas the LL deposit more nutrients into skeletal muscle, especially breast muscle [41]. A consistent feature of metabolism in the FL chickens is a glucose-insulin imbalance, where plasma glucose levels are lower and insulin levels are slightly elevated [12, 39].

Figure 1.3 The fat line (FL) and lean line (LL) chickens. Two 11 wk old cockerels from each the FL (right) and LL (left) are pictured.

Differential abundance of lipogenic genes in liver of the FL and LL chickens has been determined by differential mRNA display [42], quantitative RT-PCR [43, 44] and targeted low-density array [45]. A preliminary time course (1-11 wk) analysis of the liver transcriptome in the FL and LL chickens during juvenile development revealed 1,805 differentially expressed (DE) genes [3]. The higher rate of lipogenesis observed in liver of FL chickens contributes to a greater accumulation of abdominal

fat in this genotype regardless of age or nutritional state [46, 47]. Quantitative trait loci (QTL) analyses of a FL x LL intercross identified a major QTL for abdominal fatness at the distal end of chromosome 5 (*GGA*5) [17, 18, 48]. Furthermore, the expression quantitative trait loci (eQTL) analysis of *GGA*5, involving a three generation intercross of the FL x LL chickens, identified variations in expression of 660 hepatic genes that were correlated with abdominal fatness traits [20].

1.2.2 The high growth and low growth chickens

These growth models are populations of Rhode Island Red broiler chickens that were divergently selected at the Station de Recherches Avicoles, Institut National de la Recherche Agronomique (INRA), Nouzilly, France for either high (fast-growing line, HG) or low (slow- growing line, LG) body weight at 8 and 32 wks of age [49, 50]. Genetic selection for high growth rate (HG chickens) in these chickens is accompanied by extreme visceral fat accretion, whereas selection for slow growth rate (LG chickens) greatly diminishes abdominal fat mass. An image of the HG and LG chickens is given as Figure 1.4. It is remarkable that HG cockerels weigh nearly 3-fold more than the LG at 11 wk, as there are only subtle differences in physical appearance. The difference in weight between genotypes is a result of increased skeletal muscle and abdominal fat mass [49, 50]. The divergence in abdominal fatness in these birds is a phenotype that was incident to the selection for increased or decreased growth rate. This makes the HG and LG chickens an interesting model to compare to the FL and

LL chickens to elucidate the changes in transcription responsible for fatness or leanness.

Figure 1.4 The high growth (HG) and low growth (LG) chickens. Pictured on the right is a LG chicken and on the left is a HG chicken. Both cockerels are 11 wks old. The LG is ~1/3 the weight of the HG chicken.

Very few transcriptional studies have been completed on these HG and LG chickens. The most significant is the analysis of liver using the Del-Mar 14K Chicken Integrated Systems cDNA microarray, which uncovered 557 DE genes including transcripts for many metabolic enzymes, acute phase proteins, immune factors and transcription factors (Cogburn LA; manuscript in preparation). This study also determined that plasma levels of GH are increased in LG chickens throughout juvenile

development which appears to be hindered by ineffective binding of GH to hepatic GHR in LG chickens. Further, an F_2 intercross between HG and LG chickens at 7 and 9 wk identified 21 QTL including co-localized QTL for adiposity and glucose level (on GGA3 and GGA5), and for bodyweight, adiposity and body temperature (on GGA4) [51]. The QTL regions for glucose level and adiposity contained genes for which alleles have been associated with increased susceptibility to metabolic disorders in humans, suggesting that these chickens may be a good comparative model to provide insight into the genetic underpinnings of these diseases.

1.3 The Abdominal Fat Contribution to Adiposity

The liver has long since been referred to as the primary lipogenic tissue in birds, with a minor contribution from abdominal fat [13]. Thus, there have been a limited number of transcriptional studies of adipose tissue in the chicken. A comparison of abdominal fat was completed between meat-type (broiler) and egg-type chickens (layer) at a single physical age (10 wk) which corresponds with different physiological ages [52]. The authors focused attention only on the up-regulation of *LPL* in broiler chickens and higher expression of *APOA1* in layers. Another study using abdominal fat samples taken at 7 wk from a different population of divergently selected fat and lean chickens reported the differentially expression of 230 genes [53]. Their major conclusion that *TNFA* plays a key role in lipid metabolism of the chicken is surprising, since this adipokine has not been mapped to the chicken genome

sequence. While these studies have been relatively uninformative, recent studies have brought adipose tissue into light as a significant lipogenic tissue in both humans [54, 55] and in chickens [22]. For example, in a multi-phase dietary intervention in obese humans, some of the most remarkable changes in adipose tissue gene expression were seen in enzymes involved in unsaturated fatty acid synthesis [54]. In a recent study of adipose tissue in juvenile chickens, a short term period of fasting (5h) resulted in altered expression of 1,780 genes [22]. This relatively short period of fasting was sufficient to down regulate genes associated with fatty acid synthesis, elongation and desaturation. These findings coupled with the earlier observation of an insensitivity of avian adipose tissue to insulin [23] and the fact that chicken adipose is an endocrine organ which is void of several crucial adipokines (discussed above) establish that there is an intense need for deeper analysis of abdominal fat in chickens.

1.4 Important Processes Regulating Fatness and Leanness in Abdominal Fat

1.4.1 Ligand activated transcription factor signaling

A transcription factor is a protein that binds DNA and regulates the rate of transcription of a specific gene or set of genes. Nearly all processes are regulated by transcription factors and the interaction between transcription factors. Ligand activated transcription factor signaling is at the interface of environmental response and gene expression. For example, PPARG is naturally activated by long-chain fatty acids [56]

nitrolinoleic acid and prostaglandin J2 [57]. In the presence of these ligands, PPARG will either inhibit or activate the transcription of genes which have a PPARG response element in its promoter sequence. As another example, thyroid hormone responsive spot 14 (THRSP) is responsive to thyroid hormone and lipid derivatives [58, 59]. THRSP is a highly lipogenic transcription factor which has many lipogenic targets, thus activation of this gene is associated with fatness.

1.4.1.1 Retinoid metabolism, signaling and transcription factor activation

Retinol and its derivatives are a prime example of the crucial role in the regulation of adiposity through transcriptional activation and inhibition. Retinol or Vitamin A is a fat soluble regulator of cellular homeostasis. Vitamin A is taken in from the diet and is transported by retinol binding protein (RBP4) to the extracellular receptor STRA6 (on hepatic stellate cells and adipocytes) where it enters the cell to be stored primarily as retinyl palmitate or to be converted to retinoic acid. All retinol metabolites have a low solubility and low chemical stability so they must be transported by cellular binding proteins such as RBP7 and CRABP-I and II. In order to be an active ligand for transcription factor signaling, retinol must be converted to retinal and then into retinoic acid (Figure 1.5).

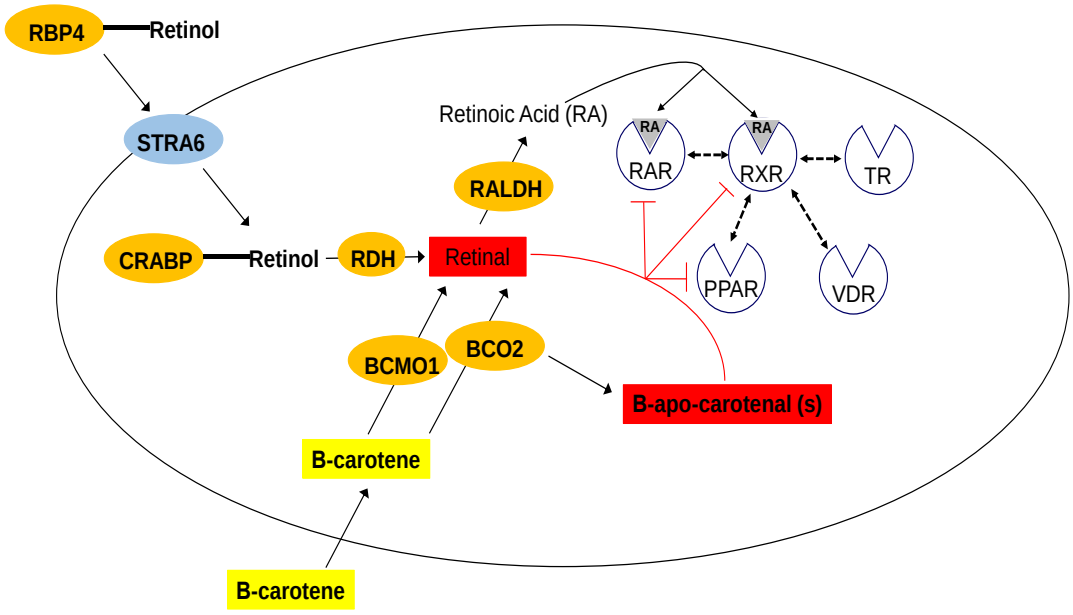

Figure 1.5 Retinol and β-carotene metabolism and signaling

Retinal can be obtained either through the reversible alcohol dehydrogenase dependent catabolism of retinol, or through the enzymatic conversion of beta-carotene (the major dietary retinoid source) by beta-carotene monooxygenase 1 and dioxygenase 2 (BCMO1 and BCO2 respectively). Enzymatic cleavage of beta-carotene by BCMO1 will yield retinal whereas cleavage by BCO2 will yield beta-apo-10'-carotenal which can be converted in to retinal or into beta-apo-14'-carotenal. Beta-apo-14'-carotenal and retinal can act up stream to block the activation of RXR and PPARG [61]. Recently, mutations in the proximal promoter of *BCMO1* we discovered were discovered, which are responsible for variation in the color of breast

meat in meat-type chickens [60]. Regardless of origin, retinal will be converted to retinoic acid (i.e., 9-cis, 13-cis and all-trans) by retinaldehyde dehydrogenases and serve as the ligand for two transcription factor receptors: retinoid X receptor and retinoic acid receptor (RXR and RAR). 9-cis retinoic acid is the natural ligand for RXR whereas all-trans-retinoic acid (ATRA) can ligate either RAR or RXR [62]. Upon ligand activation, RAR will form a homodimer, or a heterodimer with RXR, and the dimer will bind DNA to effect transcription of genes involved in a host of pathways. For example, RARA has been shown to cause the induction of genes involved in circadian rhythm and vasculature [63] and RARG is expressed lower in obese humans suggesting it could be associated with leanness [64]. The RXRs (alpha and gamma) are promiscuous nuclear transcription factors and can form homodimers or heterodimers with many different transcription factors (i.e., LXR, TR, PPAR, VDR, etc.). These dimers then bind DNA response elements to either induce or suppress the transcription of genes involved in many pathways including insulin signaling, thyroid hormone metabolism, vitamin d3 metabolism, and regulation of adiposity.

1.4.2 Hemostasis

Hemostasis is a well-defined process that causes blood flow to halt at the site of vascular injury followed by resuming normal activity once the injury is resolved. It is generally broken into three categories which occur simultaneously. Primary hemostasis begins with vasoconstriction of the vessel wall followed by platelet

adhesion, activation and aggregation ultimately resulting in the formation of a platelet plug. Secondary hemostasis results in the formation of fibrin (through the intrinsic and extrinsic coagulation pathways) which will be cross-linked to strengthen the platelet plug. Tertiary hemostasis involves the dissolution of the clot through the generation of plasmin. Hemostasis itself is a highly regulated process that involves a tight balance of the three categories of hemostasis. Importantly, the individual molecules within each of these categories have many functions outside of hemostasis, including the regulation of adiposity.

Obesity in humans is described as chronic low-grade inflammation where expression of hemostatic genes [e.g., serine peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (*PAI-1*), thrombin, fibrinogen and von Willebrand factor (VWF)] are positively associated with greater deposition of adipose tissue [65, 66]. Little is known about the expression of blood coagulation genes in visceral fat or their role in the development of adiposity in chickens. Hemostatic proteins have several functions including: 1.) removal of signal peptides, 2.) activation of zymogens, 3.) transport of enzymes, or 4.) degradation of active enzymes. Given that many adipokines are functionally and structurally similar to the classic coagulation factors and other hemostatic factors, it is possible that these proteases act on pre-pro-adipokines expressed in adipose tissue.

A prime example of proteolytic processing of adipokines is chemerin (or *RARRES2*), which links hemostatic processing to retinol/transcription factor regulation (see above). Chemerin is a recently discovered adipokine that regulates adipogenesis;

and chemerin can be transformed into a pro-inflammatory protein, a cell adhesion factor or an anti-inflammatory peptide, depending upon cleavage by specific proteases [67-69]. After removal of the N-terminal signal peptide, pro-chemerin is processed at the C-terminal end by serine proteases to generate an active pro-inflammatory adipokine, which can be cleaved further at its C-terminal end by cysteine proteases to generate an anti-inflammatory peptide [70]. Active chemerin appears to exert its action by binding its extracellular receptor *CMKLR1* on adipocytes and/or *CCRL2* on activated macrophages, which then forms an adhesive bridge between these two resident cells in adipose tissue during the inflammatory response [70]. Adipocyte-derived chemerin causes insulin resistance in skeletal muscle cells [71]; and as a secreted adipokine, chemerin regulates myogenesis by providing negative cross-talk between adipose tissue and skeletal muscle [72]. Consequently, chemerin functions as a chemokine for leukocytes, an adipokine that regulates angiogenesis, and a biomarker of metabolic syndrome and obesity in humans [73-75].

Adipose tissue also exhibits local and endocrine control of primary hemostasis including vasculature constriction and dilation which has important consequences on adipogenesis [76] and angiogenesis as well as the regulation of blood pressure [77]. This vascular regulation is highly complex and thus is controlled through several mechanisms. One of these mechanisms is the renin-angiotensin system (RAS), which was originally identified as a systemic regulator of blood pressure and it has now been determined that all components of this system are expressed in adipose tissue [78]. Angiotensin II (produced locally in adipose tissue or released into circulation from

other tissues and acting in adipose tissue) is the active ligand in the RAS which has both an involvement in lipid metabolism through regulating insulin signaling in adipose tissue [76, 79] and also plays an important role in the regulation of local and systemic blood pressure through the constriction of peripheral arterioles.

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Chapter 2

LONGITUDINAL MICROARRAY ANALYSIS OF ABDOMINAL FAT IN GENETICALLY FAT AND LEAN CHICKENS

2.1 Introduction

The models used for this study are two experimental lines of meat-type chickens that were divergently selected over seven generations for either high (fat line; FL) or low (lean line; LL) abdominal (visceral) fatness [1, 2]. These chickens exhibit a 2.5-fold difference in abdominal fat weight at 9 weeks (wk) of age, albeit their body weight and feed intake are similar [3]. This study serves a dual purpose to explore the abdominal fat transcriptome of juvenile FL and LL chickens and to identify major gene networks controlling adiposity and lipogenesis in these divergently selected models. Using the Del-Mar 14K Chicken Integrated Systems cDNA microarray, we took transcriptional snapshots of gene expression in abdominal fat across two genotypes and six ages during juvenile development (1-11 weeks of age). Interestingly, our transcriptional analysis revealed numerous differentially expressed (DE) genes that are involved in hemostasis, adipokine signaling, retinol metabolism, and the synthesis, oxidation, and transport of lipids. The liver is widely considered as

the major site of lipogenesis in chickens and other birds. However, the present transcriptional analysis of visceral fat has identified many lipogenic DE genes, including *FASN, SCD, SREBF1, SREBF2* and *THRSPA* that are expressed higher in FL chickens. The greater abundance of thrombogenic enzymes and related protease inhibitors in abdominal fat of the LL chickens suggests enhanced proteolytic processing of adipokines and other endocrine factors, with local and/or humoral actions, that could contribute to their leaner phenotype. Although abdominal fat is generally considered as a passive depot for lipids, the present descriptive study in FL and LL chickens supports the idea that it does contribute to lipid synthesis and serves as an endocrine organ, which liberates a host of adipokines and endocrine factors with intrinsic and/or extrinsic activity.

2.2 Methods

2.2.1 Animals and tissue collection

The birds were bred and raised at INRA UE1295 Pôle d'Expérimentation Avicole de Tours, F-37380 Nouzilly, France. At hatching, FL and LL cockerels were wing-banded and vaccinated against Marek's disease virus. Birds were reared together in floor pens (4.4 x 3.9 m) and provided *ad libitum* access to water and conventional starter feed for three weeks [3,050 kcal of metabolizable energy (ME)/kg and 22% protein] and thereafter with a grower ration (3,025 kcal ME/kg and 17.9% protein). Chicks were held under continuous light (24 h, 24LL) for the first two days after

hatching, followed by a 14 h light/10 h dark cycle (14L:10D) for the remainder of the experiment. Infrared gas heaters provided supplemental heat and ambient temperature was decreased weekly from 32° C at hatching until 22° C was reached at 3 wk of age. Eight birds from each genotype were randomly selected at six ages (1, 3, 5, 7, 9, and 11 wk), weighed, bled into heparinized syringes, and killed by cervical dislocation. Abdominal fat was quickly dissected and weighed; a sample was immediately snap frozen in liquid nitrogen and stored at -75° C until further processing. All animal procedures were performed under the strict supervision of a French government veterinarian and in accordance with protocols approved by the French Agricultural Agency, the Scientific Research Agency, and the Institutional Animal Care and Use Committees at INRA, Nouzilly, France. These procedures were also in compliance with the United States Department of Agriculture guidelines on the use of agricultural animals in research and approved by the University of Delaware Agricultural Animal Care and Use Committee.

2.2.2 Microarray analysis

Four birds per genotype and age were randomly selected from the total of eight birds sampled per genotype and age for microarray analysis of abdominal fat. Total cellular RNA was extracted from abdominal fat using guanidine thiocyanate and CsCl gradient purification [4], followed by a separate step for DNase I treatment. The RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies; Wilmington, DE). RNA integrity was examined using an RNA 6000 Nano Assay kit and the Model 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) to assess the quality of the RNA samples (RIN ≥ 9 considered acceptable). Twenty μg of total RNA was indirectly labeled using SuperScript Plus Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA). The first strand cDNA synthesis was performed in a 30 μl final volume containing 1x first-strand buffer, 5 μg of anchored oligo(dT_{20}), DTT, dNTP mix (including aminoallyl- and aminohexyl-modified nucleotides), 40 U of RNaseOUT and 800 U of SuperScript III reverse transcriptase with an incubation at 46° C for 3 h. The original RNA template was removed by NaOH hydrolysis, and followed by neutralization with HCl. The cDNA was purified using a low-elution volume spin cartridge (Invitrogen; Carlsbad, CA) and labeled with either Alexa Fluor® 555 or Alexa Fluor® 647 succinimidyl ester in the dark at room temperature for 2 h. After purification of labeled cDNA with a low-elution-volume spin cartridge, the efficiency of dye incorporation was determined using the Microarray Module on the NanoDrop ND-1000 spectrophotometer and the Base:Dye Ratio Calculator on the Invitrogen website [5].

Twenty-four Del-Mar 14K Chicken Integrated Systems microarrays (NCBI GEO Platform # GLP1731) were hybridized with 48 labeled samples using a balanced block design, where half of the birds from each genotype and age were labeled with Alexa Flour 647 (red dye) and the other half with Alexa Flour 555 (green dye; Figure 2.1).

Age (wk)	Red (F635)	Green (F532)	Red (F635)	Green (F532)	Red (F635)	Green (F532)	Red (F635)	Green (F532)
	Slide 74		Slide 75		Slide 76		Slide 77	
1	LL1	FL1	FL2	LL2	LL3	FL3	FL4	LL4
	Slide 62		Slide 63		Slide 64		Slide 65	
3	LL5	FL5	FL6	LL6	LL7	FL7	FL8	LL8
	Slide 66		Slide 67		Slide 68		Slide 69	
5	LL9	FL9	FL10	LL10	LL11	FL11	FL12	LL12
	Slide 19		Slide 20		Slide 21		Slide 22	
7	LL13	FL13	FL14	LL14	LL15	FL15	FL16	LL16
	Slide 58		Slide 59		Slide 60		Slide 61	
9	LL17	FL17	FL18	LL18	LL19	FL19	FL20	LL20
	Slide 54		Slide 55		Slide 56		Slide 57	
11	LL21	FL21	FL22	LL22	LL23	FL23	FL24	LL24

Figure 2.1 Experimental design for hybridization of 48 abdominal fat samples from FL and LL cockerels. Twenty-four microarrays were hybridized to 48 abdominal fat RNA samples (4 birds/genotype x 2 genotypes x 6 ages).

Hybridized slides were scanned using a GenePix 4000B scanner with GenePix Pro 4.1 software (Molecular Devices, Union City, CA) at wavelengths of 635 nm (Alexa 647-labeling) and 532 nm (Alexa 555-labeling) generating a combined TIFF image file for each slide. The laser power was set at 100% with the photomultiplier tube (PMT) setting being adjusted for each scan to produce a PMT count near unity. All slides were manually checked for quality and all spots with inadequacies in signal, background or morphology were eliminated. The image analysis results were merged with Excel files in GenePix Report (GPR) format, which contains clone identification, spot location on slide, and most current gene name/function (based on BLASTX/BLASTN score).

The microarray GPR files were analyzed as a linear model using the Limma (version 3.4.5) package in R (version 2.11.1) for analyzing microarray data [6]. Median intensities for each dye were Loess normalized (without background

subtraction) within array and between array ("Aquantile" method) to correct for dye and slide biases. A two-way ANOVA was used on Loess normalized intensity values to determine a main effect of genotype, main effect of age (each age compared to wk 1; total of 5 contrasts), and the interaction of age and genotype (A x G). The Benjamini-Hochberg procedure [7] was used to control the experiment wise false discovery rate (FDR) from multiple testing. The minimum information about microarray experiments (MIAME)-compliant microarray data were deposited into the NCBI Gene Expression Omnibus (GEO) under the accession number GSE37585.

2.2.3 Quantitative RT-PCR analysis

Candidate DE genes were selected for verification of expression by quantitative RT-PCR (qRT-PCR) analysis. First strand cDNA synthesis was performed by incubation of a 13 μl reaction volume (containing 1 μg of total DNase-treated RNA, 1 μl of 100 μM oligo dT_{20}, 1 μl of 10 mM dNTP mix, and water to 13 μl total volume) for 5 min at 70° C and then placed on ice for 2 min. A master mix containing 5 μl of 5x first-strand synthesis buffer, 1 μl of 0.1 M dithiothreitol (DTT), 1 μl of RNaseOUT, and 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) was added to the RNA in a final reaction volume of 20 μl. The cDNA was diluted to achieve a concentration of 50 ng/μl. Primers were designed for qRT-PCR using Primer Express v2.0 software (Applied Biosystems, Foster City, CA).

Detailed information for each primer pair including gene name, gene symbol, primer sequences (forward and reverse), accession number and amplicon size are provided in the Appendix.

The qRT-PCR assay was performed in an ABI Prism Sequence Detection System 7900HT, using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 400 nM of each primer (forward and reverse; Sigma-Aldrich, St. Louis, MO) in duplicate wells. Disassociation curves of each sample were analyzed to validate specific amplification and verify absence of primer dimers. PCR products were analyzed using agarose gel electrophoresis to compare approximate product size to expected amplicon size. The cycle time (Ct) for each sample was normalized to the corresponding sample geometric mean of three housekeeping genes [protein kinase, AMP-activated, beta 2 non-catalytic subunit (*PRKAB2*), protein kinase, AMP-activated, gamma 1 non-catalytic subunit (*PRKAG1*), and serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (*PAI-2* or *SERPINE2*)]. These housekeeping genes were selected using the RefFinder website [8] as the most stably expressed genes (i.e., genes with the lowest M-value) in the experiment. The $2^{-(\Delta\Delta Ct)}$ formula was used to calculate relative transcript abundance [9]. The statistical analysis was performed using a general linear model procedure in SAS v9.3. The data (log2 transformed normalized expression values) was analyzed using a two-factor analysis of variance to determine significant effects of genotype (G), age (A), and the interaction of age x genotype (A x G).

2.3 Results

2.3.1 Phenotypic measurements

Body weight (BW, kg), abdominal fat weight (g), and relative abdominal fat content (percent of body weight, %BW) in juvenile FL and LL chickens are presented in Table 2.1. The BW of FL and LL cockerels was similar for all ages between 1 and 11 wk. The absolute and relative abdominal fat weights of the FL chickens were 2.5-fold higher ($P \leq 0.05$) on average than those of the LL at all ages between 3 and 11 wk of age.

Table 2.1 Phenotypic measurements from juvenile FL and LL cockerels

	Age (wk)					
	1	3	5	7	9	11
Body weight (kg)						
FL	0.115	0.544	1.297	1.983	2.693	3.222
LL	0.123	0.551	1.204	1.964	2.787	3.281
Abdominal fat (g)						
FL	0.5	13*	38*	88*	124*	150*
LL	0.4	5 *	15*	31*	54*	59*
FL/LL ratio	1.2	2.6	2.5	2.8	2.3	2.5
Abdominal fat (%BW)						
FL	0.4	2.3*	2.9*	4.4*	4.6*	4.6*
LL	0.3	1.0*	1.2*	1.6*	1.9*	1.8*
FL/LL ratio	1.3	2.3	2.4	2.8	2.4	2.6

Values represent the least square means (LSMEANS) of eight birds/genotype and age with a common standard error (not shown). Significance (*) between FL and LL was determined at $P \leq 0.05$ using Fisher's least significance difference test.

2.3.2 Abdominal fat gene expression

Differentially expressed (DE) genes were defined as those having a significant [i.e., false discovery rate [FDR≤0.05 for genotype (G), age (A) and the interaction of A x G (FDR≤0.001 for A)] log2 expression ratio (FL/LL). Limma [10] accounted for main effects of genotype (344 DE genes) and age (3,235 DE genes) and their interaction (254 DE genes). The Venn diagram shows the intersection of these DE gene lists (Figure 2.2).

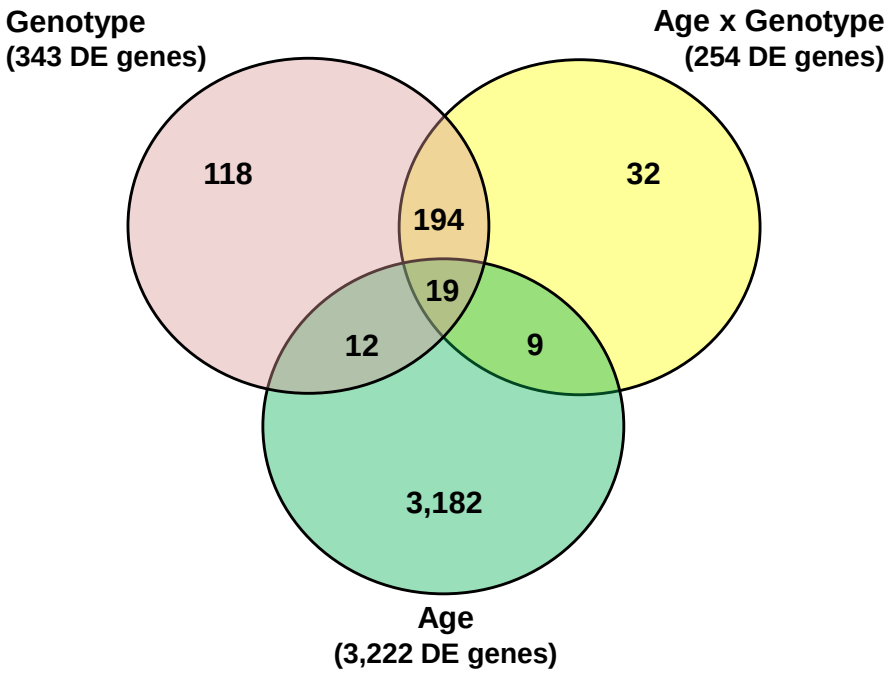


Figure 2.2 Venn diagram showing unique and shared genes among main effect of age (A) or genotype (G), and their interaction (A x G). This diagram shows the number of differentially expressed genes that are common across contrasts and those that are unique to the main effect of age ($P \leq 0.001$) or genotype ($P \leq 0.05$), and the interaction of age x genotype ($P \leq 0.05$).

The number of unique genes are indicated for the main effect of genotype (118 DE genes), age (3,182 DE genes) and the interaction of age x genotype (32 DE genes). There were 213 DE genes in common between the main effect of genotype and the age x genotype interaction. Thirty-one DE genes were shared between main effects of age and genotype, whereas 28 DE genes were in common between the main effect of age and the age x genotype interaction. Overall, 19 DE genes were found in common among all three effects.

2.3.3 Ingenuity Pathway Analysis (IPA) of differentially expressed gene sets

Significant genes (cDNA clone IDs) from the microarray analysis were annotated using the GeneBase tool on our website [11], which provides protein IDs (from GenBank or Swiss-Prot databases) of microarray cDNA probes derived from BLASTX analysis. Lists of DE genes containing the protein ID and log2 ratio for each gene were then submitted to the IPA knowledgebase [12] for functional annotation and mapping to canonical metabolic and regulatory pathways. “Analysis ready” genes were mapped by IPA for the genotype (100 DE genes), age (2,301 DE genes), and age x genotype interaction (212 DE genes) lists. The IPA Upstream Regulator Analysis was used to identify transcription factor (TF) interaction networks, predicted activation or inhibition of TF, and their direct targets from DE gene lists.

A summary of the IPA "Diseases and Disorders" category under "Biological Functions" is presented in Table 2.2. The subcategories of major interest were "Developmental Disorder" (33 genes), "Hereditary Disorder" (71 genes), "Inflammatory Disease" (7 genes, out of which 6 were up regulated in LL chickens), "Metabolic Disease" (41 genes), and "Organismal Injury and Abnormalities" (31 genes). A group of 33 genes were classified as "inborn error of metabolism" in three of the above subcategories (Developmental Disorder, Hereditary Disorder, and Metabolic Disease).

Table 2.2 Top biological functions identified by IPA analysis of the abdominal fat transcriptome in juvenile FL and LL chickens (1-11 weeks)

Diseases and Disorders	**P-Value**	**# Genes**	
Developmental Disorder	2.76E-07	33	
Hereditary Disorder	3.01E-06	71	
Inflammatory Disease	7.14E-06	11	
Organismal Injury and Abnormalities	4.51E-05	31	
Metabolic Disease	4.77E-05	41	
Molecular and Cellular Functions			
Lipid Metabolism	6.06E-05	46	
Small Molecule Biochemistry	6.06E-05	43	
Physiological System Development and Function			
Hematological System Development and Function	1.87E-05	34	
Organ Morphology	2.56E-05	7	
Renal System Development /Function	2.56E-05	10	
Embryonic Development	1.06E-04	23	
Cardiovascular System Function	1.75E-04	8	
Top Canonical Pathways	**P-Value**	**Genes**	**Ratio**
Coagulation System	2.56E-08	(7/38)	0.184
Intrinsic Prothrombin Activation Pathway	1.75E-04	(6/34)	0.176
Extrinsic Prothrombin Activation Pathway	4.65E-04	(3/20)	0.15
Acute Phase Response Signaling	5.11E-08	(15/178)	0.08

Ingenuity Pathway Analysis (IPA) software was used for functional annotation and mapping of the G and A x G lists of DE genes to gene interaction networks and to canonical metabolic/ regulatory pathways.

One gene interaction network identified by IPA was heavily populated with a large number of hemostatic genes, which were up regulated in abdominal fat of the LL chickens (Figure 2.3). These genes are involved in coagulation [*F2*, *A2M*, carboxypeptidase B2 (*CPB2*), fibrinogen alpha (*FGA*), *PLG*, protein C (*PROC*) and serine peptidase inhibitor, clade D, member 1 (*SERPIND1*)] and inflammation [*CD163* and retinoic acid receptor responder 2 (*RARRES2*) or chemerin]. Another group of DE genes [taste receptor, type 1, member 1 (*TAS1R1*), motilin receptor (*MLNR*), vasoactive intestinal peptide receptor 1 (*VIPR1*), and omega-3 fatty acid receptor 1 (*O3FAR1*)] are G-coupled receptors linked through the chemokine ligand *CXCL12*. Three genes shown in this pathway are related to steroid metabolism [hydroxysteroid (17-β) dehydrogenase 2 (*HSD17B2*) and hydroxysteroid (17-β) dehydrogenase 7 (*HSD17B7*)] and action [nuclear receptor subfamily 5, group A, member 1 (*NR5A1*)]. The transcription factor HNF1A regulates several hemostatic genes (*FGA, PLG, PROC* and *SERPINA1*) in visceral fat of LL chickens. PPARG directly regulates three adipokines (*RARRES2, RBP4* and *SERPINA1*) expressed at higher abundance of LL and several additional genes up regulated in the FL (*AGT, CTSL1, MCM7, SIM1, TGFBR1* and *TUBB*), including three metabolic enzymes (*FASN*, *PDE3B* and *PYGL*).

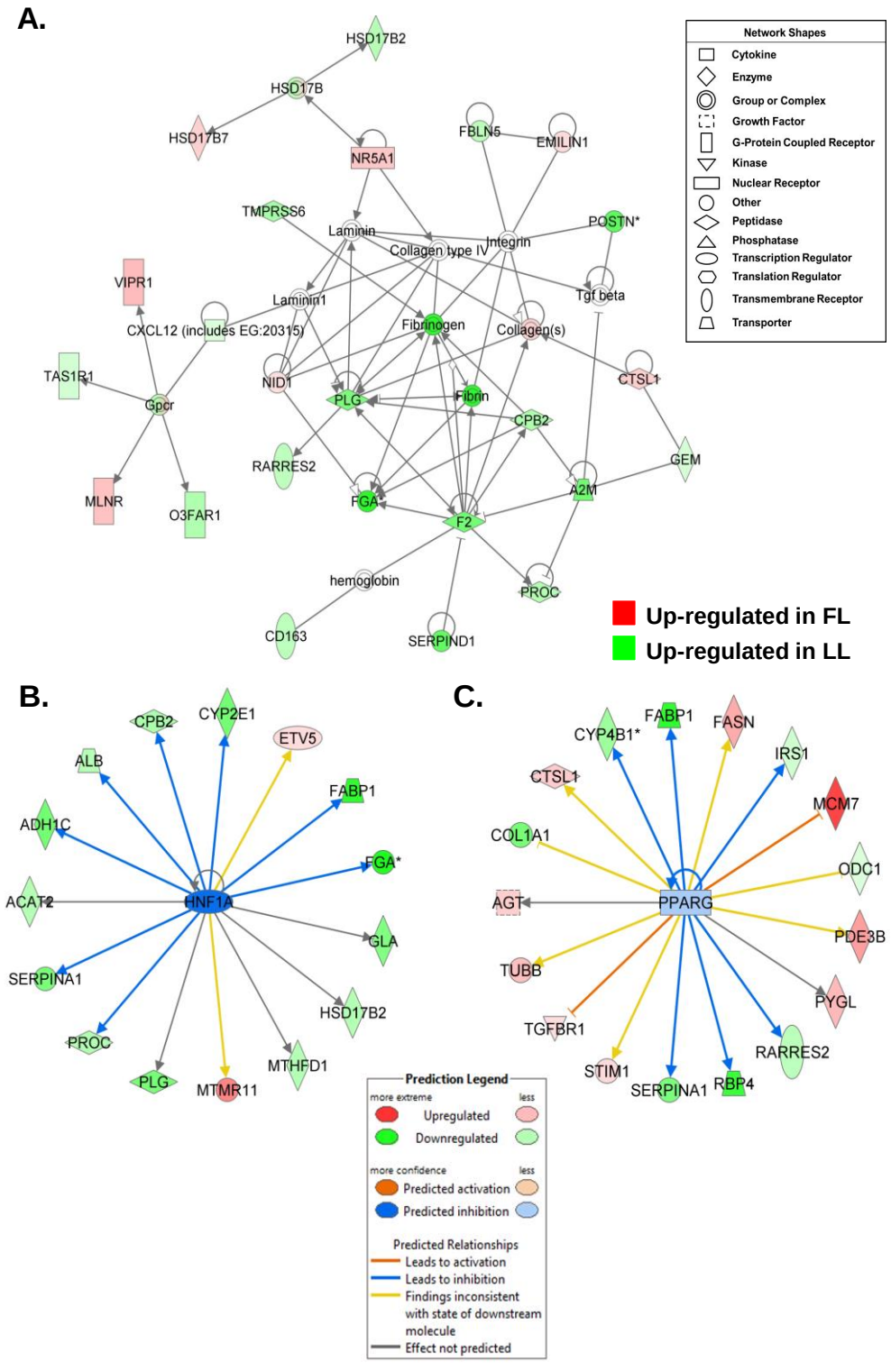

A.
HSD17B2
HSD17B
HSD17B7
NR5A1
FBLN5
EMILIN1
TMPRSS6
Laminin
Collagen type IV
Integrin
POSTN*
VIPR1
Laminin1
Tgf beta
CXCL12 (includes EG:20315)
Fibrinogen
Collagen(s)
TAS1R1
NID1
CTSL1
Gpcr
PLG
Fibrin
CPB2
RARRES2
GEM
MLNR
O3FAR1
FGA
A2M
F2
hemoglobin
PROC
CD163
SERPIND1
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Up-regulated in FL
Up-regulated in LL
B.
CPB2
CYP2E1
ETV5
ALB
FABP1
ADH1C
FGA*
ACAT2
HNF1A
GLA
SERPINA1
HSD17B2
PROC
MTHFD1
PLG
MTMR11
C.
CYP4B1*
FABP1
FASN
IRS1
CTSL1
MCM7
COL1A1
ODC1
AGT
PPARG
PDE3B
TUBB
PYGL
TGFBR1
RARRES2
STIM1
SERPINA1
RBP4
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted

Figure 2.3 Gene interaction network in abdominal fat of LL chickens associated with hemostasis. Functional gene interactions networks were identified by Ingenuity Pathway Analysis (IPA) software. This network shows direct gene interactions related to "Hematological System Development and Function" (A.). The IPA Upstream Regulator Analysis identified transcription factors with direct actions on differentially expressed target genes. This analysis of upstream regulators (based on expected responses from literature and observed responses in the data set) predicts inhibition (higher expression in LL chickens; blue color) of hepatic nuclear factor 1A (HNF1A) (B.) and peroxisome proliferator-activated receptor gamma (PPARG) (C.). Red gene symbols indicate higher expression in the FL and green gene symbols indicate higher expression in the LL.

The higher expression of select hemostatic genes found in abdominal fat of LL chickens was verified by qRT-PCR analysis (Figure 2.4): serine proteases [*F2*, coagulation factor IX (*F9*) and protein C (*PROC*)] and protease inhibitors [*A2M*, annexin A5 (*ANXA5*), and *SERPIND1*]. Thrombin (*F2*) was more abundant in abdominal fat of the LL at all ages, except 11 wk. The expression of *PROC* was 3-fold higher in the LL at 1 and 3 wk, and over 10-fold higher at 5 wk. The coagulation factor *F9* (Christmas factor) was over expressed in visceral fat of the LL by 3-fold, 24-fold, and 29-fold at 1, 5 and 7 wk, respectively. The expression patterns of two serine proteases (*F9* and *PROC*) were similar with the greatest differences at 1 and 5 wk.

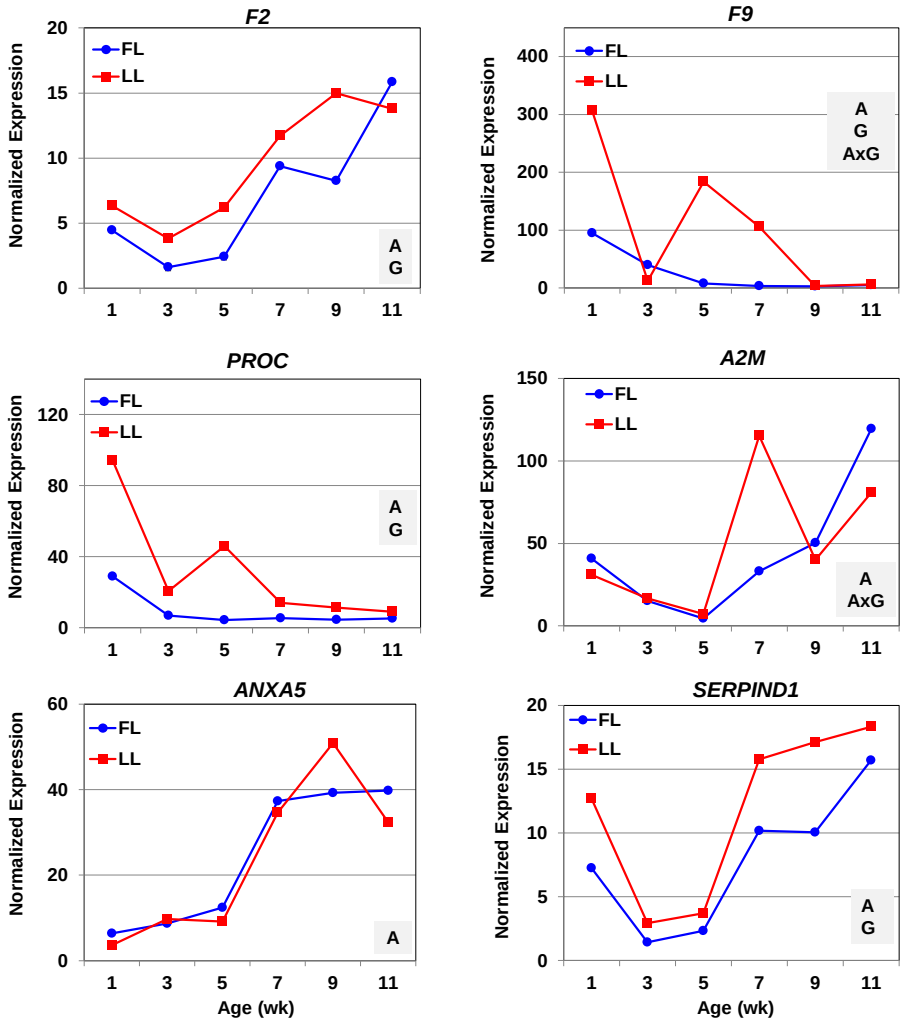


Figure 2.4 Verification of differential expression of hemostatic genes by qRT-PCR analysis. The abundance of six genes associated with hemostasis was determined by qRT-PCR analysis. Each data point represents LSMEANS (n = 4/genotype) of normalized expression values. A two-factor ANOVA was used to determine significance ($P \leq 0.05$). The shaded box in each panel indicates a significant main effect of age (A), or genotype (G) and interaction of age x genotype (A x G).

The qRT-PCR analysis shows similar expression patterns between some hemostatic factors and adipokines (Figure 2.5). For example, the expression of *ANXA5*, *F2*, adiponectin (*ADIPOQ*), adiponectin receptor 1 (*ADIPOR1*) and attractin (*ATRN*) were highest in abdominal fat of the LL at 9 wk. Similarly, expression profiles of *A2M*, retinol binding protein 4 (*RBP4*) and angiopoietin-like 4 (*ANGPTL4*) were greatest in the LL at 7 wk. The adipokine visfatin [or nicotinamide phosphoribosyltransferase (*NAMPT*)] was not differentially expressed in adipose tissue of juvenile FL and LL chickens. Both *ADIPOQ* and *ANGPTL4* were identified in the main effect of age (A) by microarray analysis, although the log2 expression ratios were only slightly higher in the FL. The qRT-PCR analysis shows that the expression of *ADIPOQ* was higher ($P \leq 0.05$) in the LL between 7 and 11 wk of age, while the abundance of *ANGPTL4* was elevated at 1, 5, 7 and 11 wk of age, albeit only age (A) produced a significant main effect.

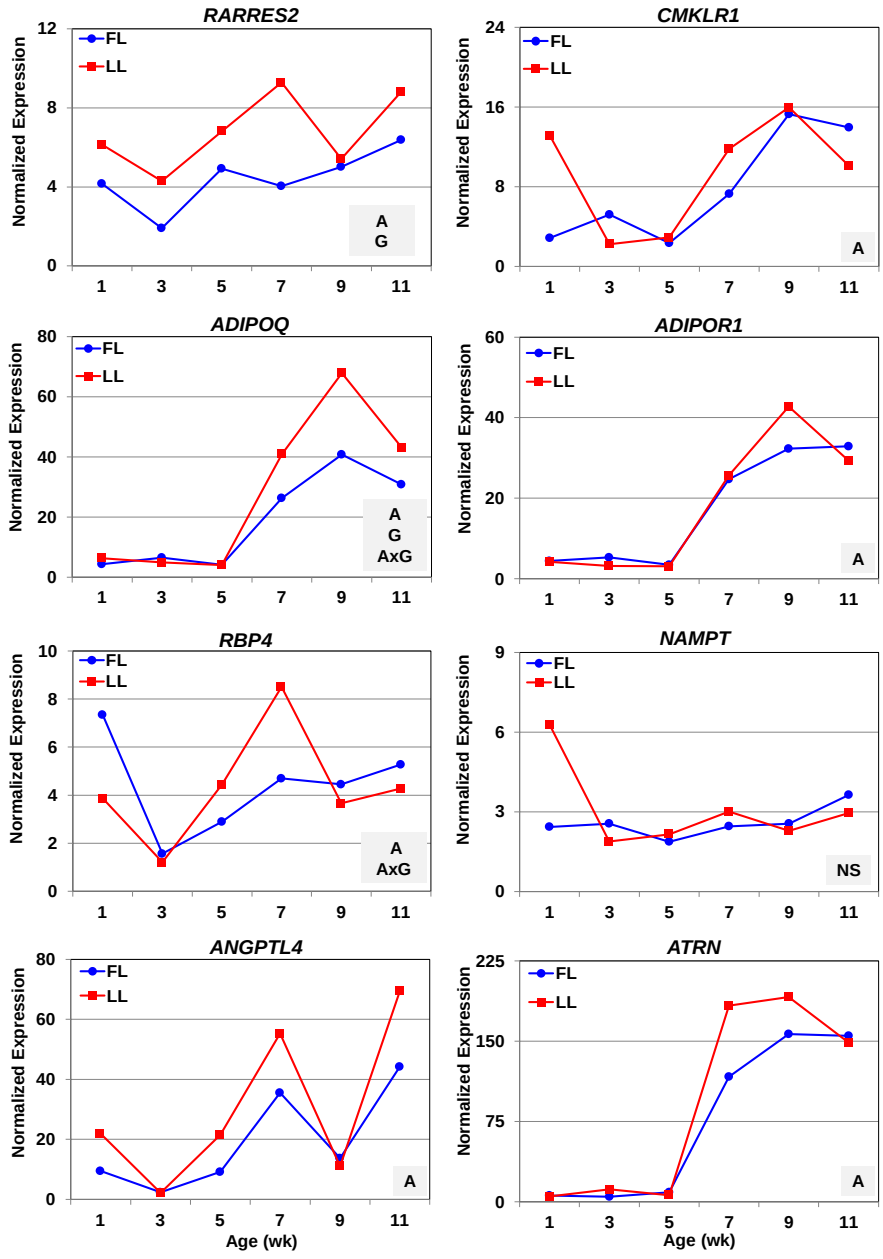


Figure 2.5 **Verification of differential expression of adipokine genes by qRT-PCR analysis.** The abundance of eight adipokines was determined by qRT-PCR analysis. Data points represent LSMEANS (n = 4/genotype) of normalized expression values. A two-factor ANOVA was used to determine significance ($P \leq 0.05$). The shaded box in each panel indicates a significant main effect of age (A) or genotype (G), and the interaction of age x genotype (AxG).

The top canonical pathways identified by IPA (Table 2.2) reflect the prevalence of hemostatic genes in adipose tissue of LL chickens. The IPA software provided functional assignments of DE genes to "Coagulation System" (7 genes), "Acute Phase Response Signaling" (15 genes) and "Intrinsic Prothrombin Activation" (6 genes) pathways. These adipose genes include serine proteases [*F2*, *PLG*, *PROC*, and complement factor B (*CFB*)], protease inhibitors [*A2M*, serine peptidase inhibitor clade A member 1 (*SERPINA1*), and *SERPIND1*] and transporters of retinol [retinol binding protein 4 (*RBP4*) and 7 (*RBP7*)]. The IPA functional category "Lipid Metabolism" shows high representation of numerous genes involved in "oxidation of lipid" (11/12 genes higher in LL chickens), "transport of lipid" (9/12 genes higher in LL chickens), "synthesis of lipid" (18/37 genes up regulated in FL chickens) and "metabolism of retinoid" (5/5 genes higher in LL chickens).

2.3.4 Higher expression of lipogenic genes in adipose tissue of FL chickens

The abdominal fat of FL chickens exhibits higher expression of lipogenic transcription factors [sterol regulatory element binding transcription factor 1 (*SREBF1*), thyroid hormone responsive Spot 14 protein (*THRSP*) and sirtuin 2 (*SIRT2*)] (Figure 2.6-A). Several other regulators of transcription [THRSP-like (THRSPL) or MID1 interacting protein 1 (*MID1IP1*); the nuclear liver X receptor-β (*LXRβ* or *NR1H2*); and the proto-oncogene *jun* (*JUN*)] were more abundant in abdominal fat of the LL. As shown in this IPA gene interaction network, *SREBF1*

directly up regulates several genes in the FL that are involved in lipid biosynthesis [*FASN*, stearoyl-CoA desaturase (*SCD*), fatty acid desaturase 2 (*FADS2*), sterol-C5-desaturase (*SC5DL*), mevalonate decarboxylase (*MVD*), 7-dehydrocholesterol reductase (*DHCR7*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) and lanosterol synthase (*LSS*)] and ketogenesis [3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*)]. Some of these genes are also targets of *SIRT2* and *THRSPA* and differentially expressed in adipose tissue of the FL. In addition, *SREBF1* directly affects numerous genes that are expressed higher in the LL [fatty acid desaturase 1 (*FADS1*), acetyl-CoA carboxylase alpha (*ACACA*), acetoacetyl-CoA synthetase (*AACS*), farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*), solute carrier family 2 (*SLC2A2*; or facilitated glucose transporter 2, *GLUT2*), succinate-CoA ligase, alpha subunit (*SUCLG1*), and phosphomevalonate kinase (*PMVK*)]. Two *JUN* targets, prostaglandin D2 synthase (*PTGDS*) and *MID1IP1* (which regulates transcription of *ACACA*), were over-expressed in adipose tissue of the LL. Insulin-like growth factor binding protein 4 (*IGFBP4*) is another target of *JUN* that was expressed at higher levels in FL adipose tissue. The IPA Upstream Regulator Analysis predicts that JUN and SREBF1 lead to activation (indicated by orange arrows) of numerous up-regulated target genes (red symbols) in abdominal fat of the FL chickens (Figure 2.6-B).

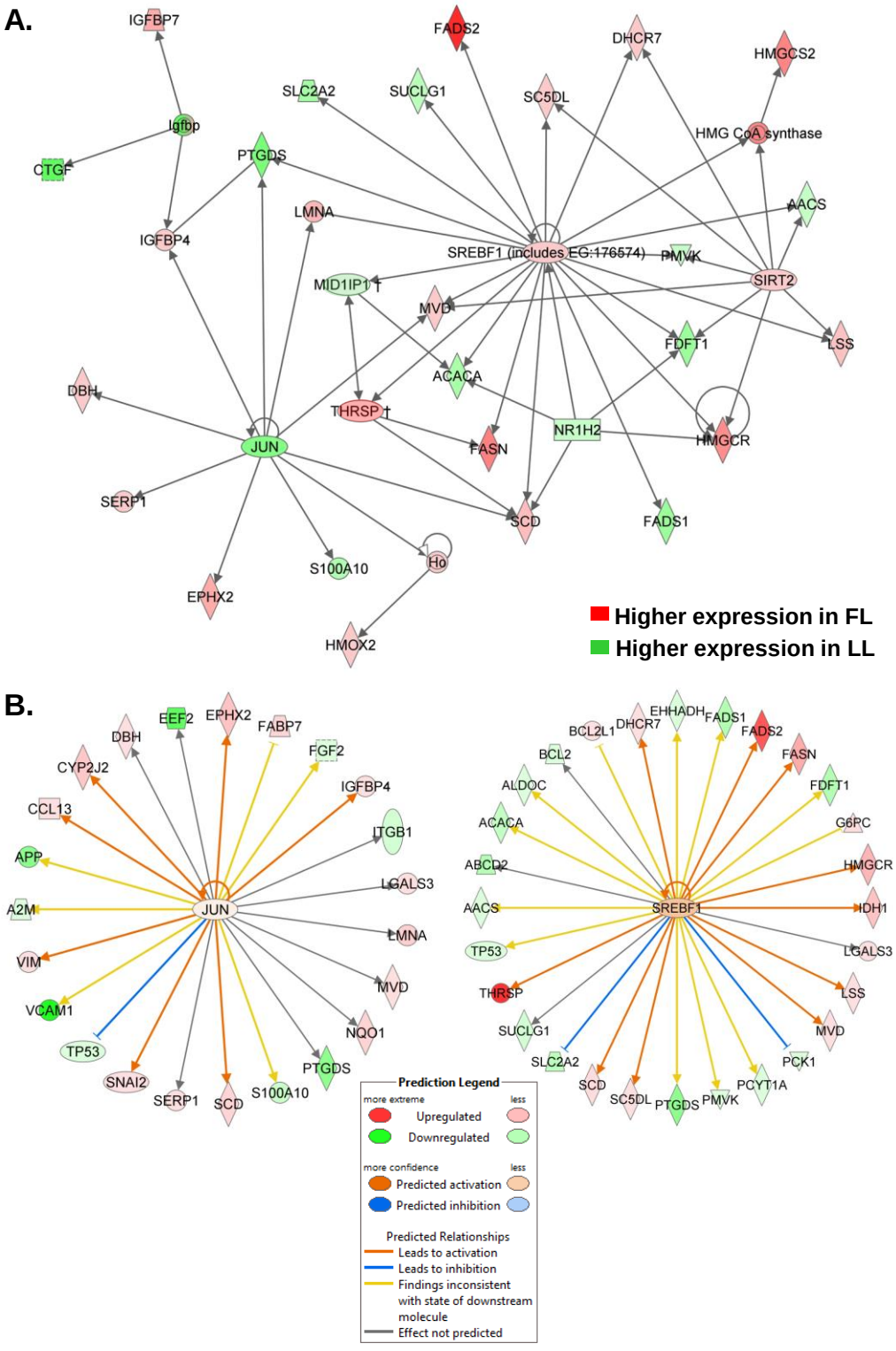

A.
IGFBP7
FADS2
DHCR7
HMGCS2
SLC2A2
SUCLG1
SC5DL
Igfbp
HMG CoA synthase
CTGF
PTGDS
LMNA
AACS
IGFBP4
SREBF1 (includes EG:176574)
PMVK
MID1IP1
SIRT2
MVD
FDFT1
LSS
ACACA
DBH
THRSP
NR1H2
HMGCR
JUN
FASN
SERP1
SCD
FADS1
S100A10
Ho
EPHX2
HMOX2
Higher expression in FL
Higher expression in LL
B.
EEF2
EPHX2
FABP7
DBH
FGF2
CYP2J2
IGFBP4
CCL13
ITGB1
APP
LGALS3
A2M
JUN
LMNA
VIM
MVD
VCAM1
NQO1
TP53
PTGDS
SNAI2
SERP1
SCD
S100A10
EHHADH
FADS1
BCL2L1
DHCR7
FADS2
BCL2
FASN
ALDOC
FDFT1
ACACA
G6PC
ABCD2
HMGCR
AACS
SREBF1
IDH1
TP53
LGALS3
THRSP
LSS
SUCLG1
MVD
SLC2A2
PCK1
SCD
SC5DL
PTGDS
PMVK
PCYT1A
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted

Figure 2.6 Transcriptional regulation of gene interaction network in abdominal fat of FL and LL chickens controlling lipogenesis. Functional gene interactions and up-stream regulators were identified by IPA (gene symbols and color schemes as described in Figure 1.3). Direct interactions were found among transcription regulators [JUN, SREBF1, SIRT2, MID1IP1, NR1H2 (LXRB) and THRSP] and lipogenic genes (A.). THRSP† was added to this network based on qRT-PCR analysis. This analysis of upstream regulators predicts activation of JUN (B.) and sterol response element binding factor 1 (SREBF1), leading to regulation of DE target genes. Gene symbol color indicates higher expression in the FL (red) or higher expression (green) in the LL.

The expression profiles of eight genes mainly associated with lipid metabolism were examined by qRT-PCR analysis (Figure 2.7). A main effect ($P \leq 0.05$) of genotype (G) was observed for *FASN* (4-fold increase in FL at wk 7), *SCD* (4-fold and 3-fold increase in FL at wk 3 and 7, respectively), and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*, over expressed in LL chickens from 7 to 11 wk). A significant age by genotype (A x G) interaction ($P \leq 0.05$) was observed for facilitated glucose transporter, member 1 (*GLUT1*), perilipin 2 (*PLIN2*) and lipoprotein lipase (*LPL*). A main effect of age (A; $P \leq 0.05$) was also observed for *FASN*, *GLUT1*, *PLIN2*, *PDK4*, *LPL*, facilitated glucose transporter, member 8 (*GLUT8*) and superoxide dismutase 3 (*SOD3*).

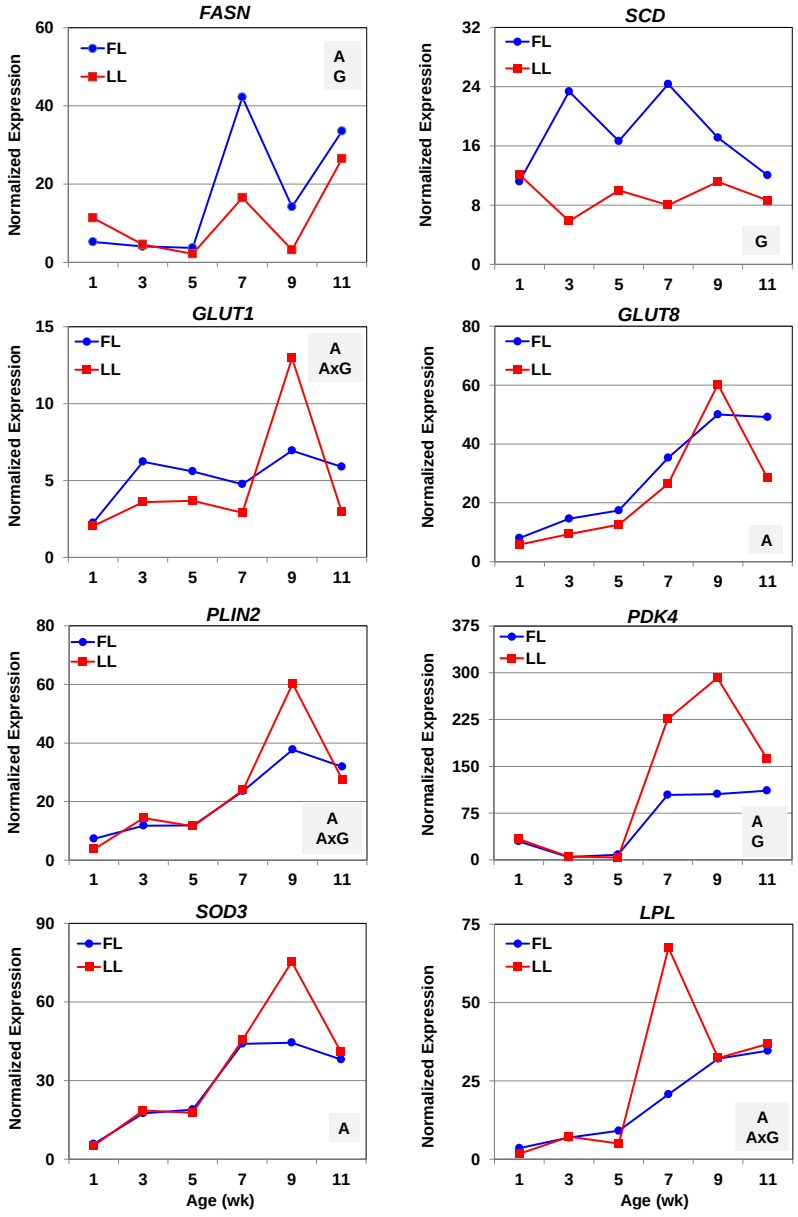


Figure 2.7 Verification of differential expression of genes associated with lipid metabolism by qRT-PCR analysis. mRNA expressions of eight genes involved in lipid metabolism were determined by quantitative reverse transcription PCR (qRT-PCR). Each data point represents LSMEANS (n = 4/genotype) of normalized expression values. A two-factor ANOVA was used to determine significance ($P \leq 0.05$). The shaded box in each panel indicates a significant effect of age (A) or genotype (G), and the interaction age x genotype (AxG).

Another network populated by numerous DE genes (up regulated in the FL) that control lipid metabolism shows the interaction of four transcription regulators (*SIRT2, PPARD, EGR1* and *CUX1*), also up regulated in visceral fat of the FL (Figure 2.8-A). Peroxisome proliferator-activated receptor delta (*PPARD*) interacts directly with patatin-like phospholipase domain containing 2 (*PNPLA2*), long chain acyl-CoA dehydrogenase (*ACADL*), aminoacylase 1 (*ACY1*), aldehyde dehydrogenase 2 (*ALDH2*), peroxiredoxin 6 (*PRDX6*), fatty acid binding protein 7 (*FABP7*), sorbitol dehydrogenase (*SORD*) and chemokine (C-C motif) ligand 13 (*CCL13*). Early growth response 1 (*EGR1*) interacts with *CCL13* and 3-hydroxy-3-methylglutaryl CoA reductase (*HMGCR*), the rate-limiting enzyme in biosynthesis of cholesterol, which is a target of the histone deacetylase sirtuin 2 (SIRT2). The ketogenic enzyme 3-hydroxy-3-methylglutary-Coenzyme A synthase 2 (*HMGCS2*) is a downstream target of both SIRT2 and PPARD. Three additional metabolic enzymes [lanosterol synthase (LSS) and 7-dehydrocholesterol reductase (DHCR7)] were also expressed at higher levels in the FL, while acetoacetyl-CoA synthetase (*AACS*), fatty acid desaturase 1 (*FADS1*), and phosphomevalonate kinase (*PMVK*) were more abundant in abdominal fat of the LL. The Ingenuity Upstream Regulator Analysis identified several additional targets of PPARD (Figure 2.8-B), which were expressed at higher levels in either the FL (*FASN*, *FLT1*, *SCD* and *TLR5*) or the LL (*ACAA*, *APOA4*, *BCL2*, *GPD2*, *SLC27A1*, *SLC27A2*, *UCP3* and *VLDLR*) chickens.

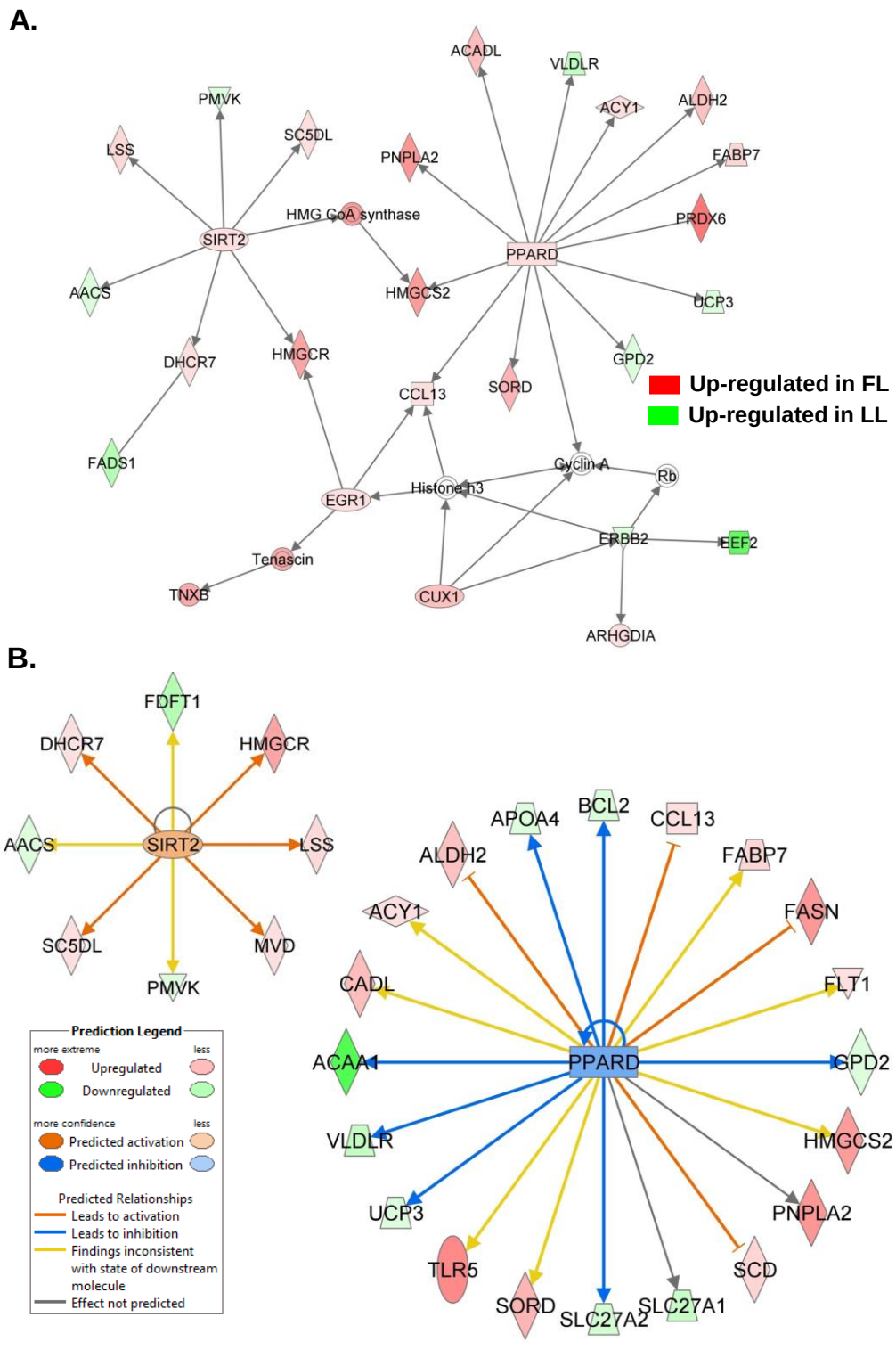

A.
ACADL
VLDLR
PMVK
ACY1
ALDH2
SC5DL
LSS
PNPLA2
FABP7
HMG CoA synthase
PRDX6
SIRT2
PPARD
AACS
HMGCS2
UCP3
DHCR7
HMGCR
GPD2
CCL13
SORD
Up-regulated in FL
Up-regulated in LL
FADS1
Cyclin A
Rb
Histone h3
EGR1
ERBB2
EEF2
Tenascin
TNXB
CUX1
ARHGDIA
B.
FDFT1
DHCR7
HMGCR
AACS
SIRT2
LSS
SC5DL
MVD
PMVK
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
BCL2
APOA4
CCL13
ALDH2
FABP7
ACY1
FASN
CADL
FLT1
ACAA1
PPARD
GPD2
VLDLR
HMGCS2
UCP3
PNPLA2
TLR5
SCD
SORD
SLC27A2
SLC27A1

Figure 2.8 Transcriptional regulators of differentially expressed genes controlling lipogenesis in abdominal fat of FL and LL chickens. A large number of DE lipogenic genes interact with two transcriptional regulators, SIRT2 and PPARD (A.). The IPA Upstream Regulator Analysis (B.) predicts that the up-regulation of SIRT2 leads to activation of five lipogenic genes (orange-edged arrows), whereas, the predicted inhibition of PPARD would lead to down regulation (blue-edged arrows) of seven DE target genes in the FL or up-regulation (green gene symbols) in the LL. The activation of major lipogenic genes (ALDH2, CCL13, FASN and SCD) would be blocked by PPARD in LL chickens.

The IPA software predicts that PPARD is inhibited (higher expression in LL chickens; blue color) based on prior knowledge of PPARD action in mammals and the observed higher expression of PPARD-activated targets in the LL (green symbols). IPA predicts that SIRT2 is activated (higher expression in FL chickens; orange color) and has a direct positive action on five target genes (*DHCR7, HMGCR, LSS, MVD* and *SC5DL*), which were up regulated in adipose tissue of the FL chickens.

2.3.5 Ligand activated nuclear receptors and other transcription factors

Of special interest are genes involved in ligand-activated gene transcription (e.g., retinol and thyroid hormone signaling) which regulate lipid metabolism (Table 2.3). Functional annotation of DE genes by IPA analysis identified five genes [alcohol dehydrogenase 1C (*ADH1C*), alcohol dehydrogenase 5 (*ADH5*), cytochrome P450, family 2, subfamily E, polypeptide 1 (*CYP2E1*), *RARRES2* and *RBP4*] related to "metabolism of retinoid". An additional four retinol-related genes [*RBP7,* nucleolar

protein 7 (*NOL7*), transthyretin (*TTR*) and retinol dehydrogenase 1 (*RDH1*)] were found by microarray analysis as higher expressed in LL chickens.

Table 2.3 Major categories of functional genes identified in abdominal fat of FL and LL chickens

Functional Category	Symbol	Gene Name	Microarray (FC)*	qRT-PCR (FC)*
Hemostasis	*A2M*	Alpha-2-macroglobulin	-1.89	-1.1
	AGT	Angiotensinogen	1.2	-
	ANG	Angiogenin	-2.51	-
	CFB	Complement factor B	-1.49	-
	CPB2	Carboxypeptidase B2	-1.43	-
	CPM	Carboxypeptidase M	-1.32	-
	F2	Thrombin	-1.85	-1.35
	F9	Christmas factor	-1.51	-4.04
	FGA	Fibrinogen alpha	-2.61	-
	PLG	Plasminogen	-1.79	-
	PROC	Protein C	-1.39	-3.54
	SERPINA1	Antitrypsin	-1.75	-
	SERPIND1	Heparin cofactor	-2.00	-1.5
	THBS2	Thrombospondin 2	-1.17	-
Adipokines	*ADIPOQ*	Adiponectin	1.03	-1.48
	ANGPTL4	Angiopoietin-like 4	1.01	-1.58
	ATRN	Attractin	-1.12	-1.22
	CFD	Adipsin	1.24	-
	LPL	Lipoprotein lipase	-	-1.41
	NAMPT	Visfatin	-	-1.2
	RARRES2	Chemerin	-1.32	-1.54
	RBP4	Retinol binding protein 4	-2.33	-1.11
	APOE	**Apolipoprotein E**	**Unidentified in Birds**	
	CCL2	**Chemokine (C-C motif) ligand 2**	**Unidentified in Birds**	
	ITLN1	**Omentin**	**Unidentified in Birds**	
	LEP	**Leptin**	**Unidentified in Birds**	
	PAI-1	**Plasminogen activator inhibitor type 1**	**Unidentified in Birds**	
	RETN	**Resistin**	**Unidentified in Birds**	
	TNFA	**Tumor necrosis factor, alpha**	**Unidentified in Birds**	
Lipogenesis	*DHCR7*	7-Dehydrocholesterol reductase	1.11	-
	FADS2	Fatty acid desaturase 2	1.21	-
	FASN	Fatty acid synthase	1.36	1.6
	G6PC	Glucose-6-phosphatase, catalytic subunit	1.46	-
	scGH	Growth hormone, chicken short form	1.15	-
	HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase	1.09	-
	INSIG2	Insulin induced gene 2	1.74	-
	LCAT	Lecithin-cholesterol acyltransferase	1.32	-
	MVD	Mevalonate (diphospho) decarboxylase	1.2	-
	SCD	Stearoyl-CoA desaturase	1.48	1.88
	SREBF1	Sterol regulatory element binding factor 1	1.12	1.32

	THRSPA	Thyroid hormone responsive spot 14 A	-	1.64
	TXNIP	Thioredoxin interacting protein	-	1.8
Lipolysis	***LIPE***	**Hormone sensitive lipase**	**Unidentified in Birds**	
	ACAT1	Acetyl-CoA acetyltransferase 1	-3.18	-
	ADH1C	Alcohol dehydrogenase 1C, gamma polypeptide	-1.81	-
	APOA1	Apolipoprotein A-I	-1.16	-
	APP	Amyloid beta (A4) precursor protein	-1.15	-
	BCMO1	beta-carotene 15,15'-monooxygenase	-	-
	BCO2	beta-carotene oxygenase 2	-1.15	-1.13
	CYP27A1	Cytochrome P450 family 27 subfamily A polypeptide 1	-1.14	-1.48
	CYP2E1	Cytochrome P450 family 2 subfamily E, polypeptide 1	-1.8	-
	EHHADH	Enoyl-CoA hydratase3-hydroxyacyl CoA dehydrogenase	-1.09	-
	GAMT	Guanidinoacetate N-methyltransferase	-1.21	-
	HADHB	trifunctional protein, beta subunit	-1.1	
	HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	-1.92	-
	HSD17B6	Hydroxysteroid (17-beta) dehydrogenase 6	-1.19	-
	IRS1	Insulin receptor substrate 1	-1.59	-
	PDK4	pyruvate dehydrogenase kinase, isozyme 4	-	-
	PHYH	Phytanoyl-CoA 2-hydroxylase	-1.56	-1.99
	SLC2A2	Facilitated glucose transporter 2 (*GLUT2*)	-2.23	-
	SOD3	superoxide dismutase 3, extracellular	-1.1	-
	TP53	Tumor protein p53	-1.29	-1.2
	UCP3	Uncoupling protein 3 (mitochondrial, proton carrier)	-1.21	-

*(+) Fold-change (FC) values are higher in FL and (-) FC values are higher in LL chickens. FC values represent averages across 6 ages.

The qRT-PCR analysis of six candidate transcription factors is presented in Figure 2.9. Four genes [peroxisome proliferator-activated receptor alpha (*PPARA*), peroxisome proliferator-activated receptor gamma (*PPARG*), *PPARD* and sterol regulatory element binding transcription factor 2 (*SREBF2*)] showed only a main effect of age (A). A main effect of genotype (G) was observed for *SREBF1* due to higher expression in the FL at 1 and 5 wk. Similarly, the abundance of retinoid X receptor, gamma (*RXRG*) was higher in adipose tissue of the FL at 1, 5 and 11 wk, which produced a main effect of genotype (G).

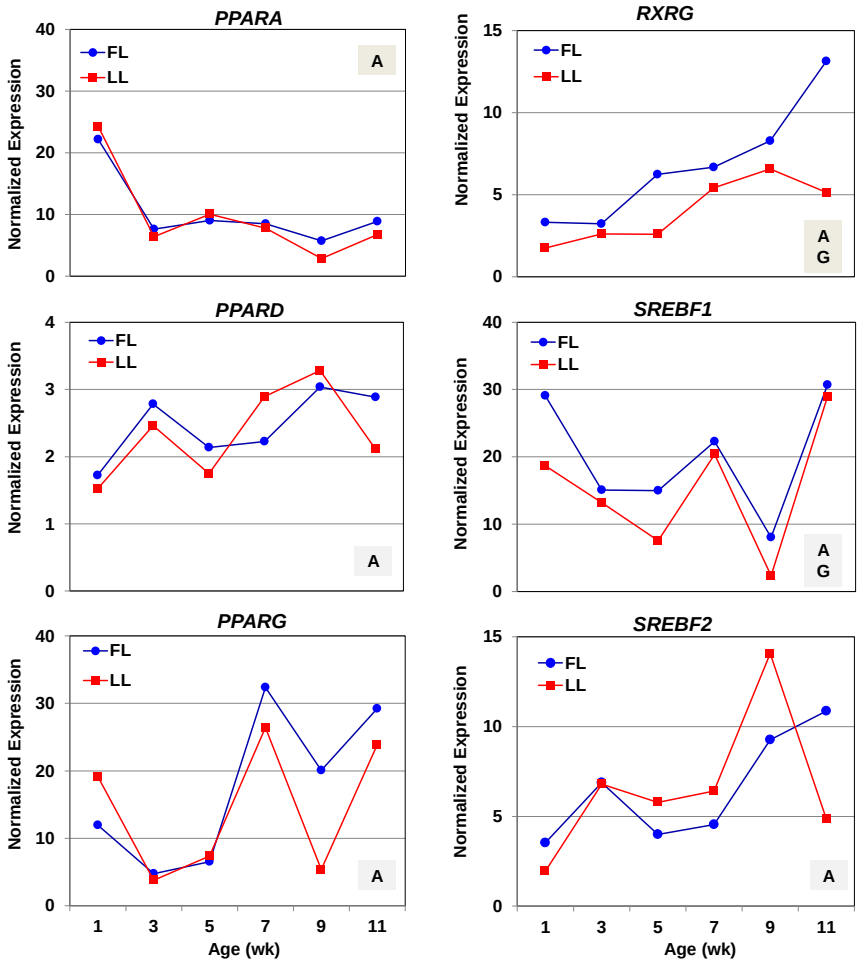


Figure 2.9 Verification of differential expression of transcription factors by qRT-PCR analysis. Each data point represents LSMEANS (n = 4/genotype) of normalized expression values. A two-factor ANOVA was used to determine significance ($P \leq 0.05$). The shaded box in each panel indicates significant main effects of age (A) or genotype (G), and the interaction of age x genotype (A x G).

The abundance of eight additional genes controlling metabolism and signaling of thyroid hormone and retinol was examined by qRT-PCR analysis (Figure 2.10).

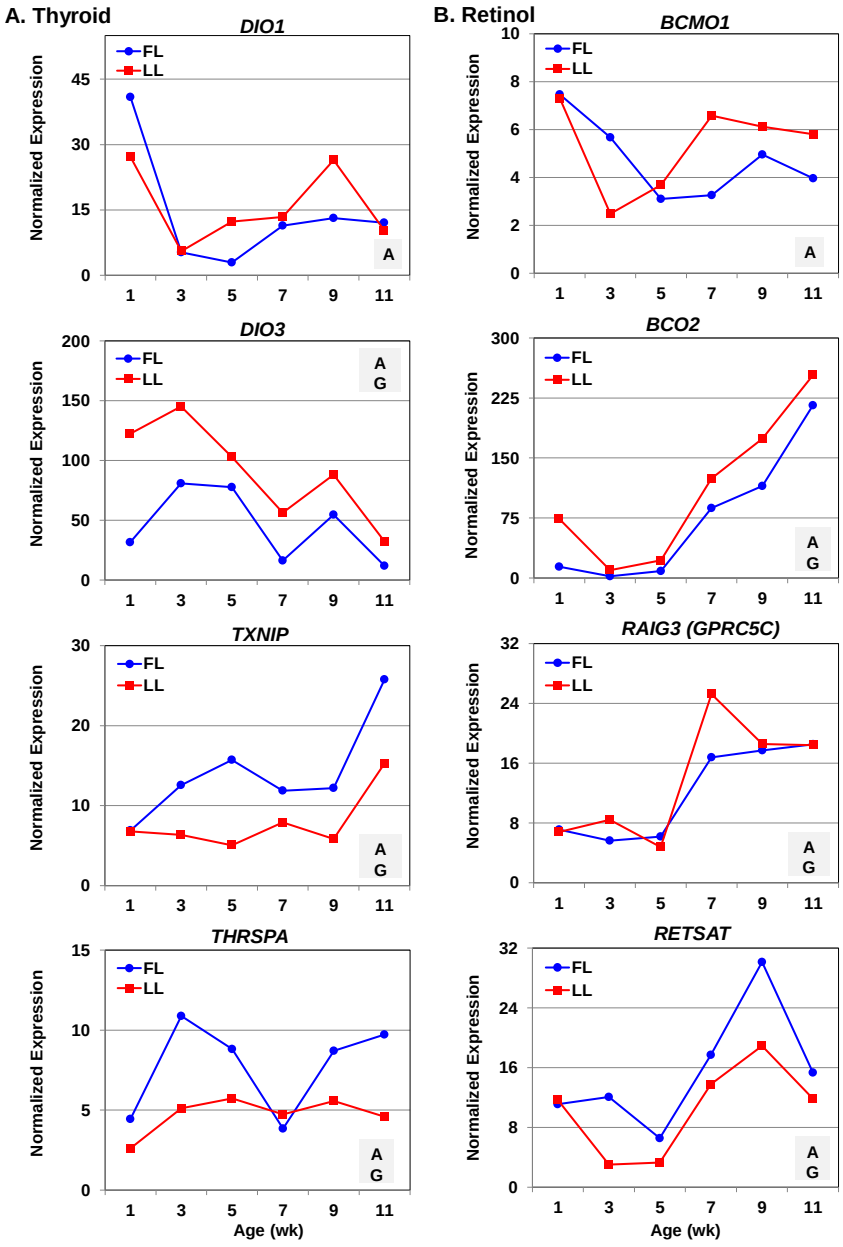


Figure 2.10 qRT-PCR analysis of genes involved in thyroid hormone and retinol metabolism and signaling. The abundance of genes involved in signaling and metabolism of thyroid hormone (A., left side of figure) and retinol (B., right side of figure) was verified by qRT-PCR analysis. Each data point represents LSMEANS (n = 4) of normalized expression values. A two-factor ANOVA was used to determine significance ($P \leq 0.05$). The shaded box in each panel indicates significant main effects of age (A) or genotype (G), and the interaction of age x genotype (AxG).

Type I iodothyronine deiodinase (*DIO1*), which converts the prohormone T_4 to metabolically active T_3, showed only a main effect of age, whereas type III iodothyronine deiodinase (*DIO3*) presented main effects of age and genotype due to a consistently higher abundance in abdominal fat of LL chickens (Figure 2.10-A). In contrast, the transcriptional regulator *THRSPA* and thioredoxin interacting protein (*TXNIP*) showed a main effect of age (A) and genotype (G) with higher expression in visceral fat of FL chickens at five of the six ages examined. Four genes involved in retinol metabolism [beta-carotene 15, 15'-monooxygenase (*BCMO1*), beta-carotene oxygenase 2 (*BCO2*), retinol saturase (*RETSAT*)] and the retinoic acid-induced gene 3 (*RAIG3*) [or G protein-coupled receptor, family C, group 5, member C (*GPRC5C*)] were also examined by qRT-PCR analysis (Figure 2.10-B). Although higher in the LL between 7 and 11 wk of age, *BCMO1* produced only a main effect of age (A). The expression of *BCO2* sharply increased with age (main effect) and was consistently higher in abdominal fat of the LL birds (main effect of genotype). Similarly, *RAIG3* showed main effects of age and genotype, with higher expression in the LL at 7 wk of age. The abundance of *RETSAT* was higher in visceral fat of the FL at 3 and 9 wk. Furthermore, the retinoid ligand-activated transcription factor *RXRG* was up-regulated in the FL, especially at 11 wk of age (Figure 2.9).

Another gene interaction network (Figure 2.11) identified by IPA shows interactions of several ligand-activated nuclear receptors and transcription regulators [*RXRG, CEPBZ, NR1H4* (farnesoid X receptor, FXR), *THRA, THRSP, MID1IP1*, nuclear receptor coactivator 1 (*NCOA1*), forkhead box J1 (*FOXJ1*), and CCCTC-binding factor (*CTCF*)].

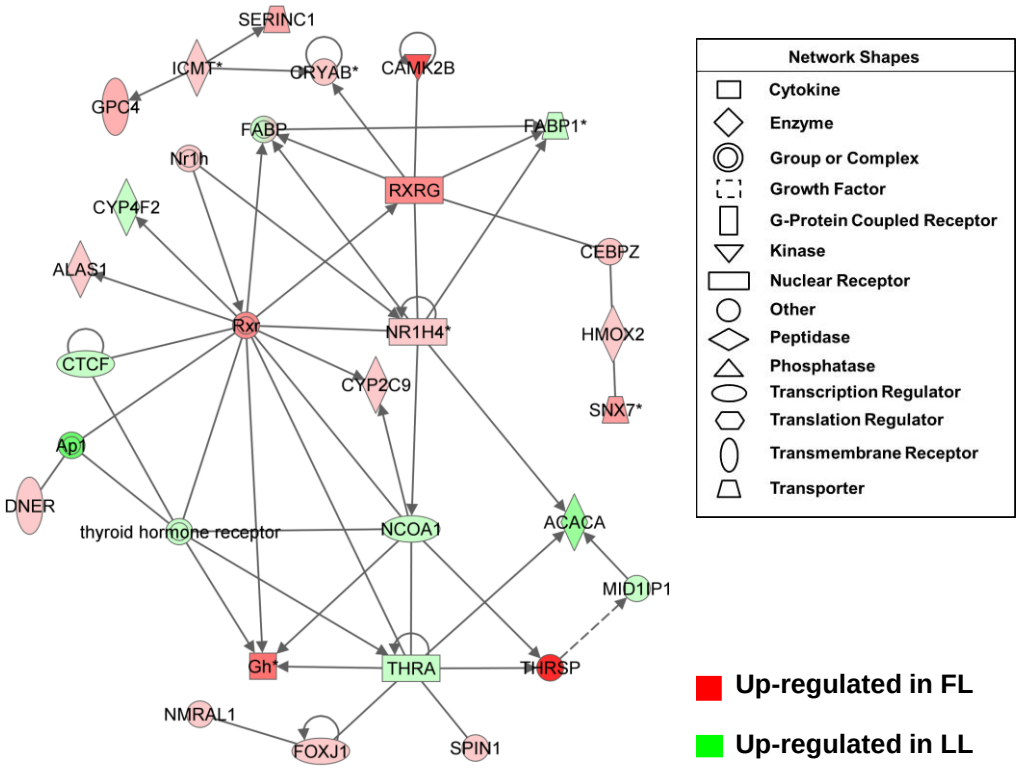
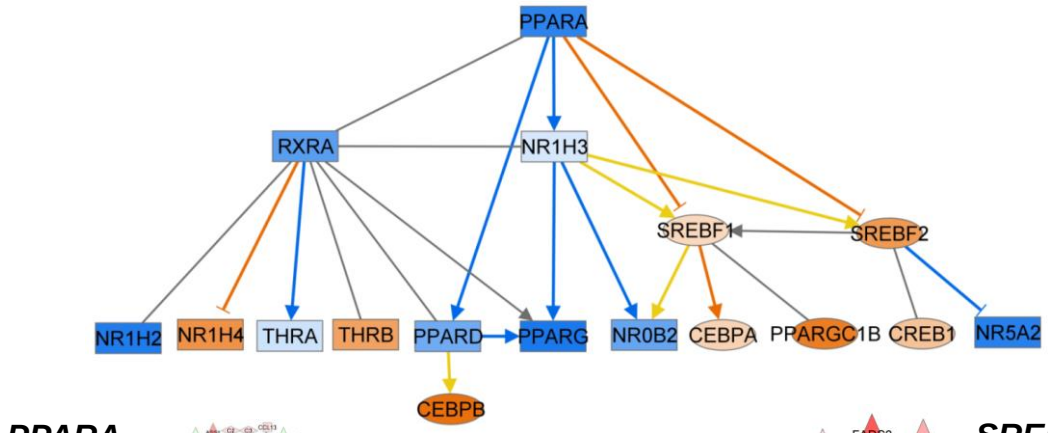


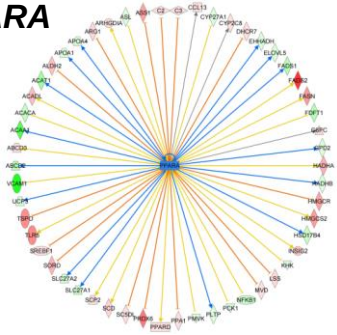
Figure 2.11 Gene interaction network of nuclear receptors, co-activators and regulators of gene transcription in abdominal fat of juvenile FL and LL chickens. This gene network shows direct interactions of seven transcriptional regulators [CEBPZ, RXRG, NR1H4 or farnesoid X receptor (FXR), NCOA1 or steroid receptor coactivator 1 (SRC-1), THRA, THRSP and MID1IP1 (or THRSP-like, THRSPL)] and their target genes. Gene symbol color indicates higher expression in the FL (red) or higher expression (green) in the LL.

The target genes of these upstream regulators were up regulated in abdominal fat of the FL [*GH, DNER, CYP2C9, ALAS1, CRYAB, ICMT, GPC4, SERINC1, CAMK2B, HMOX2* and *SNX7*] or LL chickens (*CYP4F2, FABP1* and *ACACA*).

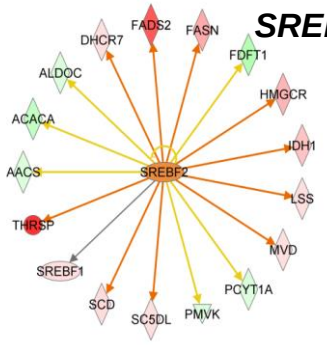
A final non-redundant list of lipid metabolism genes were identified by IPA from the main effects of genotype, age or the interaction of genotype x age and then used for Ingenuity® Upstream Regulator Analysis. This analysis illustrates the interaction of numerous ligand-activated nuclear receptors and other transcription factors (TF), providing predictions of either an activated (orange color) or inhibited (blue color) state (Figure 2.12). These predictions are based on prior knowledge of transcriptional responses and the responses of downstream targets found in the DE gene data set. For example, this mechanistic network of transcription regulators indicates whether the TF (orange color) and target gene (red gene symbol) are both activated (increased expression in FL) or if the activity of the TF is inhibited (blue color), which would be associated with increased expression in the LL (green gene symbol).



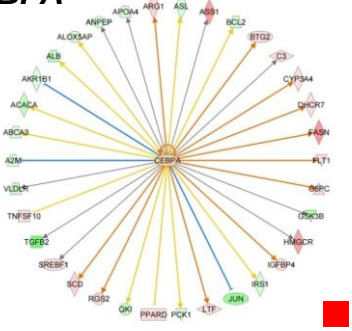
PPARA



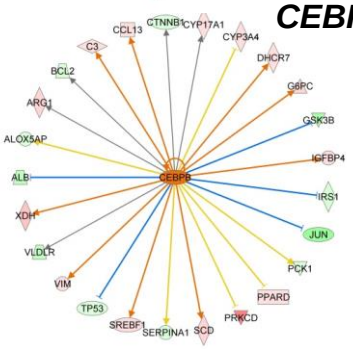
SREBF2



CEBPA



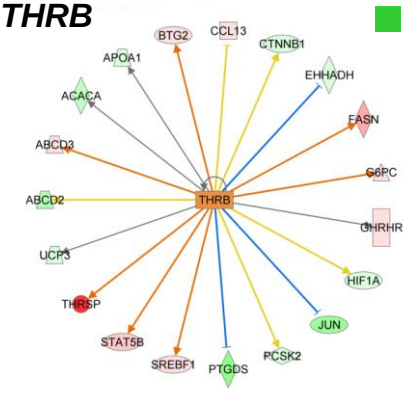
CEBPB



Higher expression in FL

Higher expression in LL

THRB



CREB1

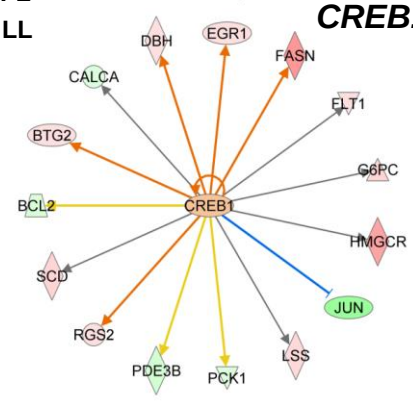


Figure 2.12 Upstream regulators of gene transcription in abdominal fat of juvenile FL and LL chickens. Ingenuity® Upstream Regulator Analysis revealed many transcriptional regulators (see Table 2.4) controlling lipid metabolism genes in abdominal fat (A.). This IPA analysis shows "up-stream regulators" and their downstream targets found among DE lipid metabolism genes identified in abdominal fat of the FL and LL chickens. DE gene targets regulated by six transcription factors are shown (B.). The IPA prediction of activation (orange lines and symbols) or (blue lines and symbols) inhibition states is based on knowledge from literature and expression values of DE genes identified. Gene symbol color indicates up-regulation in the FL (red) or up-regulation in the LL (green).

This mechanistic network predicts inhibition (blue lines and symbols) of eight transcription factors (PPARA, RXRA, NR1H2, NR1H3, PPARD, PPARG, NROB2 and NR5A2) and the activation (orange lines and symbols) of an additional eight transcription factors (NR1H4, THRB, CEBPA, CEBPB, CREB1, PPARGC1B, SREBF1 and SREBF2) (Table 2.4). The gene targets are presented for two transcription factors (PPARA and CEBPA) predicted to be inhibited and four transcription factors (THRB, SREBF2, CEBPB and CREB1) that were predicted to be activated by the IPA Upstream Regulator Analysis. This mechanistic analysis shows that three transcription factors (PPARA, CEBPA and CEBPB) exert direct actions on target genes up-regulated in the LL, while three other transcription factors (THRB, SREBF2 and CREB1) mainly target up-regulated genes in the FL, which are involved in the synthesis, transport or metabolism of lipids.

Table 2.4 Upstream regulators of genes controlling lipid metabolism in abdominal fat of FL and LL chickens (1-11 weeks)

Symbol	NCBI Entrez Gene Name	Activation z-score	P-value of overlap	# Targets
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	0.379	1.61E-15	34
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1.935	1.75E-10	25
CREB1	cAMP responsive element binding protein 1	0.527	1.64E-05	15
NR0B2	nuclear receptor subfamily 0, group B, member 2 (SHP)	-0.84	3.74E-08	10
NR1H2	nuclear receptor subfamily 1, group H, member 2 (LXRB)	-1.512	2.64E-10	12
NR1H3	nuclear receptor subfamily 1, group H, member 3 (LXRA)	-0.2	4.69E-11	14
NR1H4	nuclear receptor subfamily 1, group H, member 4 (FXR)	1.076	1.40E-06	11
NR5A2	nuclear receptor subfamily 5, group A, member 2 (LRH1)	-1.412	3.57E-04	7
PPARA	peroxisome proliferator-activated receptor alpha	-1.339	1.78E-32	54
PPARD	peroxisome proliferator-activated receptor delta	-0.767	3.13E-13	21
PPARG	peroxisome proliferator-activated receptor gamma	-1.629	1.38E-27	48
PPARGC1B	PPARG, coactivator 1 beta	1.488	1.37E-12	12
RXRA	retinoid X receptor, alpha	-0.932	3.32E-18	31
SREBF1	sterol regulatory element binding transcription factor 1	0.511	2.91E-20	29
SREBF2	sterol regulatory element binding transcription factor 2	1.171	2.78E-15	17
THRA	thyroid hormone receptor, alpha	-0.246	2.08E-08	12
THRB	thyroid hormone receptor, beta	1.135	7.18E-11	19

Ingenuity Upstream Regulator Analysis® identified multiple transcription factors regulating differential expression of target genes enriched for lipid metabolism in abdominal fat of juvenile FL and LL chickens (Figure 2.12). The activation Z-score indicates whether the observed gene responses to upstream regulators agree with expected changes derived from the literature. A Fisher's Exact Test was calculated to determine the significance for enrichment of known targets of each upstream regulator in the observed datasets. *Abbreviations*: small heterodimer partner (SHP), liver X receptor alpha (LXRA), liver X receptor beta (LXRB), farnesoid X nuclear receptor (FXR), and liver receptor homolog 1 (LRH1).

2.3.6 Correlation of gene expression between microarray and qRT-PCR analyses

Four genes (*RBP4*, *FASN*, *DIO1*, and *F9*) were chosen to determine the correlation between microarray and qRT-PCR analyses (Figure 2.13). The log2 ratios (FL/LL) for each week from the microarray and qRT-PCR analyses were compared using Spearman's rank correlation coefficient at a significance level of $P \leq 0.05$. All

four genes show significant correlations (r_s = 0.77–0.94) between the microarray and qRT-PCR analyses.

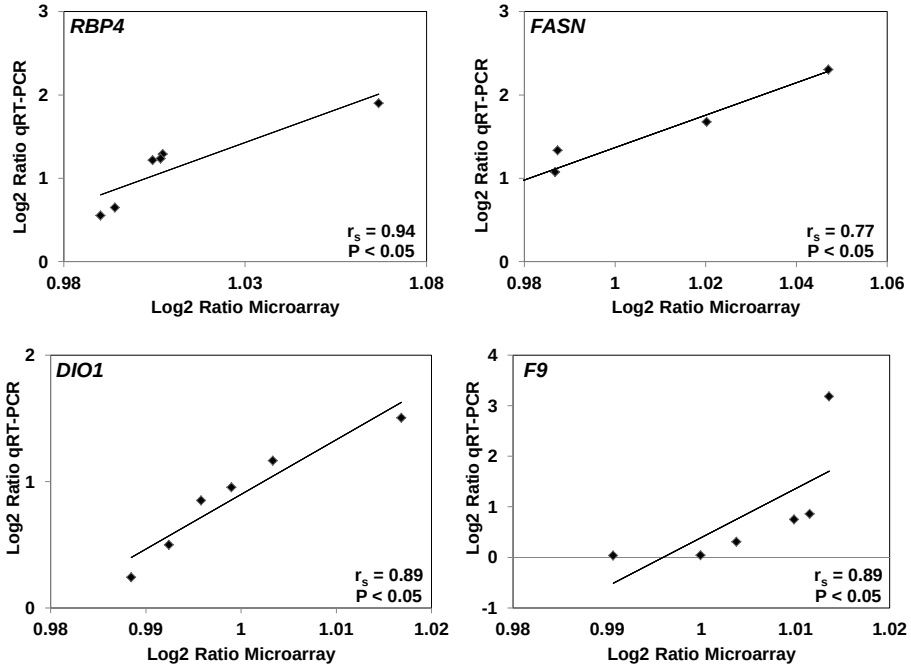


Figure 2.13 Correlation between Microarray and qRT-PCR Analyses. Plotted correlation between microarray and qRT-PCR analyses for four genes between 1-11 wk. The log2 ratios for each week from the microarray and qRT-PCR analysis were assigned a Spearman's rank correlation coefficient and then used for a correlation analysis at a significance level of $P \leq 0.05$.

2.4 Discussion

The divergent FL and LL chickens were originally developed as experimental models to study genetic and endocrine mechanisms controlling excessive abdominal fatness [13]. Indeed, juvenile FL and LL chickens exhibit a 2.5-fold difference in

abdominal fatness between 3 and 11 wk of age while maintaining similar rates of growth (Table 2.1), feed intake, and energy metabolism. The search for major genes contributing to the divergence in adiposity between the FL and LL has primarily focused on the liver [14-19]. In the present study, the Del-Mar 14K Integrated Systems microarray was used to examine gene expression profiles of abdominal fat in juvenile FL and LL cockerels across six ages (1-11 wk). This analysis of time-course transcriptional profiles has provided the first panoramic view of the abdominal fat transcriptome in the FL and LL chickens and given functional insight into the 2.5-fold divergence of adiposity. In particular, we have discovered numerous DE genes that are involved in hemostasis (blood coagulation), adipokine signaling, thyroid hormone and retinol action, and lipogenesis in abdominal fat of FL and LL chickens during juvenile development. These findings are unlike previous microarray studies of adipose tissue in meat-type chickens.

2.4.1 Higher expression of hemostatic factors in adipose tissue of LL chickens

A large number of genes involved in hemostasis were differentially expressed in adipose tissue of LL chickens (Table 2.3). Several coagulation factors identified in our transcriptional analysis of adipose tissue are either proteases (*i.e.*, *F2*, *F9*, *PLG*, *PROC*, and *CFB*) or protease inhibitors (*i.e.*, *A2M*, *ANXA5*, *SERPINA1*, and *SERPIND1*). We also found higher expression of carboxypeptidases [carboxypeptidase B2 (*CPB2* or thrombin-activatable fibrinolysis inhibitor) and

carboxypeptidase M (*CPM*)] in abdominal fat of LL chickens. Our discovery of a higher abundance of several genes encoding blood clotting factors in LL chickens is quite peculiar given that fattening, rather than leanness, in mammals is usually associated with the prothrombotic state [20-23]. In fact, obesity in humans is described as chronic low-grade inflammation where expression of hemostatic genes [e.g., serine peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (*PAI-1*), thrombin, fibrinogen and von Willebrand factor (VWF)] are positively associated with greater deposition of adipose tissue [20, 24]. The adipokine *PAI-1* (*SERPINE1*) encodes a secreted regulator of fibrinolysis, which serves as a biomarker for metabolic syndrome in humans [24]. Although *PAI-1* has not been mapped to the chicken genome, we did find higher expression of the plasminogen activator inhibitor 1 RNA-binding protein (*SERBP1*) in abdominal fat of the FL chickens. The SERBP1 protein greatly increases the degradation of *PAI-1* mRNA in rat hepatoma cells [25]. In addition, SERBP1 functions as a partner with the progesterone receptor membrane component 1 (PGRMC1) in mediating the anti-apoptotic action of progesterone on the female reproductive tract of humans [26]. Our identification of *SERBP1* and its up-regulation in adipose tissue of the FL suggest that a functional homolog of *PAI-1* does exist in the chicken. Another related member of the same clade as *PAI-1*, *SERPINE2* was not differentially expressed in abdominal fat of FL and LL chickens according to microarray analysis. Since *SERPINE2* was one of the most stably expressed genes in our qRT-PCR analysis, it was used as a housekeeping gene to normalize gene expression. Another hemostatic gene up

regulated in abdominal fat of LL chickens was thrombospondin 2 (*THBS2*), which inhibits adipogenesis in mammals [27].

Hemostatic proteins have several functions, some of which include removal of signal peptides, activation of zymogens, transport of enzymes, or degradation of active enzymes. Given that many adipokines have a high functional and structural similarity to the classic coagulation factors and other hemostatic factors (e.g., *ANGPTL4* contains a fibrinogen-like domain), it is reasonable to assume that these proteases act on pre-pro-adipokines or other secreted proteins expressed in adipose tissue. Little is known about the expression of blood coagulation genes in visceral fat or their role in the development of adiposity in chickens. Using K-means clustering (data not shown), we found that the expression profiles for most genes involved in coagulation were clustered with those of adipokines; this general trend was verified by qRT-PCR analysis (Figures 2.4 and 2.5). For example, secreted proteins *ADIPOQ* and *ATRN* have expression patterns that are similar to those of serine proteases (e.g., *F2*) and protease inhibitors (e.g., *ANXA5*). Further, the developmental profiles of *RBP4* and *ANGPTL4* were similar to that of *A2M*, a protease inhibitor and transporter of cytokines. The positive correlation of expression patterns between coagulation factors and adipokines is not surprising given that many adipokines are associated with hemostatic or inflammatory processes (e.g. *RARRES2*); and conversely, several genes involved in coagulation are considered as adipokines (e.g. *PAI-1, A2M, F2* and *FGA*). Furthermore, a similar transcriptional analysis of liver from the same individual FL and LL birds failed to reveal differential expression of these blood coagulation factors

[18]. The lack of a parallel effect of genotype on hepatic expression of coagulation factors in the FL and LL chickens suggests that their ectopic expression in abdominal fat is specific and without consequence to systemic hemostasis.

2.4.2 Adipokines in abdominal fat of FL and LL chickens

A prime example of proteolytic processing of adipokines is chemerin (or *RARRES2*), which is expressed at higher levels in abdominal fat of LL chickens. Chemerin is a recently discovered adipokine that regulates adipogenesis; and chemerin can be transformed into a pro-inflammatory protein, a cell adhesion factor or an anti-inflammatory peptide, depending upon cleavage by specific proteases [28-30]. After removal of the N-terminal signal peptide, pro-chemerin is processed at the C-terminal end by serine proteases to generate an active pro-inflammatory adipokine, which can be cleaved further at its C-terminal end by cysteine proteases to generate an anti-inflammatory peptide [31]. Active chemerin appears to exert its action by binding its extracellular receptor *CMKLR1* on adipocytes and/or *CCRL2* on activated macrophages, which then forms an adhesive bridge between these two resident cells in adipose tissue during the inflammatory response [31]. Adipocyte-derived chemerin causes insulin resistance in skeletal muscle cells [32]; and as a secreted adipokine, chemerin regulates myogenesis by providing negative cross-talk between adipose tissue and skeletal muscle [33]. Consequently, chemerin functions as a chemokine for leukocytes, an adipokine that regulates angiogenesis, and a biomarker of metabolic

syndrome and obesity in humans [34-36]. However, chemerin appears to be associated with leanness in the chicken.

Retinol binding protein 4 (*RBP4*), the main transporter of retinol in blood, is another adipokine that was expressed higher in abdominal fat of LL chickens at 5 and 7 wk of age. Like chemerin, RBP4 serves as a biomarker of obesity-related diseases including insulin resistance, dyslipidemia, hypertension, and visceral obesity in both adult and adolescent humans [37]. Similar to other genes involved in coagulation, chemerin and *RBP4* are expressed higher in LL, rather than FL chickens. In contrast, several adipokines (*ADIPOQ*, *ADIPOR1* and *ATRN*) found in abdominal fat of FL and LL chickens are regulated similar to mammals. For example, *ADIPOQ* is inversely related to fatness and it is associated with increased insulin sensitivity in mammals [38]. From the qRT-PCR analysis, we observed late up-regulation of *ADIPOQ* (wk 7-11) and its receptor *ADIPOR1* (wk 9) in LL chickens, which suggests that adipose tissue of FL chickens could be less sensitive to insulin at these ages. Attractin (*ATRN*) is a neuropeptide involved in melanocortin signaling and regulation of food intake, which suppresses diet-induced obesity [39]. Our qRT-PCR analysis shows that the expression of *ATRN* markedly increases in abdominal fat after 5 wk of age in both the FL and LL; furthermore, the expression pattern of *ATRN* is strikingly similar to that of *ADIPOQ* and *ADIPOR1*. The adipokine *ANGPTL4* was expressed higher in LL chickens at 1, 5, 7 and 11 wk of age, although this difference was not statistically significant by qRT-PCR analysis. Originally, ANGPTL4 was identified as a secreted "fasting-induced adipose factor (FIAF)" in the mouse that was sharply up regulated by

fasting and a target gene of the transcription factor PPARA [40]. In fact, ANGPTL4 is a potent irreversible inhibitor of lipoprotein lipase (LPL) activity, which leads to hypertriglyceridemia [41]. Our qRT-PCR analysis shows a 3-fold increase in expression of LPL in the LL at 7 wk. Of particular interest, ANGPTL4 promotes the cleavage of LPL, while the proteolytic cleavage of ANGPTL4 by proprotein convertase releases a more potent inhibitor of LPL activity—the N terminal domain [42]. Thus, abdominal fat of chickens is enriched with adipokines, which can exert either local (autocrine/paracrine) or systemic (endocrine) actions after proteolytic processing and secretion into circulation (Table 2.3).

Our initial survey of global gene expression in abdominal fat of juvenile FL and LL chickens highlights another important feature of the avian endocrine system—the virtual absence of several important adipokines normally found in mammals (Table 2.3). A few examples of adipokines not yet mapped to the current draft of the chicken genome (galGAL4), include leptin (*LEP*), omentin (*ITLN1)*, resistin (*RETN)*, tumor necrosis factor alpha (*TNFA*), and *PAI-1*. The existence of the chicken *LEP* gene remains a great and unresolved controversy [43-47], especially since extensive expressed sequence tag (EST) [48] and whole genome sequencing projects have failed to identify a *bona fide LEP* gene in the chicken. Furthermore, the *LEP* gene is absent from the genome of all birds sequenced so far (i.e., chicken, turkey, zebra finch and budgerigar). However, the leptin receptor (*LEPR*) gene is expressed in several chicken tissues [48-52]; and chicken LEPR is capable of activating the JAK-STAT pathway *in vitro* [53, 54]. Similarly, components of TNF signaling are up regulated in

the hypothalamus of LL chickens [52], although *TNFA* is yet to be identified in chickens. Despite the absence of several mammalian adipokines (i.e., *LEP*, *TNFA*, *RETN*, *PAI-1*, and *ITLN1*) and metabolic enzymes (i.e., *LIPE*), adipogenesis and lipid metabolism in the chicken are regulated by mechanisms that are similar to those described in mammals.

2.4.3 Retinol metabolism and retinoic acid signaling in adipose tissue

Another remarkable observation from the present study was the over expression of 13 genes in abdominal fat of LL chickens that control metabolism of retinol; the precursor of retinoic acid (RA), which itself is a major chemical activator of multiple transcription factors controlling lipogenesis. The primary source of retinol is dietary plant-based β-carotene, which is symmetrically cleaved by the enzyme β-carotene monooxygenase 1 (BCMO1) into two molecules of retinal. Recently, we discovered mutations in the proximal promoter of *BCMO1*, which are responsible for variation in the color of breast meat in another F_2 resource population of meat-type chickens [55]. Another enzyme, β-carotene oxygenase 2 (BCO2), asymmetrically cleaves one molecule of β-carotene to generate one molecule of retinal and a by-product (e.g., β-apo-14'-carotenal), which acts downstream to block signaling of PPARG [56]. The *BCO2* gene in chickens was originally identified as the *yellow skin* gene, which controls the β-carotene content and thereby yellow pigmentation of the skin [57]. Our qRT-PCR analysis of these two β-carotene degrading enzymes (Figure

2.10), showed only a main effect of age on expression of *BCMO1*, whereas the abundance of *BCO2* was greater in abdominal fat of LL chickens, producing a main effect of genotype. Another study found increased expression of *BCO2* in adipocytes from *BCMO1* knockout mice and that dietary β-carotene reduces adiposity of mice—but only in the presence of a functional BCMO1 enzyme [58]. This study also demonstrates the importance of BCMO1 in generating the precursor (retinal) for RA, which inhibits activation of PPARG and its lipogenic target genes that are mainly metabolic enzymes, adipokines and transport proteins. Our study shows higher expression of both *BCMO1* and *BCO2* in abdominal fat of the LL chickens after 5 wk of age, which presumably would lead to generation of more retinal and RA. This idea is supported by the differential expression of several genes involved in retinol metabolism and RA signaling in adipose tissue of FL and LL chickens. These genes are involved in transport of retinol (*RBP4*, *TTR* and *RBP7*), metabolism of retinol (*RDH1*, *RETSAT*, *ADH1C, ADH5,* and *CYP2E1*), and respond to RA (*RARRES2*, *GPRC5C,* and *NOL7*). In 3T3-L1 preadipocytes, RA inhibits adipogenesis via up-regulation of the transcriptional modulator SMAD3 [59]. Interestingly, two members of the SMAD family (*SMAD5* and *SMAD6*) were up-regulated (main effect of age) in adipose tissue of LL chickens. The ligand (RA) activates its nuclear receptors (RAR and RXR), which can form heterodimers with other ligand-dependent transcription factors (e.g., LXR, PPARG and THR) to initiate transcription of numerous downstream target genes. Thus, RA seems to play an important role in reduction of adipogenesis and adiposity in the LL chickens.

2.4.4 Visceral adipose tissue as a major site of lipogenesis in chickens

Because the liver is widely considered as the primary site of *de novo* lipid synthesis in birds, most transcriptional studies of lipogenesis in the chicken have focused on liver, rather than adipose tissue. A targeted low-density array enabled an initial transcriptional analysis of liver [at a single age (8 wk)] in the FL and LL chickens [17]. This study showed up-regulation of several lipogenic genes (*ACACA*, *FASN, SCD, APOA1*, *SREBF1*, and *MDH2*) in the FL chickens. Examination of hepatic gene expression at three ages (1, 4, and 7 wk) in another population of chickens divergently selected on abdominal fatness revealed differential expression of several genes involved in lipid metabolism, including *ACAT1*, *CEBPγ*, *FABP1*, *APOA1*, *MDH1*, *APOD* and *PPARG* [60]. A time-course (1-11 wk) transcriptional study of liver in juvenile FL and LL chickens revealed 1,805 DE genes, mostly between 7 and 11 wk [18]. These functional genes identified in the liver of juvenile FL and LL birds were transcription factors, metabolic enzymes, transport proteins, differentiation factors, signaling molecules and adipokines.

In contrast, there have been only a few transcriptional studies of adipose tissue in the chicken. For example, a comparison of abdominal fat between meat-type (broiler) and egg-type chickens (layer) at a single physical age (10 wk), albeit at different physiological ages, focused attention on the up-regulation of *LPL* in broiler chickens and higher expression of *APOA1* in layers [61]. Another study using abdominal fat samples taken at 7 wk from a different population of divergently

selected fat and lean chickens reported the differentially expression of 230 adipose genes (153 were up-regulated in the fat chickens, while 77 were up-regulated in the lean birds) [62]. Their conclusion that *TNFA* plays a key role in lipid metabolism of the chicken is surprising, since this adipokine has not been mapped to the chicken genome sequence. A recent transcriptional study of chicken abdominal fat compared a short-term (5 hr) fasting-to-refeeding response with acute insulin immunoneutralization [63]. Surprisingly, fasting provoked larger changes in adipose gene transcription (1,780 DE genes) than did insulin neutralization with only 92 DE genes, which confirms the insensitivity of chicken abdominal fat to insulin [64]. In contrast, more than a thousand genes were either differentially expressed in liver or leg muscle of the same birds following insulin immunoneutralization [65]. Nonetheless, short-term fasting in the chicken depressed the expression of 40 genes in abdominal fat that are involved in the synthesis and storage of lipid, while a number of adipose genes that control lipolysis and oxidation of fatty acids were up-regulated by fasting or insulin neutralization [63].

The present study has identified a large number of lipogenic genes that are up regulated in abdominal fat of FL chickens (Table 2.3). A prime example of this lipogenic group is our clone for GH1 (GenBank accession BI390457) that corresponds to the short form of chicken GH (scGH), which lacks a signal peptide and is highly expressed in ocular tissue [66, 67], pituitary gland and heart of chick embryos [67]. This short alternatively spliced (16.5 kDa) isoform of full length GH (20 kDa) functions as an “intracrine” factor within the cell [67]. Our discovery of higher

expression of scGH in abdominal fat of the FL chicken supports the idea of a local lipogenic action of GH on adipose tissue, rather than the lipolytic response usually observed in mammals [68]. In fact, our earlier work clearly established the lipogenic action of exogenous GH in juvenile chickens [69-72].

Up-regulation of two transcription factors (*SREBF1* and *THRSPA*) and the histone deacetylase *SIRT2* in abdominal fat of the FL were accompanied by higher expression of multiple genes involved in the generation and metabolism of lipids (*DHCR7, FADS2, FASN, HMGCR, HMGCS2, LSS, MVD, SCD* and *SC5DL*). The higher expression of the transcription factor *SREBF1* and 12 lipogenic target genes in the FL strongly supports our idea that the divergence in abdominal fatness of FL and LL chickens could be related to differential expression of several lipogenic genes in abdominal fat of the FL. For example, *FADS2*, which catalyzes the rate limiting step in synthesis of highly unsaturated fatty acids, was highly up regulated in abdominal fat of FL chickens; binding sites for both SREBF1 and PPARA are found in the promoter region of *FADS2* [73]. Likewise, SREBF1 regulates transcription of several genes that control synthesis of fatty acids, including acetyl-CoA carboxylase alpha (*ACACA*), which catalyzes the rate-limiting step in fatty acid synthesis [74, 75]. Furthermore, MID1 interacting protein 1 (MID1IP1) [or THRSP-like (THRSPL)] enhances ACACA polymerization and its enzymatic activity [76]. Adipose tissue from FL chickens shows higher expression of *THRSPA*, a transcriptional regulator of several lipogenic genes in the chicken [18, 51, 72, 77]. Earlier, we discovered a 9-base pair deletion near the putative DNA-binding domain of chicken *THRSPA* and

demonstrated association of this insertion/deletion polymorphism with abdominal fatness traits in multiple resource populations of chickens [77]. Mutations in the *THRSPA* gene of chickens [77-79], ducks [80] and geese [81] are associated with fatness traits and are perhaps of potential use as molecular markers in poultry breeding programs. Furthermore, THRSP is a major regulator of adipogenesis in skeletal muscle of beef cattle [82, 83] and of lipogenesis in the lactating mammary gland of the dairy cow [84-86]. Interestingly, the THRSP-null mouse shows reduced lipogenesis in the mammary gland [87] and pups from the THRSP-null mouse exhibit reduced body weight gain due to diminished milk triglycerides [88]. In humans, amplification of the THRSP locus is associated with lipogenic breast cancer [89]; and, as such, THRSP serves as a marker of aggressive breast cancer and a potential target of anti-cancer drugs [90]. In humans, expression of *THRSP* in adipose tissue is depressed by transition from a lipogenic fed state to a lipolytic state induced by a 48 hr fast [91]. These observations support the idea that THRSP is a transcriptional activator of several lipogenic enzymes (ACLY, FASN and ME) in the mouse [92]. THRSP is activated in response to T_3, glucose and insulin and inhibited by polyunsaturated fatty acids [93], cyclic AMP or glucagon [94]. Recent work has shown that induction of THRSP increases expression of FASN in cultured hepatocyte cells and RNAi-mediated knock-down of THRSP depresses expression of FASN [95]. Another study showed that FASN co-precipitates with THRSP in nuclear extracts from the mouse (referenced in [86]). The exact mechanism by which THRSP and MID1IP1 interact and work as regulators of gene transcription is currently unknown. These genes are

highly expressed in fatty tissues of birds and mammals, where they regulate the expression and activity of multiple lipogenic enzymes. The proximal (4 kb) promoter region of *THRSPA* contains four putative binding sites for PPARG and four SREBF sites (L.A. Cogburn, unpublished observations). In the present study, we found higher expression of *THRSPA* in abdominal fat of FL chickens at all ages, except at 7 wk. In the rat, the far-upstream region of the *THRSP* promoter contains three T_3-THR response elements (TREs) [96]. Thus, *THRSPA* is responsive to metabolically active thyroid hormone (T_3) generated by the activation enzyme *DIO1*, whereas the enzyme DIO3 is responsible for degradation of metabolically active T_3 and conversion of the prohormone (T_4) to metabolically inactive reverse T_3 (rT_3) [97]. The up regulation of *DIO3* in adipose tissue of juvenile LL chickens (1-11 wk) suggests that less T_3 would be available to activate *THRSPA* transcription, which was observed in the LL. Thioredoxin interacting protein (TXNIP) is another important regulator of hepatic glucose metabolism [98] that also mediates hypothalamic control over energy utilization and adiposity in the mouse [99]. The up-regulation of *TXNIP* in abdominal fat of the FL during the period of maximal fatness (3-11 wk) could contribute to their enhanced lipogenesis and adiposity. Likewise, we have discovered another putative sensor of glucose, the sweet taste receptor 1 (*TAS1R1*) gene, which is differentially expressed in the hypothalamus [52] and abdominal fat (Figure 2.3) of FL and LL chickens. Our observation of higher expression of *TAS1R1* in the hypothalamus of the FL and abdominal fat of the LL suggest tissue specific regulation of this important tissue glucose sensor [100-102].

2.4.5 Increased lipolysis in abdominal fat of LL chickens

In contrast to the enhanced lipogenic state found in abdominal fat of FL chickens, the LL show higher expression of numerous genes involved in lipolysis (Table 2.3). Two cytochrome P450 family members (*CYP27A1* and *CYP2E1*) were expressed at higher levels in abdominal fat of the LL when compared to FL chickens. *CYP27A1* is involved in clearance of cholesterol via bile excretion, whereas *CYP2E1* is strongly induced in white adipose tissue of the rat by prolonged fasting [103]. The beta-subunit (*HADHB*) of mitochondrial tri-functional protein (MTP), a complex that catalyzes the final three steps of β-oxidation of long chain fatty acids, was also up regulated in adipose tissue of LL chickens. MTP knockout mice exhibit neonatal hypoglycemia and sudden neonatal death indicating its essential role in β-oxidation of long chain fatty acids [104]. Two members of the hydroxysteroid (17-β) dehydrogenase family (17β-HSD, members 4 and 6) were also expressed higher in adipose tissue of the LL. The significance of HSD17B4 in β-oxidation of branched chain fatty acids was demonstrated in HSD17B4 knockout mice, which were unable to degrade phytanic and pristanic acids [105]. Since the other 17β-HSD (HSD17B6) exhibits retinol dehydrogenase activity [106], it's up-regulation in adipose tissue of the LL chicken suggests increased availability of all trans-retinoic acid. In addition, *PDK4*, which inhibits the pyruvate dehydrogenase complex and conversion of pyruvate to acetyl-CoA [107], was highly up-regulated in abdominal fat of the LL between 7 and 11 wk. The high expression of *PDK4* in the liver of chicken embryos

[18], whose energy is derived exclusively from yolk lipids, supports a similar action of PDK4 in adipose tissue of the LL chickens. Furthermore, the expression of *PDK4* increased sharply in abdominal fat of two-week-old chickens by a 5-h fast or insulin immunoneutralization [63]. The tumor suppressor protein p53 enhances lipid catabolism and induces expression of guanidinoacetate N-methyltransferase (GAMT), which enhances β-oxidation of fatty acids [108]; both of these genes were up regulated in visceral fat of the LL chickens. Another gene up regulated in the LL that protects against oxidative stress is *SOD3,* which is expressed at higher levels in the liver of low-growth (leaner) chickens [18]. Thus, the present time-course transcriptional analysis of abdominal fat in juvenile FL and LL chickens provides compelling evidence for enhanced lipolysis in adipose tissue of the LL.

2.5 Chapter Summary

The present study adds a new dimension to function of visceral fat as a proteolytic processor of adipokines and other endocrine signals that control lipid metabolism. In particular, the LL cockerels exhibit high expression of several blood coagulation factors in adipose tissue. Some of these changes in the LL occur before the divergence in fatness. These hemostatic proteases and protease inhibitors could be involved in activation of adipokines, chemokines and other metabolic ligands that contribute to suppression of lipogenesis and adipogenesis in the LL. Furthermore, abdominal fat of the LL chickens has increased expression of genes involved in

mobilization, utilization and export of lipids. Several transcription factors have a larger number of target genes expressed higher in the LL that could also favor suppression of abdominal fat accretion. In contrast, abdominal fat of the FL chickens expresses a greater abundance of numerous target genes involved in lipogenesis and adipogenesis, which could contribute to their greater adiposity. The higher expression of these target genes in FL chickens appears after the onset of divergence in fatness. Therefore, visceral fat of the chicken could play a more significant role in lipogenesis and adiposity than previously considered. The assumption that the liver of birds serves as the major site of lipogenesis needs to be re-examined.

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Chapter 3

DEEP RNA SEQUENCE ANALYSIS OF ABDOMINAL FAT IN GENETICALLY FAT AND LEAN CHICKENS AT 7 WEEKS OF AGE

3.1 Introduction

High-throughput and massively parallel RNA sequencing (RNA-Seq) technology is a powerful method for gene expression profiling, which can be used to gain an understanding of the mechanisms controlling major processes under genetic and physiological perturbations. One of the most significant advantages of RNA-Seq over earlier methods (i.e., differential mRNA display and microarray) is the ability to sequence all RNA species present in a sample without having to identify target genes *a priori*. In chickens, RNA-Seq has been used for several applications including unraveling the complex mechanism underlying embryonic digit development [1, 2], evaluation of coverage at different sequencing depths in lung tissue [3] and identification of long non-coding RNA in skeletal muscle [4]. To our knowledge, there has been no attempt to use RNA-Seq technology for gene expression analysis of chicken adipose tissue.

The present study had several purposes: (1) to define the most prevalent biological processes transcriptionally up-regulated in abdominal fat, (2) to compare major differences in the abdominal fat transcriptomes of FL and LL chickens and (3) to confirm our finding of higher lipogenic gene expression in abdominal fat of FL chickens by microarray analysis (see Chapter 2). Remarkably, in the present study, functional analysis of the highest expressed genes in abdominal fat identified several significant categories: endocrine system disorders (82 genes), metabolic diseases (65 genes), lipid metabolism (165 genes), energy metabolism (46 genes) and carbohydrate metabolism (50 genes). Analysis of differential expression revealed 25 DE genes that are involved in lipid metabolism, which were also among the highest expressed genes in abdominal fat of FL and LL chickens. Another intriguing finding is that hemostasis is a highly differentially regulated process, which could have a role in the expansion of adipose mass in FL chickens. Furthermore, the high abundance of ectopically expressed genes in abdominal fat of FL and LL chickens indicates that visceral fat functions autonomously as well as an endocrine organ in the regulation of lipid metabolism and perhaps feed intake. Collectively, this evidence supports our idea that abdominal fat in FL and LL chickens makes a substantial and underappreciated contribution to adipocyte hypertrophy through *in situ* lipogenesis.

3.2 Methods

3.2.1 Animals and tissue preparation

The birds were bred and raised at INRA UE1295 Pôle d'Expérimentation Avicole de Tours, F-37380 Nouzilly, France. Hatchling FL and LL cockerels were wing-banded and vaccinated against Marek's Disease virus. Chicks were reared together in floor pens (4.4 X 3.9 m) and provided *ad libitum* access to water and a conventional starter diet for three weeks (3,050 kcal/kg metabolizable energy and 22% protein) and a grower ration (3,025 kcal/kg and 17.9% protein) for the remainder of the experiement. Chicks were held under continuous light (24 h light LL) for the first two days after hatching, followed by a 14 h light/10 h dark cycle (14L/10D) thereafter. Infrared gas heaters were used to provide supplemental heat, and ambient temperature was decreased from 32° C at hatching until 22° C was reached at 3 wk of age and held for the remainder of the experiment. At 7 wk, eight randomly selected chicks from each line were bled into heparinized syringes, killed by cervical dislocation and abdominal adipose was removed and weighed. Abdominal fat samples were immediately snap frozen in liquid nitrogen and stored at -75° C until further processing. All animal procedures were performed under the strict supervision of a French government veterinarian and in accordance with protocols approved by the French Agricultural Agency, the Scientific Research Agency, and the Institutional Animal Care and Use Committees at INRA, Nouzilly, France. These procedures were

also in compliance with the United States Department of Agriculture guidelines on the use of agricultural animals in research and approved by the University of Delaware Agricultural Animal Care and Use Committee.

3.2.2 RNA extraction, library preparation and RNA sequencing

Aliquots of adipose tissue from each of eight individuals (4 FL and 4 LL) were homogenized and cellular RNA was extracted from abdominal fat using guanidine thiocyanate and CsCl gradient purification [5], followed by a separate step for DNase I treatment. Sample quality was analyzed with an RNA 6000 Nano Assay kit and the Model 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA). An rRNA ratio (28S/18S) was observed and all samples were determined to have an RNA integrity number (RIN) greater than 9.0. Sequencing libraries were made from 5 µg of total adipose RNA with the Illumina RNA Sample Prep Kit v3 following standard Illumina protocols. Individual RNA samples were indexed (bar-coded) after the fragmentation step to enable multiplexing of libraries within sequencing lanes. Libraries were pooled and sequenced using an Illumina HiSeq 2000 Sequencing System (http://www.illumina.com/systems/hiseq_2000.ilmn, Illumina, InC., San Diego, CA) at Delaware Biotechnology Institute, University of Delaware. Three separate sequencing schemes were employed to determine the achievable depth of coverage from 1 (n = 2 sequencing samples), 4 (n = 8 sequencing samples), and 8 (n = 16 sequencing samples) multiplexed libraries per sequencing lane.

3.2.3 RNA sequence analysis

RNA-Seq analysis was completed in CLC Genomics Workbench 5.1 (http://www.clcbio.com/index.php?id=1240, CLC bio, Cambridge, MD). The data analysis included sequence data filtering, read mapping, transcript and gene identification, differential expression analysis, and functional annotation.

3.2.3.1 Sequence data filtering

Twenty-four short read (101 base pairs) sequencing samples (12 FL and 12 LL from the 3 sequencing schemes) were de-multiplexed and imported into CLC Genomics Workbench separately. Several quality control trimming methods were used within the CLC Genomics Workbench including quality trimming, ambiguity trimming and adapter trimming with default settings applied before mapping to the reference genome.

3.2.3.2 Read mapping and transcript/gene identification

The reference genome for the chicken (*Gallus gallus*, build 2.1) in FASTA format and the corresponding annotation file in GTF format were obtained from Ensembl (ftp.ensembl.org/pub/release-64), which cover 17,934 genes and 22,298 transcripts. Two hundred flanking upstream and downstream residues around known

genes were included in the analysis. The short read sequences were mapped to the reference genome, with mapping parameters enforcing: (1) a maximum of two mismatches and (2) that reads must map with ≥ 90% of the bases aligned to the reference sequence with ≥ 80% similarity. Non-specific matches (reads mapped to multiple places in the reference genome) were not included in the analysis. The RNA-Seq reads have been deposited into NCBI GEO database under the accession #GSE42980.

3.2.3.3 Differential expression analysis

The unique exon reads count (including the exon-exon and exon-intron junctions) for the reads mapped to a gene and its flanking regions were used as the raw expression value for that gene. This raw expression value was normalized to the median of the total mapped reads across the 24 samples to account for variation in original library concentration and multiplexing number.

The 24 sequencing samples (4 individuals x 2 genotypes across 3 sequencing depths) were divided into two treatment groups (FL and LL), resulting in 12 replicates in each group. Normalized expression values were analyzed as a beta-binomial model [6] to detect differential expression. The two-sided P-value was corrected using false discovery rate (FDR) to address the issue of multiple testing [7]. Genes with a FDR adjusted P-value ($P \leq 0.05$) and fold change ≥ 1.2 were considered to be differentially expressed.

3.2.4 Quantitative RT-PCR verification analysis

For verification of expression, quantitative RT-PCR (qRT-PCR) analysis was preformed on a set of DE genes identified by the RNA-Seq analysis. cDNA was prepared by first strand cDNA synthesis, performed by incubation of a 13 μl reaction (containing 1 μg of total DNase-treated RNA, 1 μl of 100 μM oligo dT_{20}, 1 μl of 10 mM dNTP mix, and water to 13 μl total volume) for 5 min at 70° C and placed on ice for 2 min. A master mix containing 5 μl of 5x first-strand synthesis buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT, and 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) was added (final reaction volume of 20 μl). The resulting cDNA was diluted to achieve a concentration of 50 ng/μl. Primers were designed for qRT-PCR using Primer Express v2.0 software (Applied Biosystems, Foster City, CA). Detailed information for each primer pair including gene name, gene symbol, primer sequences (forward and reverse), GenBank accession number and amplicon size are provided in the Appendix.

An ABI Prism Sequence Detection System 7900HT was used to perform the qRT-PCR assay, using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 400 nM of each primer (forward and reverse; Sigma-Aldrich, St. Louis, MO) in duplicate wells. A disassociation step was used to validate specific amplification and verify absence of primer dimers. PCR products were analyzed using agarose gel electrophoresis to compare product size to the expected amplicon size.

The cycle time (Ct) for each sample was normalized to the corresponding sample geometric mean of two housekeeping genes: pantothenate kinase 1 (*PANK1*) and ribosomal protein L14 (*RPL14*). These housekeeping genes were selected based on invariability in the RNA-Seq experiment and were also determined to be the most stably expressed genes assessed by qRT-PCR analysis using RefFinder (http://www.leonxie.com/referencegene.php). The $2^{-(\Delta\Delta Ct)}$ formula was used to calculate relative transcript abundance [8]. The statistical analysis was performed using a general linear model procedure in SAS v9.3.

3.3 Results

3.3.1 Mapped reads: genes and transcripts detected

Sequence data from 24 de-multiplexed short read samples (two libraries were removed due to having low quality control scores) were filtered and mapped to the reference genome (*Gallus gallus*, build 2.1). Table 3.1 shows the summary of the mapped reads and the corresponding genes and transcripts detected by the RNA-Seq analysis. On average across all depths (averaging across the FL and LL chickens), 45% (38.5 M) of reads were mapped which equates to 74% of genes (13,265) and 66% of transcripts (14,724) identified and a 14X coverage. Comparing sequencing depths (averaging across the FL and LL chickens), multiplexing 8 libraries per lane (Scheme A) resulted in 71% of genes and 63% of transcripts being detected achieving 7X coverage. These percentages increased to 73% of genes and 65% of transcripts and

coverage increased to 11X when multiplexing number was reduced to 4 libraries/lane (Scheme B). The greatest percentages were achieved by sequencing 1 library per lane (Scheme C) where 78% of genes and 70% of transcripts were detected, with 26X coverage. Genes from the reference genome were mapped to UniProtKB accession numbers by PIR ID mapping service [9] and were assigned fold change values based on the number of normalized reads from the RNA-Seq analysis.

Table 3.1 Summary of RNA-Seq coverage in abdominal fat across three sequencing depths in FL and LL chickens

Scheme (samples/lane)	Sample	Read Trimming		Read Mapping		Expressed Genes	Expressed Transcripts
		Total input reads	Paired-end reads after trimming	Total reads mapped	Total reads unmapped		
A (n=8)	FL	40.26M	34.22M	19.12M	21.14M	12810	14081
	LL	35.71M	35.34M	17.67M	18.04M	12612	13902
B (n=4)*	FL	59.13M	58.71M	32.54M	26.59M	12959	14358
	LL	67.11M	27.54M	36.02M	31.09M	13187	14599
C (n=1)	FL	123.68M	122.2M	54.64M	69.05M	13890	15550
	LL	187.85M	182.7M	71.05M	116.8M	14134	15853
Average Across Schemes	FL	74.36M	71.71M	35.43M	38.92M	13220	14663
	LL	96.89M	81.86M	41.58M	55.31M	13311	14785
Average Across Genotypes (A,B,C)		85.62M	76.78M	38.51M	47.12M	13265	14724

*One library per sequencing lane had a low QC score and was removed from the analysis; M = Million

3.3.2 Abdominal fat transcriptome of FL and LL chickens at 7 wk

We identified 1,687 DE genes with FDR corrected P-value ≤ 0.05 and a fold change larger than 1.2. Of these DE genes, 1,182 were up-regulated in LL chickens and only 505 were expressed higher in FL chickens. The number of normalized reads per gene ranged from 0 to ~840,000 reads (averaged across genotype). The highest expressed (HE) genes averaged across genotype (top five percent; 900 genes) were also analyzed. A list of 610 genes associated with lipid metabolism (lipogenesis, lipolysis, lipid transport, etc.) was compiled from the RNA-Seq analysis for comparison with HE and DE genes. The Venn diagram shows the intersection between the DE genes, the HE genes in abdominal fat and the genes associated with lipid metabolism (Figure 3.1). There were 164 DE genes which were also expressed within the top five percent of genes in abdominal fat and 108 DE genes that are associated with lipid metabolism. Further, 87 of the HE genes in abdominal fat are associated with lipid metabolism. Interestingly, 25 DE genes were amongst the top five percent of genes expressed and are also associated with lipid metabolism.

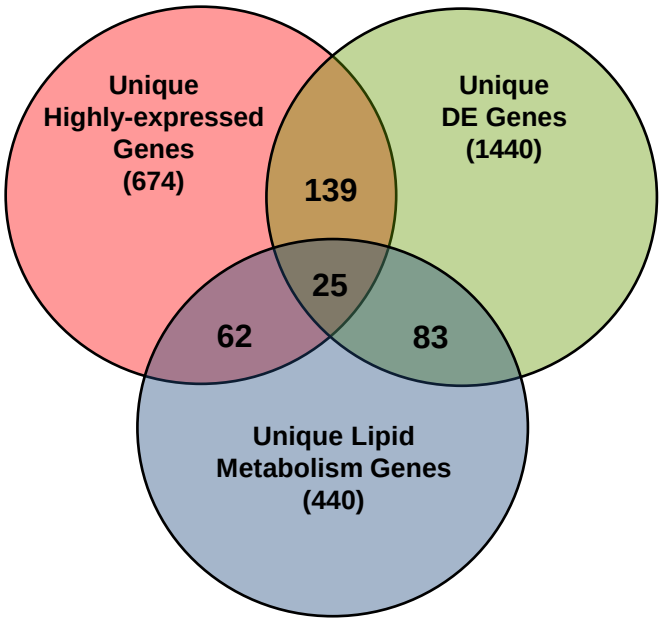


Figure 3.1 Venn diagram illustrating overlap between several functional gene lists in abdominal fat of FL and LL chickens at 7 weeks. Venn diagram showing the intersections of differentially expressed genes, genes that are highly expressed in adipose tissue and genes that are involved in lipid metabolism

3.3.3 Ingenuity Pathway Analysis (IPA) of gene interactions and functional pathways

3.3.3.1 Analysis of the highest expressed processes in abdominal fat of FL and LL chickens

The 900 HE genes in abdominal fat were submitted to the IPA knowledgebase (http://www.ingenuity.com/) for functional annotation and mapping to canonical metabolic and regulatory pathways. There were 850 genes identified as “Analysis

Ready" by IPA. The summary of the overrepresented "Top Biological Functions" that are highly expressed in abdominal fat of FL and LL chickens is shown in Table 3.2. The subcategories reveal the most prevalent processes occurring in abdominal fat, including: regulation of adiposity ["Endocrine System Disorders" (82 genes), "Metabolic Disease" (65 genes), "Lipid Metabolism" (165 genes), "Energy Metabolism" (46 genes), "Carbohydrate Metabolism" (50 genes), "mTOR Signaling" (32 genes), "IGF-1 Signaling" (16 genes), and "Glycolysis I" (8 genes)] hemostasis ["Hematological Disease" (139 genes), "Hematopoesis" (63 genes), "Hematological System Development and Function" (130 genes), "Connective Tissue Development" (118 genes) and "Integrin Signaling" (43 genes)] and cellular growth and development ["Developmental Disorder" (140 genes), "Cellular Growth and Proliferation" (351 genes), "Organismal Development" (223 genes) and "Embryonic Development" (124 genes)]. Genes in these lists can be grouped into several functional categories including transcription factors, metabolic enzymes, adipokines, hemostatic and growth factors.

Table 3.2 Top biological functions of highest expressed genes in adipose tissue of FL and LL chickens

Diseases and Disorders	**P-Value**	**# Genes**
Endocrine System Disorder	2.41E-07	82
Metabolic Disease	3.01E-06	65
Developmental Disorder	1.30E-06	140
Hematological Disease	6.63E-09	139
Reproductive System Disease	4.72E-10	225
Molecular and Cellular Functions		
Lipid Metabolism	1.19E-09	165
Energy Production	7.41E-06	46
Carbohydrate Metabolism	7.41E-06	50
Cellular Growth and Proliferation	1.72E-26	351
Hematopoesis	4.06E-05	63
Physiological System Development and Function		
Hematological System Development and Function	7.69E-10	130
Organismal Development	1.63E-18	223
Connective Tissue Development	1.98E-14	118
Embryonic Development	1.71E-12	124
Cardiovascular System Development	9.35E-19	188
Top Canonical Pathways	**P-Value**	**Genes**
Integrin Signaling	8.31E-17	(43/207)
mTOR Signaling	3.40E-08	(32/198)
IGF-1 Signaling	3.22E-05	(16/102)
Oleate Biosynthesis II (Animals)	9.26E-03	(3/13)
Glycolysis I	3.46E-05	(8/28)

Ingenuity pathway Analysis (IPA) Software was used analyze functional categories of highly expressed genes identified by RNA-Seq analysis of abdominal fat at 7 wk of age. For "Top Canonical Pathways" the ratio given for "Genes" is the number of genes in the highest expressed list out of the number of genes known (in the IPA knowledgebase) to be involved in that process.

The lipogenic influence of abdominal fat is supported by the abundance of highly expressed transcriptional regulators of lipogenesis and adipogenesis (Figure 3.2).

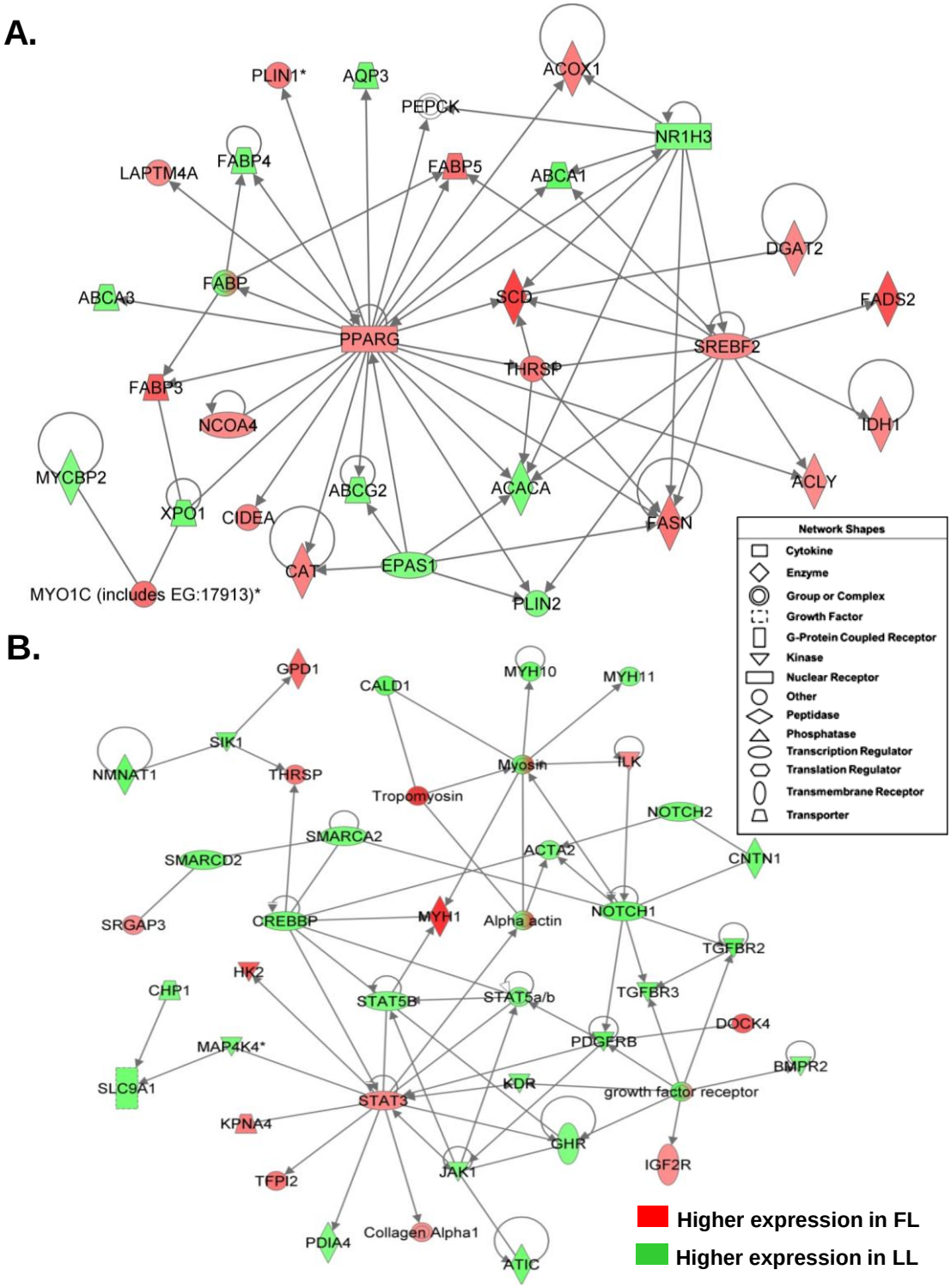

A.
PLIN1*
AQP3
ACOX1
PEPCK
NR1H3
LAPTM4A
FABP4
FABP5
ABCA1
DGAT2
ABCA3
FABP
SCD
FADS2
PPARG
SREBF2
THRSP
FABP3
NCOA4
IDH1
MYCBP2
ABCG2
ACACA
ACLY
XPO1
CIDEA
FASN
CAT
EPAS1
MYO1C (includes EG:17913)*
PLIN2
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
B.
GPD1
CALD1
MYH10
MYH11
SIK1
NMNAT1
THRSP
Myosin
ILK
Tropomyosin
NOTCH2
SMARCA2
SMARCD2
ACTA2
CNTN1
SRGAP3
CREBBP
MYH1
Alpha actin
NOTCH1
TGFBR2
HK2
CHP1
STAT5B
STAT5a/b
TGFBR3
DOCK4
MAP4K4*
PDGFRB
BMPR2
SLC9A1
KDR
growth factor receptor
STAT3
KPNA4
GHR
IGF2R
JAK1
TFPI2
PDIA4
Collagen Alpha1
ATIC
Higher expression in FL
Higher expression in LL

Figure 3.2 Interaction of highly expressed genes associated with lipogenesis and adipogenesis in abdominal fat of FL and LL chickens at 7 weeks. Functional gene interaction networks were identified by Ingenuity Pathway Analysis (IPA). Genes are colored based on fold change values from RNA-Seq analysis, where the red color signifies a higher expression in FL chickens and green color indicates higher expression in LL chickens. No false discovery rate (FDR)-cutoff was used in this analysis. Each gene was assigned a shape and function shown in the "Network Shapes" box. A. This interaction network was annotated as "Lipid Metabolism, Molecular Transport, and Small Molecular Biochemistry" and places an emphasis on transcriptional regulation of adipogenesis and lipogenesis. B. Gene interaction network of genes annotated by IPA as "Cellular Development, Cellular Growth and Proliferation, and Cellular Movement".

As an example of this regulation by transcription factors, sterol regulatory element binding factor 2 (*SREBF2*), thyroid hormone responsive spot 14 (*THRSP*), nuclear receptor subfamily 1, group H, member 3 (*NR1H3* or *LXR*α) and proliferator-activated receptor gamma (*PPARG*) interact with each other and ultimately effect the transcription of many target genes (Figure 3.2-A). Some targets of *SREBF2* which are also expressed very highly in abdominal fat include fatty acid desaturase 2 (*FADS2*), acetyl-CoA carboxylase alpha (*ACACA*), stearoyl-CoA desaturase (*SCD*), fatty acid synthase (*FASN*), ATP citrate lyase (*ACLY*), perilipin 2 (*PLIN2*), isocitrate dehydrogenase 1 (*IDH1*) and *THRSP*. *THRSP*, which itself targets *ACACA*, *FASN* and *SCD*, is also targeted by *PPARG*. Further, *PPARG* has numerous highly expressed target genes that regulate lipid metabolism [*ACOX1*, fatty acid binding protein 3-5 (*FABP3*, *FABP4* and *FABP5*), catalase (*CAT*), perilipin 1 (*PLIN1*), lysosomal-associated protein transmembrane 4A (*LAPTM4A*) and *NRIH3*].

The genes regulated by transcription factors presented in Figure 3.2 fall into the group of genes functionally associated with "Lipid Metabolism" (165 genes; 19% of input list). Some genes associated with "Lipid Metabolism" are involved in: fatty acid synthesis, elongation, and desaturation [*ACACA*, *ACOX1*, acyl-CoA dehydrogenase, long chain (*ACADL*), acyl-CoA synthetase long-chain family member 1 (*ACSL1*) and diacylglycerol O-acyltransferase 2 (*DGAT2*), fatty acid elongase 1 (*ELOVL1*), *FASN, FADS2,* and *SCD*], fatty acid transport [fatty acid binding protein 4 (*FABP3, FABP4* and *FABP5*)], and adipokine signaling [adiponectin (*ADIPOQ*) and lipoprotein lipase (*LPL*)]. Some other highly expressed genes involved in lipid metabolism include diacylglycerol kinase, zeta (*DGKZ*), insulin-like growth factor 2 receptor (*IGF2R*), insulin-like growth factor binding protein 2 and 7 (*IGFBP2* and *IGFBP7*, respectively), malate dehydrogenase 2, NAD (*MDH2*), and somatostatin receptor 2 (*SSTR2*).

Signal transducers and activators of transcription (STATs; *STAT3* and *STAT5B*) are also highly expressed in adipose tissue (Figure 3.2-B). *STAT3* is a binding partner of *STAT5B*, *GHR*, and mitogen-activated protein kinase kinase kinase kinase 4 (*MAP4K4*) and directly targets hexokinase 2 (*HK2*), tissue factor pathway inhibitor 2 (*TFPI2*) and protein disulfide isomerase family A, member 4 (*PDIA4*). Conversely, several genes that target *STAT3* are also highly expressed [Janus kinase 1 (*JAK1*), kinase insert domain receptor (*KDR*), platelet-derived growth factor receptor, beta polypeptide (*PDGFRB*), and CREB binding protein (*CREBBP*): which also targets *THRSP*]. Another gene regulated through *THRSP*, highly expressed in

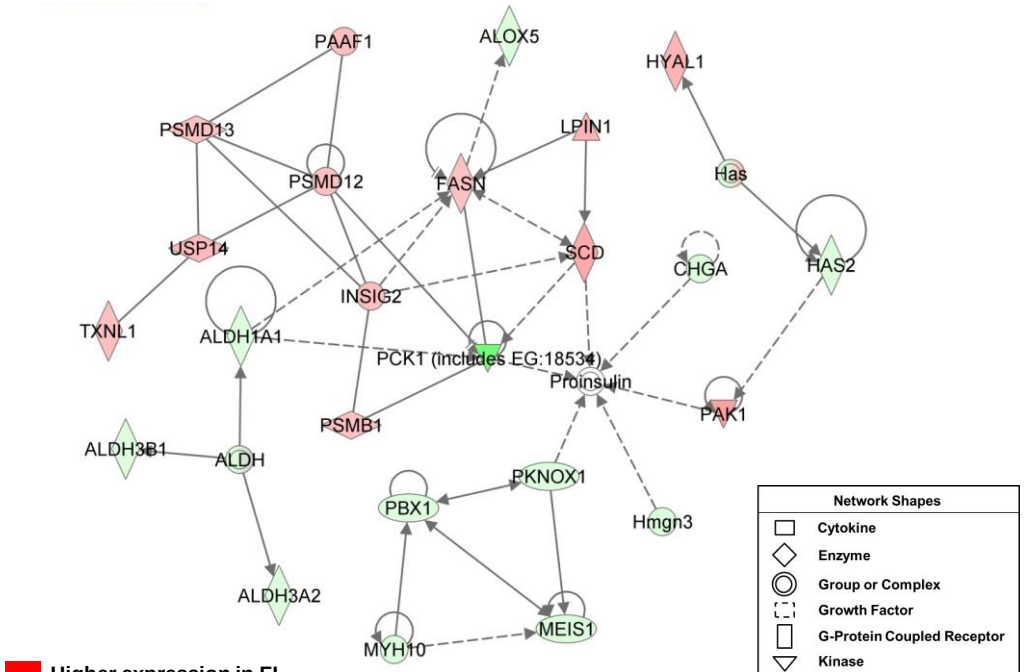
abdominal fat at 7 wk, is glycolytic enzyme glycerol-3-phosphate dehydrogenase 1 (*GPD1*), an important regulator of adiposity. Furthermore, NOTCH cellular signaling and growth regulation is a significant process occurring in abdominal fat of FL and LL chickens. Notch 1 (*NOTCH1*) has interactions with *PDGFRB*, transforming growth factor, beta receptor II and III (*TGFBR2* and *TGFBR3*), actin, alpha 2 (*ACTA2*), contactin 2 (*CNTN2*) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (*SMARCA2*).

3.3.3.2 Analysis of differential expression between FL and LL chickens

Differentially expressed genes were submitted to the IPA knowledgebase (http://www.ingenuity.com/) for functional annotation and mapping to canonical metabolic and regulatory pathways. A total of 1,479 genes were identified as "Analysis ready" by IPA. Some of the major "Diseases and Disorders" identified include "Connective Tissue Disorders" (184 DE genes), "Developmental Disorder" (255 DE genes) and "Cardiovascular Disease" (195 DE genes). A large number of DE genes can be mapped to several interesting canonical pathways. For example, 14 percent (32/231 known genes) of genes in the "LPS/IL-1 Mediated Inhibition of RXR Function" pathway were DE between FL and LL chickens. "PTEN signaling" and "Protein Kinase A Signaling" pathways also had numerous DE genes (20/132 and 47/395, respectively). Other interesting canonical pathways were "TR/RXR Activation" (15/89 genes were DE) and "Coagulation System" (8/35 genes were DE).

Differential regulation of the molecular and cellular function subcategory "Lipid Metabolism" (183 genes; 25 of which are HE genes) is of particular interest to this study. A large number of DE genes involved in lipid metabolism were identified by functional gene interaction analysis (Figure 3.3). Proinsulin is indirectly affected by PBX/knotted 1 homeobox 1 (*PKNOX1*), high mobility group nucleosomal binding domain 3 (*HMGN3*), p21 protein (Cdc42/Rac)-activated kinase 1 (*PAK1*), chromogranin A (parathyroid secretory protein 1; *CHGA*), phosphoenolpyruvate carboxykinase 1, cytosolic (*PCK1*) and *SCD* (Figure 3.3-A). Lipin 1 (*LPIN1*), which is up-regulated in FL chickens, directly interacts with *FASN* and *SCD* (higher in the FL). Both *SCD* and *FASN* also interact with insulin induced gene 2 (*INSIG2*) and *PCK1*. Further, several genes associated with the proteasome [proteasome 26S subunit, non-ATPase, 12 (*PSMD12*), proteasome 26S subunit, non-ATPase, 13 (*PSMD13*), proteasomal ATPase-associated factor 1 (*PAAF1*), and ubiquitin specific peptidase 14 (*USP14*)] were up-regulated in FL chickens. Aldehyde dehydrogenase family 3 members A2 and B1 (*ALDH3A2* and *ALDH3B1*, respectively), and family 1 member A1 (*ALDH1A1*) were expressed higher in LL chickens. Also up-regulated in LL chickens were meis homeobox 1 (*MEIS1*), responsible for maintenance of a proliferative state of hematopoietic progenitor cells, and hyaluronan synthase 2 (*HAS2*), which is involved in the synthesis of hyaluronan. Conversely, hyaluronoglucosaminidase 1 (*HYAL1*), which degrades hyaluronan, was higher in FL chickens.

A.


PAAF1
ALOX5
HYAL1
PSMD13
LPIN1
PSMD12
FASN
Has
USP14
SCD
CHGA
HAS2
INSIG2
TXNL1
ALDH1A1
PCK1 (includes EG:18534)
Proinsulin
PAK1
PSMB1
ALDH3B1
ALDH
PKNOX1
PBX1
Hmgn3
ALDH3A2
MEIS1
MYH10
Higher expression in FL
Higher expression in LL
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter

B.

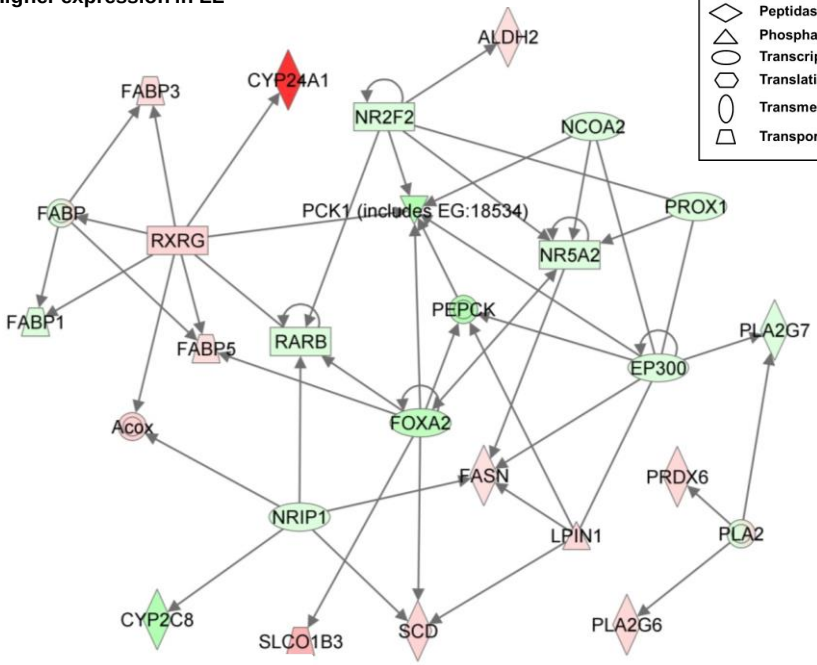

ALDH2
CYP24A1
FABP3
NR2F2
NCOA2
FABP
RXRG
PCK1 (includes EG:18534)
PROX1
NR5A2
FABP1
PEPCK
PLA2G7
FABP5
RARB
EP300
Acox
FOXA2
FASN
PRDX6
NRIP1
LPIN1
PLA2
CYP2C8
SLCO1B3
SCD
PLA2G6

Figure 3.3 Interaction of differentially expressed genes associated with lipid metabolism in abdominal fat of FL and LL chickens at 7 weeks. Functional gene interactions were identified by IPA of DE genes (FDR≤0.05). Genes are colored based on fold change values from RNA-Seq analysis where the red color signifies a higher expression in FL chickens and the green color indicates a higher expression in LL chickens. Each gene was assigned a shape and function shown in the "Network Shapes" box. A. This interaction network was annotated as "Lipid Metabolism, and Small Molecular Biochemistry". B. Gene interaction network annotated by IPA as "Energy Production, Lipid Metabolism and Small Molecule Biochemistry".

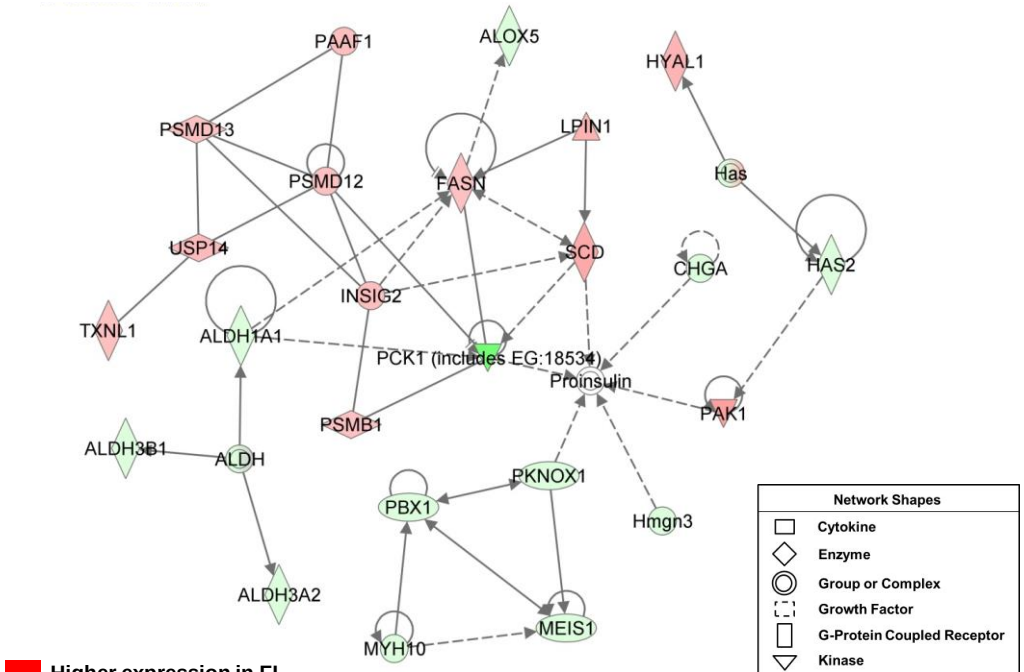
Retinoid X receptor gamma (*RXRG*), up-regulated in FL chickens, targets *FABP1*, *3*, and *5*, *CYP24A1* and the ACOX group of genes [e.g., acyl-CoA oxidase 2, branched chain (*ACOX2*)] (Figure 3.3-B). A binding partner of *RXRG*, retinoic acid receptor, beta (*RARB*) is directly targeted by nuclear receptor interacting protein 1 (*NRIP1*), nuclear receptor subfamily 2, group F, member 2 (*NR2F2*) and forkhead box A2 (*FOXA2*), which are all up-regulated in LL chickens.

The prevalence of differentially expressed G-coupled protein receptors in abdominal fat is revealed in Figure 3.4-A. Receptors for the neuropeptides melanocortin, somatostatin, and neuropeptide Y (*MC5R*, *SSTR2* and *NPY2R*, respectively) were all up-regulated in FL chickens. The urotensin 2 receptor (*UTS2R*) and endothelin receptor B (*EDNRB*) were also higher in FL chickens while the growth factor ligands for the endothelin receptor (endothelin 1 and 2; *EDN1* and *EDN2*, respectively) were higher in the LL. Other genes up-regulated in LL chickens were the G-coupled protein receptors for glucagon-like peptide 1 (*GLP1R*), lysophosphatidic acid (*LPAR3*), glutamate (*GRM8*), cannabinoid (*CNR1*), thrombin (*F2R*) and

angiotensin II [angiotensin II receptor, type 1 and 2 (*AGTR1* and *AGTR2*)]. The mineralocorticoid nuclear receptor [nuclear receptor subfamily 3, group C, member 2 (*NR3C2*)], several of its targets [sodium channel, non-voltage-gated 1 alpha subunit (*SCNN1A*), FK506 binding protein 1B (*FKBP1B*), ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (*ABCC9*), *AGTR1* and *EDN1*)], parathyroid hormone-like hormone (*PTHLH*) and homeobox A3 (*HOXA3*) were also higher in LL chickens. The plasmin (fibrinolysis) activator, *PLAU*, was up-regulated in FL chickens.

The transcript for major pancreatic hormone glucagon (*GCG*) was expressed higher in abdominal fat of LL chickens along with dipeptidyl-peptidase 4 (*DPP4*), a proteolytic enzyme which inactivates glucagon-like peptide 1 (GLP-1) (Figure 3.4-B). *GCG* indirectly inhibits phosphodiesterase 3A, cGMP-inhibited (*PDE3A*) and down-regulates aldehyde dehydrogenase 2 (*ALDH2*). *ALHD2* binds to *MDH2* (higher expression in FL chickens) and indirectly decreases expression of guanylate cyclase 1, soluble, beta 2 (*GUCY1B2*) and 2',3'-cyclic nucleotide 3' phosphodiesterase (*CNP*). Another target of *GCG*, forkhead box A2 (*FOXA2*; also up-regulated in LL chickens) directly up-regulates several genes [3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*), albumin (*ALB*), and nuclear receptor *NR5A2* (which is also a direct target of *NR2F2*)]. Key regulators of prostaglandin [prostaglandin-endoperoxide synthase 1 (*PTGS1*)] and ceramide [ceramide synthase 1 (*CERS1*)] synthesis were up-regulated in LL chickens while the enzyme that catalyzes the synthesis and degradation of ceramide into sphingosine and fatty acid [N-acylsphingosine amidohydrolase (acid ceramidase) (*ASAH1*)] was expressed higher in FL chickens.

A.



Higher expression in FL

Higher expression in LL

B.

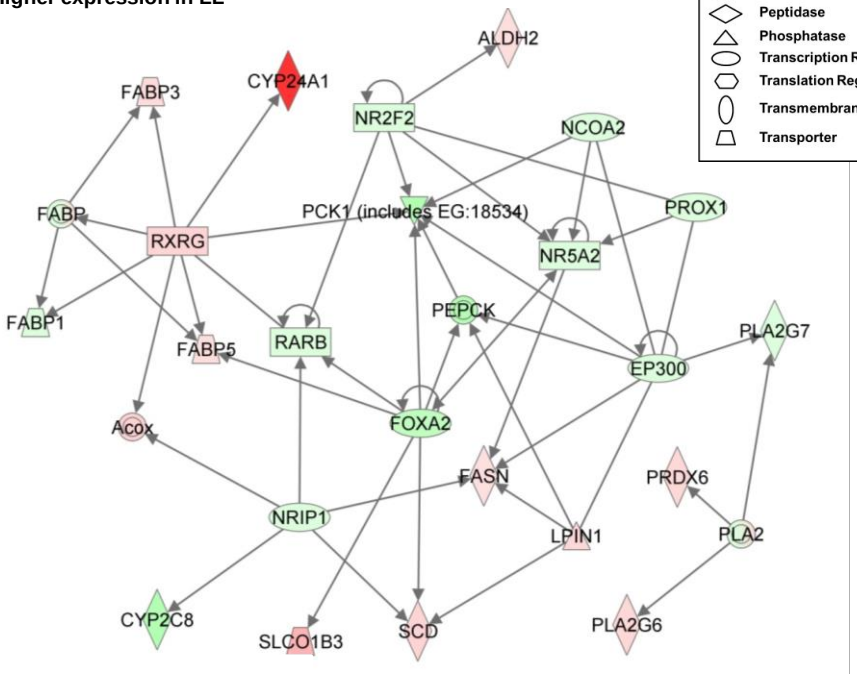


Figure 3.4 Interaction of differentially expressed genes in abdominal fat of FL and LL chickens at 7 weeks. Functional gene interactions were identified by IPA. Genes are colored based on fold change values (FL/LL) from RNA-Seq analysis where red signifies higher expression in FL chickens and green higher expression in LL chickens. Each gene was assigned a shape and function shown in the "Network Shapes" box. A. This gene interaction network was annotated by IPA as "Nucleic Acid Metabolism, Small Molecule Biochemistry and Cardiovascular System Development and Function". B. Gene interaction network annotated by IPA as "Lipid Metabolism, Small Molecule Biochemistry and Molecular Transport".

3.3.4 Verification of RNA-Seq analysis by Quantitative RT-PCR

Based on biological function, candidate genes were selected from the RNA-Seq analysis for qRT-PCR verification. The results of the qRT-PCR analysis are shown in Figure 3.5 along with the corresponding results from the RNA-Seq analysis. Of the genes shown, 41 were DE in the RNA-Seq analysis (FDR adjusted *P*-value $\leq$ 0.05 with fold change $\geq$ 1.2). All 41 genes were also significantly different ($P \leq 0.05$) by qRT-PCR analysis. Neuropeptide Y (*NPY*) was a candidate gene not significantly different between the FL and LL chickens by RNA-Seq or qRT-PCR analysis. Similarly, the glucagon receptor (*GCGR*) was not differentially expressed by RNA-Seq, however, was 1.6 fold higher in LL chickens by qRT-PCR analysis ($P \leq 0.05$). The short chicken growth hormone isoform (*scGH*) was 1.5-fold higher ($P \leq 0.05$) in FL chickens by qRT-PCR analysis while its expression was not determined by RNA-Seq analysis. Albumin (*ALB*; not shown in figure) was highly up-regulated in LL chickens by both RNA-Seq (38 fold) and qRT-PCR (73 fold) analyses. Furthermore, the fold

change values obtained across analyses were highly correlated (Pearson Correlation r = 0.90; $P < 0.001$).

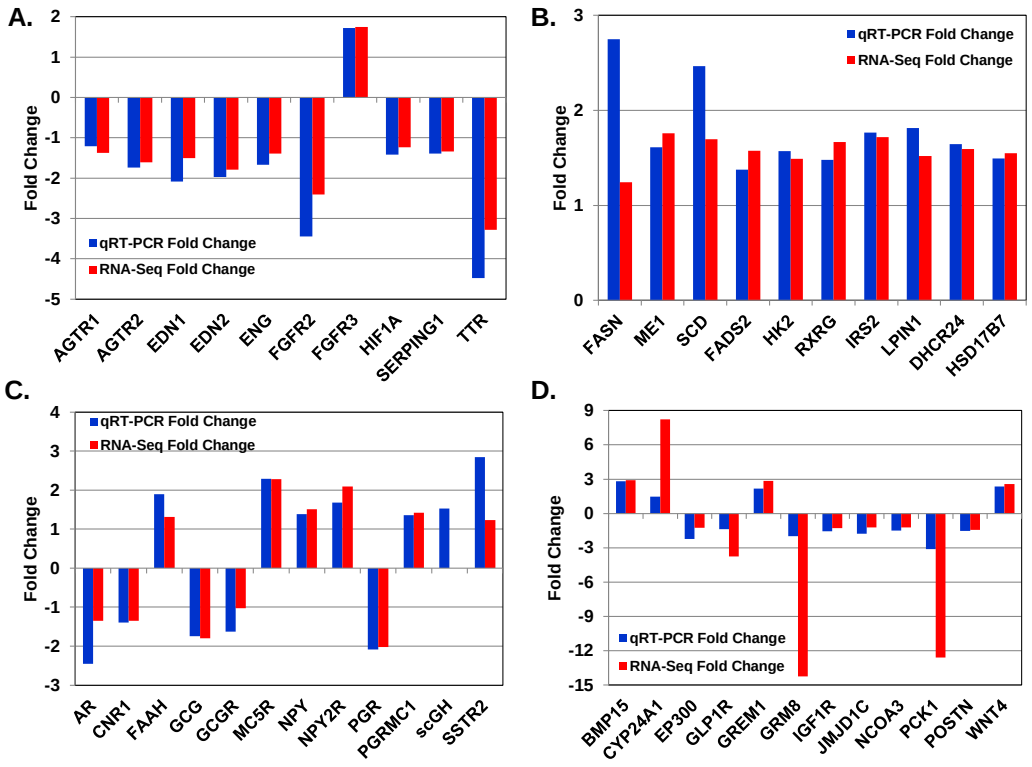


Figure 3.5 **Verification of RNA-Seq expression by qRT-PCR analysis in abdominal fat of FL and LL chickens at 7 weeks.** Quantitative RT-PCR expression ratios (blue bars) are compared expression ratios from RNA-Seq analysis (red bars). Shown are comparisons for 41 genes differentially expressed by RNA-Seq analysis, two genes not differentially expressed by RNA-Seq analysis (NPY and GCGR) and a candidate gene not measured by RNA-Seq analysis (scGH). Fold change values for genes expressed higher in FL chickens are assigned a positive value and genes expressed higher in LL chickens negative. A. Fold change comparisons for genes associated with hemostasis. B. Fold change comparisons for genes involved in up-regulation of lipogenesis and cholesterol synthesis. C. Fold change comparisons for "ectopically" expressed genes in abdominal fat. D. Fold change comparisons for other interesting DE genes in abdominal fat.

3.3.5 Power to detect differential expression in abdominal fat of FL and LL chickens

A power analysis on this RNA-Seq experiment, using expression data for all genes analyzed, was completed using the web-based software "Scotty" (http://euler.bc.edu/marthlab/scotty/scotty.php [10]). This analysis determined that our sample size (n=4), sequenced across three depths, was adequate for each genotype to detect ~70% of genes expressed at 1.5 fold difference and ~90% at a fold change of 2 [$P \leq 0.05$ and 55 million reads per sample, the average obtained by this experiment (see Table 3.1)]. Further, the "Scotty" program performed hierarchical clustering on our RNA-Seq dataset using Spearman correlation as the distance metric. This analysis grouped the two genotypes separately with the individuals from each the FL and LL being closely linked (graph not shown).

3.4 Discussion

The FL and LL chickens were developed to analyze the underlying effects of genetic selection for excessive fatness, a trait that is obfuscated by novel features of avian metabolism. At 7 wk the FL and LL chickens exhibit a 2.8-fold difference in abdominal fatness at the same body weight at 7 wk (see Chapter 2) [11] while maintaining similar feed intake [12]. To maintain a similar body weight despite large differences in abdominal fatness, FL chickens appear to favor partitioning of nutrients (particularly dietary amino acids) into abdominal fat and have a higher protein

turnover rate [12]. On the other hand, LL chickens have a higher lean muscle mass and a lower abdominal fat content than do the FL chickens. Several metabolic (liver, adipose tissue, and skeletal muscle) and regulatory (pituitary and hypothalamus) tissues of the FL and LL chickens have been analyzed using numerous transcriptional methods (i.e., differential mRNA display, quantitative RT-PCR, low and high density microarrays, etc.) to unravel the underlying mechanisms of genetic selection for excessive fatness [11, 13-17] (see Chapter 2). To our knowledge, this is the first deep RNA-Seq gene expression analysis in the FL and LL chickens, or any line of chickens genetically selected for differences in abdominal fatness. This analysis confirms our earlier observation of a substantial contribution of lipogenesis in abdominal fat to visceral fatness (see Chapter 2), reveals ectopic expression of many new candidate targets with potential roles in regulating excessive fatness, and validates (across experiments) several important genes involved in the regulation of adiposity in chickens.

Understanding the molecular mechanism of obesity requires cross-model validation of processes and gene expression. A limited number of large scale transcriptional studies have been completed in abdominal fat of chickens [18-20], the most similar to the present study being recently completed by Ji *et al* [20]. We found 80 DE abdominal fat genes that are common between the FL and LL chickens and commercial broiler chickens subjected to a brief (5h) period of fasting [20]. Further, five DE genes are shared between FL and LL chickens at 7 wk and in chickens after acute insulin immunoneutralization [20]. While there is overlap in the DE gene lists

between genetic and nutritional perturbation models, many differences exist in the processes being affected and the manner in which they are affected. This is likely due to the FL and LL chickens exhibiting prolonged accretion of adipose tissue while nutritional perturbations represent rapid changes in gene expression to meet short term energy demands. Therefore, genes common between these genetic and nutritional perturbation studies appear to be involved in both chronic and acute deposition of visceral fat. These common genes include metabolic enzymes, growth factors and several genes whose expression in abdominal fat is considered abnormal.

3.4.1 Lipid metabolism is altered in abdominal fat of FL and LL chickens

A major finding of our time course microarray analysis in abdominal fat of FL and LL chickens (see Chapter 2) was discovery of many DE genes involved in lipid synthesis, which challenges the accepted notion that adipose tissue of chickens has limited lipogenic activity [20-22]. The up-regulation of many DE lipogenic genes in FL chickens has now been confirmed by both RNA-Seq and qRT-PCR analyses of abdominal fat. Further, this finding was validated cross experimentally comparing the FL and LL chickens to chickens exposed to nutritional perturbations [20] where several lipogenic genes were differentially expressed. For example, *ME1*, which is critical for the synthesis of lipids via generation of NADPH required for the conversion of malonyl-CoA to fatty acids (by *FASN*), was down-regulated in LL chickens, which is similar to fasted chickens. Further, both the soluble (*MDH1*) and

mitochondrial (*MDH2*; expressed higher in adipose tissue and liver [16] of FL chickens) forms of malate dehydrogenase are among the top six percent of highest expressed genes in abdominal fat of FL and LL chickens. *ME1* expression responds to changes in dietary protein content (increasing dietary protein decreases *ME1* expression [23]) or administration of dexamethasone [24]. In genetically fat pigs, the expression of *MDH1*, *MDH2* and *ME1* show a strong positive correlation with adipocyte volume [25], which is also seen in the FL chickens.

Another gene differentially expressed in both genetic and nutritional perturbations is *LPIN1*, which was identified as a reciprocal regulator of triglyceride synthesis and hydrolysis in adipocytes of *LPIN1* knockout mice [26]. This gene was expressed higher in abdominal fat of our genetically fat (FL) chickens while 5 hr of food deprivation [20] and 30% energy restriction in chickens [27] also resulted in up-regulation. These findings suggest that genetic perturbation and nutritional perturbation have converse effects on the expression of *LPIN1*. This is supported by genetic knockdown of *LPIN1* expression in mice [26, 28] resulting in decreased adiposity and, on the other hand, by the decreased expression of *LPIN1* in subcutaneous and visceral adipose tissue of overweight and obese humans (presumably a nutritional perturbation [29]). Furthermore, *LPIN1* controls several specific differentially regulated processes in FL and LL chickens including adipogenesis, lipogenesis and eicosanoid signaling (discussed below).

The *LPIN1* regulation of adipogenesis is via control of phosphatidate levels which influence PPARG expression [28] and it is required for PPARG driven

adipogenesis both *in vitro* and *in vivo* [30, 31]. Further, LPIN1 has also been described as a co-regulator of transcription factor signaling interacting with PPARs (PPARA, PPARG, and PPARGC1A) and CEBPA [32, 33]. These interactions, and others like it, are part of a complex labyrinth which determines which processes will be up-regulated in a cell/organism (i.e. lipolysis/lipogenesis) during perturbation. In fact, the complex interactions between transcription factors and their ultimate effect on the expression of target genes are perhaps the most crucial elements in the regulation of lipogenesis/lipolysis in abdominal fat. An example of this elaborate regulation is depicted in FL chickens, where these interactions ultimately lead to enhanced adipogenesis and lipogenesis (Figure 3.6).This figure describes a somewhat hierarchical structure within a group of interacting transcription factors (central in figure) where PPARA regulates the expression/function of NR1H2, NR1H3, RXRG, PPARG and PPARGC1A. These activated transcription factors activate other transcription factors (i.e., SREBF1, SREBF2, CEBPA and PPARGC1B). It must be noted that reciprocal activation does occur between these transcription factors meaning that there is not truly a set hierarchy, and thus the "expression order" is controlled largely by the order of activation (driven by ligand availability, etc.) of transcription factors and which co-activators are present. Since many transcription factors are ligand activated (e.g., PPARs, RARs, RXRs, LXRs, etc.), form heterodimers, and are part of a transcriptional unit (comprised of transcription factors, co-activators and co-repressors [34, 35]) their regulation is more complex. This implies that if a ligand and/or heterodimeric partner for the transcription factor is

present in higher quantities in one genotype, the transcription factor itself would not have to be differentially expressed between the FL and LL chickens.

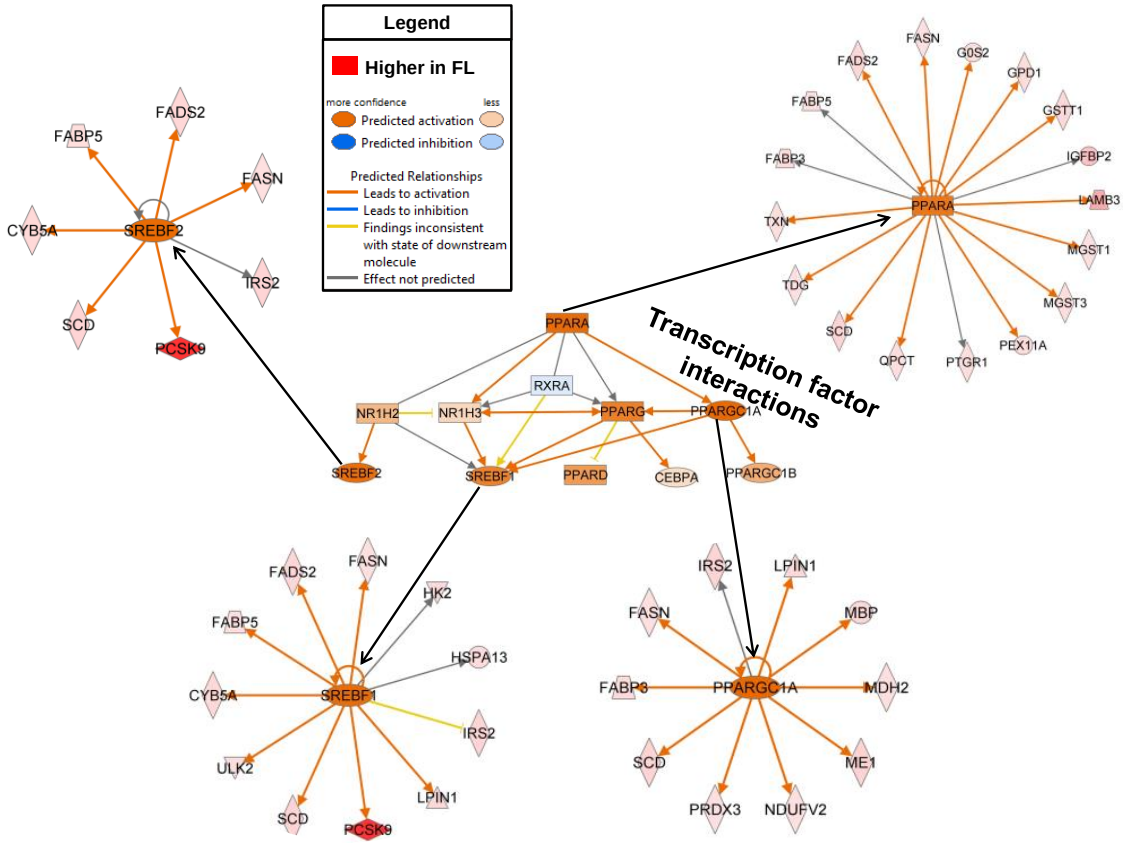


Figure 3.6 Transcription factor interactions and up-regulation of lipogenesis in abdominal fat of FL chickens at 7 weeks. Interactions and IPA predicted expression for transcription factors are shown in the center of the figure. The ultimate result of the interactions between transcription factors are shown in the periphery of the figure for PPARA, PPARGCIA, SREBF1 and SREBF2. Only values for DE genes up-regulated in FL chickens are shown.

For example, transcripts for PPARG, which is naturally activated by long-chain fatty acids [36] nitrolinoleic acid and prostaglandin J2 (reviewed by [37]), *SREBF2*, which is inhibited by high levels cholesterol, and THRSP, which is

responsive to thyroid hormone and lipid derivatives, were very highly expressed, though not differentially expressed in abdominal fat of FL and LL chickens at 7 wk. THRSP is an important lipogenic transcription factor which has many lipogenic targets and is, itself, targeted by SREBF2 and PPARG. While increased cholesterol in FL chickens [suggested by up-regulation of genes involved in cholesterol biosynthesis; e.g. *HSD17B7* [38], and *DHCR24* [39]] might directly inhibit SREBP2, it would also activate NR1H2 (LXRB; Figure 3.6) which directly targets SREBF2 [40, 41]. The potential increase in activation of SREBF2 and PPARG (evidenced by increased long-chain fatty acid through higher expression of *FASN* and *SCD*) coupled with increased levels of active thyroid hormone (T_3) in FL chickens (unpublished data) not only greatly increases THRSP expression and activation, but also amplifies the lipogenic response in abdominal fat of these chickens.

Individually, each of the transcription factors (Figure 3.6) has many targets involved in the synthesis and regulation of lipid. Differentially expressed targets expressed higher in FL chickens for several of these transcription factors (PPARA, SREBF1, SREBF2 and PPARGCIA) are shown in the periphery of the figure. Interestingly, there are numerous redundancies in targets across transcription factors (i.e., *FABP5*, *FADS2*, *FASN*, *IRS2*, *LPIN1*, *PCSK9*, *SCD*, etc.) which show an intensified lipogenic response in FL chickens. The lipogenic response to this transcription factor regulation in genetically fat and lean chickens appears to be much greater than that seen during nutritional perturbation [20]. For example, some of the highest expressed genes and thus most biologically important differences between FL

and LL chickens are emphasized by comparing the DE gene list to the highest expressed genes in abdominal fat which are involved in lipid metabolism (see Table 3.3). Of these, the most remarkable include *FABP3* and 5, *FADS2*, *FASN*, *GPD1*, *HK2*, *HSD17B7*, *IGFBP2*, *MDH2*, *PGRMC1* and *SCD* (controlled by transcription factors represented in Figure 3.6). Although not among the highest expressed genes in adipose tissue, the FL chickens also over express malonyl CoA:ACP acyltransferase (*MCAT*; 1.4-fold) acyl-CoA oxidase 2, branched chain (*ACOX2*; 1.8-fold), and acyl-CoA synthetase bubblegum family member 2 (*ACSBG2*; 1.3-fold). Ultimately, these enzymes (*HK2*, *GPD1*, *MDH2*, *MCAT*, *ACOX2*, and *ACSBG2*) aid in the generation of substrate for lipid synthesis via major lipogenic enzymes: *FASN* and *SCD*.

Table 3.3 Most interesting genes in adipose tissue of FL and LL chickens

Gene Symbol	Gene Name	Fold Change	Average Reads
Differentially Expressed and Highest Expressed Lipid Metabolism Genes			
FABP3	fatty acid binding protein 3, muscle and heart	1.4	10216
FABP5	fatty acid binding protein 5	1.3	10800
FADS2	fatty acid desaturase 2	1.6	6764
FASN	fatty acid synthase	1.2	32362
GPD1	glycerol-3-phosphate dehydrogenase 1	1.3	16641
HK2	hexokinase 2	1.5	5270
HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	1.5	4293
IGFBP2	insulin-like growth factor binding protein 2,	2.5	5886
MDH2	malate dehydrogenase 2, NAD (mitochondrial)	1.2	5214
PGRMC1	progesterone receptor membrane component 1	1.4	22381
SERINC1	serine incorporator 1	1.2	8013
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	1.7	93949
ABCA1	ATP-binding cassette, sub-family A member 1	-1.3	6388
IGFBP7	insulin-like growth factor binding protein 7	-1.4	10915
PDGFRB	platelet-derived growth factor receptor, beta	-1.3	8198
Differentially Expressed Genes			
ACOX2	acyl-CoA oxidase 2, branched chain	1.8	370
ACSBG2	acyl-CoA synthetase bubblegum member 2	1.3	2147
DHCR24	24-dehydrocholesterol reductase	1.6	3843
EDNRB	endothelin receptor type B	1.6	237
INSIG2	insulin induced gene 2	1.3	763

MCAT	malonyl CoA:ACP acyltransferase	1.4	833
PLAU	plasminogen activator, urokinase	1.7	694
PRKAG2	protein kinase, AMP-activated, gamma 2	1.4	1286
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	-2.5	646
ALOX5	arachidonate 5-lipoxygenase	-1.5	449
BMP5	bone morphogenetic protein 5	-1.5	162
BMP7	bone morphogenetic protein 7	-1.7	154
DAGLA	diacylglycerol lipase, alpha	-1.3	627
FAR1	fatty acyl CoA reductase 1	-1.8	424
FAR2	fatty acyl CoA reductase 2	-1.3	588
HPGDS	hematopoietic prostaglandin D synthase	-1.6	152
IRS4	insulin receptor substrate 4	-1.2	669
RARB	retinoic acid receptor, beta	-1.5	433
TNFSF10	tumor necrosis factor (ligand) member 10	-1.4	1641
Highest Expressed Genes (not DE)			
ACACA	acetyl-CoA carboxylase alpha		6729
ACACB	acetyl-CoA carboxylase beta		10524
ACAD9	acyl-CoA dehydrogenase family, member 9		6329
ACADL	acyl-CoA dehydrogenase, long chain		5508
ACLY	ATP citrate lyase		6716
ACOX1	acyl-CoA oxidase 1, palmitoyl		22460
ACSL1	acyl-CoA synthetase long-chain family member 1		60335
ACSS2	acyl-CoA synthetase short-chain family member 2		7764
ADIPOQ	adiponectin, C1Q and collagen domain containing		10438
ANG	angiogenin, ribonuclease, RNase A family, 5		5741
ELOVL1	ELOVL fatty acid elongase 1		6289
FABP4	fatty acid binding protein 4, adipocyte		45632
GHR	growth hormone receptor		4754
LPL	lipoprotein lipase		163988
NFKBIA	nuclear factor of kappa inhibitor, alpha		6990
PLIN1	perilipin 1		54722
PLIN2	perilipin 2		8175
PPARG	peroxisome proliferator-activated receptor gamma		6837
SREBF2	sterol regulatory element binding transcription factor 2		7325
STAT5B	signal transducer and activator of transcription 5B		9306
THRSPA	thyroid hormone responsive Spot 14 protein, alpha		42780

Comparison of highest expressed and differentially expressed genes in abdominal fat. Genes with positive fold change values are higher expressed in the FL and genes with negative fold change values are higher in LL chickens. Average reads across the FL and LL chickens from RNA-Seq analysis are given.

Hormone receptors play a major role in controlling lipid metabolism in abdominal fat of FL and LL chickens. The androgen receptor (*AR*) was expressed 40 percent higher in LL chickens compared to their fatter counterpart. Androgen signaling in adipose tissue of murine models protects against obesity and regulates insulin action and glucose homeostasis, where *AR* knockout mice are more susceptible

to high-fat diet–induced visceral obesity [42]. The up-regulation of *AR* in abdominal fat of the LL may suggest a similar mechanism for *AR* signaling in adipose tissue of chickens. Progesterone signaling in abdominal fat of FL and LL chickens provides further evidence for differential regulation of steroid hormone signaling. The receptor for progesterone (*PGR*) was up-regulated over 2-fold in LL chickens. Interestingly, progesterone administration in rats increases body and inguinal white adipose tissue mass, a response only observed in females [43]. This suggests that up-regulation of *PGR* in abdominal fat of LL cockerels indirectly impacts lipid metabolism. The membrane-bound progesterone receptor, *PGRMC1*, is one of the highest expressed genes in abdominal fat (Table 3.3) of FL chickens. *PGRMC1* has been shown to directly regulate cholesterol synthesis and hormone metabolism in mammals [44].

3.4.2 Hemostatic mechanism in abdominal fat of chickens

Adipose tissue exhibits local and endocrine control of primary hemostasis including vasculature constriction and dilation which has important consequences on adipogenesis [45] and angiogenesis as well as the regulation of blood pressure [46]. This vascular regulation is highly complex and thus is controlled through several mechanisms. One of these mechanisms is the renin-angiotensin system (RAS), which was originally identified as a systemic regulator of blood pressure and it has now been determined that all components of this system are expressed in adipose tissue [47]. Angiotensin II (produced locally in adipose tissue or released into circulation from

other tissues and acting in adipose tissue) is the active ligand in the RAS which has both an involvement in lipid metabolism through regulating insulin signaling in adipose tissue [45, 48] and also plays an important role in the regulation of local and systemic blood pressure through the constriction of peripheral arterioles. The G-coupled protein receptor for angiotensin II, *AGTR1*, was up-regulated by both insulin immunoneutralization and by fasting [20]. Similarly, we found that *AGTR1* and *AGTR2* were down-regulated in adipose tissue of FL chickens (Table 3.3). The down-regulation of angiotensin II receptors in adipose tissue of obese models (FL, fed and normal insulin activity) suggests an increased vasodilation in abdominal fat, a process that accompanies angiogenesis [49], allowing for expansion of adipose mass. Further, an overactive RAS could inhibit adipocyte differentiation [45] which suggests that adipose tissue of LL chickens is down regulating adipogenesis through this process.

Vascular tone is also mediated through the release of vasoconstrictors or vasodilators independent of the RAS including catecholamines, endothelins and nitric oxide. The decreased expression of potent vasoconstrictors *EDN1* and *EDN2* in adipose tissue of FL chickens reinforces the hypothesis of increased vasodilation in abdominal fat of fat chickens. Interestingly, while only the endothelin receptor B (*EDNRB*) was differentially expressed (1.5-fold higher in FL chickens), *EDNRA* was expressed over 10-fold higher which suggests that it is the more active isoform in adipose tissue of chickens. In mammals, the major effects of vasodilation are mediated through down regulation of vasoconstrictors and/or through the increased generation/action of nitric oxide (NO). NO production, mediated by nitric oxide

synthase (NOS), is usually driven by hypoxia [50]. Hypoxic conditions in adipose tissue in mammals leads to overproduction, and ultimately resistance to NO [51]. However, hypoxia does not seem to stimulate the over production of NO in abdominal fat of the FL chickens, where *HIF1A* is down regulated and the expression of NOS is no different from their lean counterpart. We cannot comment on the sensitivity of visceral fat of FL and LL chickens to NO, but our results do not indicate an overproduction, thus we hypothesize that there is no difference in sensitivity between the lines. Rather, regulation of NO signaling in these chickens appears to be controlled by enzymes involved in the breakdown of NO-derived cGMP and cAMP (which itself is under the regulation of cGMP [52]), which are expressed at lower levels in fat chickens (*PDE1C*, *PDE3A*, *PDE5A*, *PDE9A* and *PDE10A*). Phosphodiesterases catalyze the degradation of cGMP, ultimately inhibiting the vasodilation effects of nitric oxide, and have been implemented in the treatment of cardiovascular diseases [53], respiratory diseases [54] and metabolic syndrome [55]. For example, PDE1C, a non-selective phosphodiesterase, is amongst the highest expressed phosphodiesterases in rat β-islet cells and knockdown of this gene significantly increases insulin secretion [56]. Adipose tissue expression of another nonselective phosphodiesterase, *PDE3A*, was correlated with weight loss in humans after Roux-en-Y gastric bypass [57]. Furthermore, inhibition of cGMP specific phosphodiesterases (i.e., PDE5A and PDE9A) is effective in cardio protection [53].

The up-regulation of growth factors (*PDGFC* and *FGFR3*) and *GREM1* in FL chickens supports an angiogenic mechanism. The higher expression of *FGFR3* in FL

chickens is similar to what was observed in adipose tissue of ApoE knockout mice, which exhibit increased adiposity when fed a high fat diet [58]. The expression of *GREM1*, a bone morphogenetic protein antagonist, correlates with increased angiogenesis in humans with pancreatic neuroendocrine tumors [59], whereas the knock-down of *GREM1* in human HK-2 cells increases *BMP7* signaling activity [60]. Correspondingly, bone morphogenetic proteins 5 and 7 were down regulated in FL chickens. Similar to FL chickens, the expression of *BMP5* is down regulated under highly vascularized conditions, like those simulated by tumor cell lines (i.e., adrenocortical carcinoma and adrenocortical tumor cell lines [61]). Collectively, the expression of vasoconstrictive and angiogenic factors appear necessary for the expansion of adipose mass in FL chickens.

The serine protease thrombin (*F2*), which we identified as a differentially expressed gene in the time course microarray analysis of abdominal fat of the FL and LL chickens (see Chapter 2), is an extremely potent platelet agonist. In the RNA-Seq analysis of abdominal fat at 7 wk, *F2* was not significantly different (1.3 fold higher in LL chickens; $P = 0.07$; data not shown) between the genotypes. Interestingly we found the platelet receptor for *F2* (*F2R* or *PAR1*) expressed higher in adipose tissue of LL chickens. Very little is known about the thrombin-PAR1 interaction in within adipose tissue of any species. PAR1 and PAR4 are expressed in human adipose tissue, where treatment with thrombin induces expression and secretion of several adipokines (IL-1B, IL-6, MCP-1, and VEGF) [62]; however, this is mediated through the PAR4 rather than PAR1 receptor. In another study, Kajimoto *et al.* [63] found that the PAR1-

F2 interaction in 3T3-L1 adipocytes stimulates FABP4 which regulates the expression of interleukin 6 and vascular endothelial growth factor. There have been no studies describing the PAR1-F2 interaction in adipose tissue of chickens; however our studies do show that genes involved in this signaling are present, although further evaluation of this system is needed.

The balance of secondary and tertiary hemostasis prevents extreme vascular conditions such as uncontrolled hemorrhage or excessive clotting. The up-regulation of numerous genes involved in these processes (pro-coagulation and anti-coagulation) in LL chickens was a major finding in our time-course microarray analysis of abdominal fat (see Chapter 2). While prothrombotic genes are associated with increased fatness in humans [64-67], we suggested a novel role (rather than simply regulating systemic hemostasis) for the up-regulation of these genes in abdominal fat of lean chickens; they could catalyze the activation or deactivation of adipokines and other endocrine factors. Several of these DE were also differentially expressed in the present analysis, while RNA-Seq revealed additional differences not determined by microarray analysis. Both the time course microarray (Chapter 2) and RNA-Seq analysis demonstrate a large number of differentially expressed pro- and anti-coagulation genes in abdominal fat of FL and LL chickens.

Most remarkably, abdominal fat of LL chickens appears to be in a pro-coagulation state, since five genes driving clot formation are up-regulated (*F9*, *F2*, *FGA*, *FGB*, and *FGG*). Six genes (*F2R*, *FGB*, *FGG*, *PROS1*, *PLAU*, and *SERPINF2*) were identified as DE by RNA-Seq analysis but not in the time course microarray

analysis. The down-regulation of *PROS1*, an anticoagulant, in the LL supports the pro-coagulation state of abdominal fat in these chickens. Fibrinogen (*FGA*, *FGB* and *FGG*) is mainly produced in and secreted by the liver. Interestingly, fibrinogen expression in FL chickens is nearly undetectable whereas expression in LL chickens is greater than 10-fold higher for all three chains. Similarly, the level of FGG in plasma was inversely related to adiposity in rats fed a high-fat diet for 8 wk [68] and in subcutaneous and visceral fat in humans [69]. Aside from its function in coagulation, fibrinogen has been identified as a binding surface for several proteins involved in vascular homeostasis [70]. The expression of fibrinogen in abdominal fat of LL chicken is very similar to that of *ALB* in this experiment, and suggests that both albumin and fibrinogen are being up-regulated in LL chickens for increased molecular transport. The differential expression of a large number of hemostatic factors observed in these genetic lines was not observed during nutritional/hormonal perturbation in the chicken [20], which suggests these genes regulate chronic accumulation of adiposity.

3.4.3 Ectopically expressed genes in abdominal fat of FL and LL chickens

Perhaps the most remarkable findings of our RNA-Seq analysis of abdominal fat was the "ectopic" expression numerous genes (Table 3.4). Among these genes is *GCG*, the major regulator of glycemia in chickens [71-73], which was expressed higher in LL chickens, a finding similar to what was observed in abdominal fat of chickens exposed to acute insulin immunoneutralization [20].

Table 3.4 Ectopic expression in abdominal fat of FL and LL chickens

Biological Process	Gene Symbol	Gene Name	Fold Change	Average Reads
Glucagon Signaling				
	DPP4	dipeptidyl-peptidase 4	-1.66	705
	GCG	glucagon	-1.8	88
	GCGR	glucagon receptor	-1.63	2,765
	GLP1R	glucagon-like peptide 1 receptor	-3.75	50
	GLP2R	glucagon-like peptide 2 receptor	NS	0
	SSTR2	somatostatin receptor 2	1.24	25,729
Eicosanoid/Cannabinoid Signaling				
	CNR1	cannabinoid receptor 1 (brain)	-1.35	1,478
	DAGLA	diacylglycerol lipase, alpha	-1.25	627
	FAAH	fatty acid amide hydrolase	1.31	3,177
	FADS2	fatty acid desaturase 2	1.58	6,764
	LPIN1	lipin 1	1.52	1,830
Hypothalamic and Pituitary Signaling				
	GH	growth hormone	NS	34
	GHR	growth hormone receptor	NS	4,754
	MC5R	melanocortin 5 receptor	2.28	419
	NPY	neuropeptide Y	NS	35
	NPY2R	neuropeptide Y receptor Y2	2.09	73
	scGH	short chicken growth hormone	1.52	ND

Fold change values given as FL /LL expression (positive values) or LL /FL expression (negative values). Average reads across the FL and LL chickens from RNA-Seq analysis are given. NS = Not significant; ND = Not determined.

The endocrine pancreas is the major site of glucagon production and secretion in birds [74] and adipose tissue is a major target of *GCG*. Thus, it is likely that *GCG* produced in abdominal fat of LL chickens is not secreted and exhibits an intracrine function, acting to stimulate the release of stored energy from adipocytes, rather than serving a paracrine function as suggested by Ji *et al* [20]. Unlike the finding with insulin immunoneutralized chickens, we found *GCGR* expression to be similar (direction and fold change) to *GCG* in abdominal fat of FL and LL chickens. Furthermore, *GCGR* was among the top 9% of genes expressed in adipose tissue. In agreement with up-regulation of glucagon production in abdominal fat of LL chickens,

the transcript for somatostatin receptor (*SSTR2*), which when activated by somatostatin inhibits production of GCG in pancreatic α-cells [75, 76], is highly expressed and up-regulated in FL chickens.

Interestingly, we found that the glucagon-like peptide 1 receptor (*GLP1R*) was nearly 4-fold higher in LL chickens, whereas the glucagon-like peptide 2 receptor (*GLP2R*) was undetectable in abdominal fat. The absolute absence of *GLP2R* in our experiment could be of biological importance since its expression in avian adipose tissue has been reported [77]. The presence of *GLP1R* and absence of *GLP2R* in FL and LL chickens suggests that signaling occurs through the preproglucagon class A transcript (contains *GCG* and *GLP1*) in abdominal fat of these birds rather than the preproglucagon class B transcript (contains *GCG*, *GLP1* and *GLP2*) [77], which was up-regulated by insulin immunoneutralization [20]. Furthermore, lipids and carbohydrates are potent stimuli for the release of GLP1, and the binding of GLP1 to pancreatic β cell GLP1R stimulates insulin release in mammals [78], suggesting a role for GLP1R in regulating insulin signaling. The peculiar up-regulation of this potentially lipogenic mechanism in abdominal fat of LL chickens is likely blunted by the insensitivity of avian adipose tissue to insulin and the accompanying up-regulation of *DPP4* (1.7-fold higher in LL chickens), a potent degrading enzyme of incretins including GLP1 [78]. The lack of parallel evidence across genetic and nutritional perturbations for glucagon signaling in abdominal fat, suggests differences between acute and long term control mechanisms.

Another intriguing process found in abdominal fat of FL and LL chickens was the apparent eicosanoid activation of cannabinoid signaling. In human breast cancer MCF7 cells, loss of FADS2 function blocks normal polyunsaturated fatty acid biosynthesis resulting in the FADS1 generation of polyunsaturated fatty acids which are missing the 8–9 double bond of eicosanoid signaling precursors (i.e., arachidonic acid and eicosapentaenoic acid [79]). This suggests that *FADS2* (higher in FL chickens) is more biologically important to eicosanoid signaling than *FADS1*, which was down-regulated by food deprivation [20]. Another enzyme involved in eicosanoid signaling, *DAGLA* (higher in LL chickens), catalyzes the hydrolysis of diacylglycerol (DAG), which is produced by lipin 1 (discussed earlier). The product of this reaction is arachidonic acid precursor 2-arachidonoyl-glycerol (2-AG), a major peripheral endocannabinoid signaling molecule [80]. In adipose tissue of rodents, 2-AG activates CNR1 (also up-regulated in LL chickens), an event that is up-regulated in fat treatment groups [80-82]. Furthermore, *FAAH*, the enzyme that degrades the active endocannabinoid 2-AG [83] was up-regulated in FL chickens.

Several genes that function in the regulation of energy metabolism and food intake in the hypothalamus and pituitary gland were detected in abdominal fat of FL and LL chickens. The hypothalamic orexigenic peptide NPY has been identified in cultured adipocytes from dogs [84], rats [85, 86], humans [87, 88], and 3T3-L1 adipocytes [89]. In both rat pre-adipocytes and 3T3-L1 pre-adipocytes, NPY signals through NPY1R and stimulates both adipogenesis and angiogenesis, while NPY2R is not detectable [90]. Interestingly, in live mice, treatment of adipose tissue with

exogenous NPY stimulates adipogenesis, which appears to be mediated through the NPY2R receptor, since injection with an NPY2R antagonist decreases adiposity [91]. In the same study, a co-culture of 3T3-L1 pre-adipocytes with neuroblastoma cells markedly increased the expression of *NPY2R*, where it was expressed at extremely low levels in 3T3-L1 pre-adipocytes alone. These experiments conclude that in abdominal fat of live mice NPY signals through both NPY1R and NPY2R. In the RNA-Seq analysis of abdominal fat from FL and LL chickens, *NPY2R* was differentially expressed, while no reads were mapped for *NPY1R*. This suggests that NPY signaling occurs through the NPY2R receptor in adipose tissue of chickens, and this process is up-regulated in fat chicken models (which is further evidence for the above discussion on expansion of adipose tissue mass in FL chickens). We also found the peripheral receptor for melanocyte-stimulating hormone and adrenocorticotropic hormone, *MC5R*, expressed higher in FL chickens. MC5R has been shown to have a role in lipid metabolism in skeletal muscle of mice [92] and in hepatocytes of sea bass where a MC5R agonist stimulates lipolysis and the release of free fatty acids into culture [93]. Furthermore, MC5R has been associated with obese phenotypes in humans [94] and the extremely high synteny between chicken and human MC5R suggests a parallel action across species.

Growth hormone signaling is highly active in abdominal fat of FL and LL chickens (see Figure 3.2-B). The mechanism for GH signaling relies on feedback between the anterior pituitary gland and the liver through the production and secretion of GH and insulin like growth factor 1 (IGF1), respectively. The activation of this axis

(GH/GHR/IGF1) in humans is generally anabolic in most tissues, although it is associated with decreased fat mass and metabolic risks [95]. The action of exogenous GH in mammals is opposite to that of chickens, where chronic intravenous infusion significantly increases body fat content [96]. In the present study of abdominal fat, GH was not differentially expressed, although the short GH isoform (*scGH*) was expressed higher in FL chickens. A 3D-structural analysis of scGH and human GH showed only a 51.4% similarity, which suggests that scGH lacks major residues required for binding to the GHR [97]. However, the unaltered C-terminal sequence of scGH (compared to normal chicken GH) suggests that it binds to the chicken GHR (which was highly expressed), or has alternative biological functions within adipocytes of FL chickens by acting as an intracrine factor, since it lacks a signal peptide [98].

The up-regulation of GH signaling in abdominal fat of FL chickens could enhance IGF signaling. The over expression of the IGF binding protein, *IGFBP2* (2.5-fold higher in FL chickens; Table 3.3) agrees with this idea. Several polymorphisms in *IGFBP2* have been associated with abdominal fatness in chickens including single nucleotide polymorphisms (SNPs) in the 3'-flanking region [99] and intron 2 [100]. Insulin and IGF signaling are closely related to insulin receptor substrates acting through both INSR (not DE; among top 15% of genes expressed) and the IGF receptor (*IGF1R*, higher in LL chickens [101]). Since insulin receptor substrates signal through IGF1R, both insulin and IGF signaling could be enhanced in adipose tissue of the FL chickens via over expression of *IRS2* (see Figure 3.5-B). Another IGF binding protein (*IGFBP7*; highly expressed, up-regulated in LL chickens) has not been shown to be

associated with adiposity in chickens; however, its high expression in adipose tissue confirms an earlier observation [19]. Further investigation of this axis is needed.

3.5 Chapter Summary

This study utilized RNA sequencing of abdominal fat in 7 week old chickens genetically selected for fatness (FL chickens) or leanness (LL chickens). Our findings present the most complete and accurate depiction of the transcriptional role of adipose tissue in the chicken to date. The highest expressed genes in abdominal fat of FL and LL chickens clearly indicate that lipogenesis is a major activity in both genotypes, although the higher expression of lipogenic genes in the FL could account for their greater visceral fat mass. The principal distinguishing feature of the LL abdominal fat transcriptome is the extraordinary abundance of multiple hemostatic genes that regulate vasomotor tone and proteolytic processing of coagulation factors and perhaps endocrine factors. Furthermore, the discovery of numerous ectopically expressed genes involved in endocrine and arachidonic acid signaling in abdominal fat of FL and LL chickens indicates that visceral fat could function autonomously as an endocrine organ that regulates lipid metabolism and feed intake. The present RNA-Seq transcriptional analysis provides further evidence to support our idea that abdominal fat is an active endocrine organ, which makes a substantial and underappreciated contribution to lipogenesis and adipocyte hypertrophy in the chicken.

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Chapter 4

TRANSCRIPTIONAL ANALYSIS OF CHICKENS DIVERGENTLY SELECTED ON BODYWEIGHT UNCOVERS NOVEL MECHANISMS FOR CONTROLLING LEANNESS AND VALIDATES ABDOMINAL FAT AS A LIPOGENIC TISSUE

4.1 Introduction

The domestic chicken (*Gallus domesticus*) is a widely used biological model and an important source of high-quality dietary protein. Decades of intensive genetic selection have established the remarkable growth rate of the commercial broiler today. However, this selection has been accompanied by unfavorable increases in fat deposition, skeletal abnormalities and metabolic and reproductive disorders [1-4].

Since selection for growth mainly relies on quantitative traits, little regard has been given to identifying differentially regulated tissue specific pathways controlling incidental changes in phenotypic measurements other than growth (i.e., abdominal fatness). In fact, most of the transcriptional studies that have been completed in models of chicken growth have concentrated on skeletal muscle [5-7], to identify changes that are responsible for differences in growth rate (and potential), and

hypothalamus [8-16], to understand differences in endocrine control of growth and metabolism.

While these studies were informative in their own right, the understanding of gene regulation in metabolic tissues in these animals could also prove useful for both improvement of growth rate and reducing incidental changes in unfavorable traits, specifically the abdominal fatness that accompanies increasing growth rate. For example, one study compared intra-muscular adipose tissue between two lines of chickens selected for fast growth or slow growth [17]. This analysis determined that expression of several differentially expressed (DE) genes correlated with increased or decreased growth of breast muscle and intramuscular fat deposition indicating that adipose tissue may be a major regulator of muscle growth rate and potential, but it did not uncover any mechanisms which would result in the divergence of abdominal fatness.

The liver has long since been referred to as the primary lipogenic tissue in birds [18], however, very few studies have looked at gene expression in the liver of chickens divergently selected for growth. For example, expression of several genes has been analyzed in high weight and low weight chickens including *CPT1A/B* [5], *GHR* and *IGF1* [6]. Liver was also analyzed in the same animals used in the present study by microarray analysis using the Del-Mar 14K Chicken Integrated Systems cDNA microarray, which uncovered 557 DE genes including transcripts for many metabolic enzymes, acute phase proteins, immune factors and transcription factors (Cogburn LA; manuscript in preparation). These growth models are populations of

Rhode Island Red broiler chickens that were divergently selected at the Station de Recherches Avicoles, Institut National de la Recherche Agronomique (INRA), Nouzilly, France for either high (fast-growing line, HG) or low (slow- growing line, LG) body weight at 8 and 32 wks of age [19, 20]. Similar to the previously mentioned growth models, genetic selection for high growth rate (HG chickens) in these chickens is accompanied by extreme abdominal (visceral) fat accretion, whereas selection for slow growth rate (LG chickens) greatly diminishes abdominal fat mass.

In previous studies looking at abdominal fat of chickens genetically selected for a two to three fold difference in visceral fatness (albeit the same body weight) we observed the differential expression of many lipogenic genes, suggesting that adipose tissue may have a more significant involvement in the synthesis of lipid than classically thought (see Chapter 2 and 3). Thus, the present study aimed to identify differences in gene expression in abdominal fat of HG and LG chickens to gain a better understanding of the intra-tissue-specific contribution to the incidental divergence in abdominal fatness between these models. Our analyses revealed that abdominal fat of HG chickens overexpresses genes involved in increased adiposity, including transcriptional regulators and metabolic (lipogenic) enzymes, throughout juvenile development (1-11 wk). Conversely, low growth chickens up-regulate several energy producing processes (i.e., peroxisomal β-oxidation, mitochondrial β-oxidation, ketogenesis and oxidative phosphorylation) early on in juvenile development which are likely responsible for their extreme leanness. RNA-Seq analysis at 7 wk determined that hemostatic factors may contribute to the extreme leanness of LG

chickens. These findings serve as a cross-model validation that abdominal fat has a major contribution to the regulation of adiposity in models divergently selected for a specific trait resulting (directly or incidentally) in large differences in abdominal fatness.

4.2 Methods

4.2.1 Animal management and tissue preparation

All birds were bred and raised at INRA UE1295 Pôle d'Expérimentation Avicole de Tours, F-37380 Nouzilly, France. At hatching, cockerels were wing-banded and vaccinated against Marek's disease virus. Birds were provided *ad libitum* access to water and fed a conventional starter ration (22% crude protein and 3050 kcal ME/kg) from hatching to 3 weeks of age and then with a grower pelleted ration from 3 to 11 weeks of age (20% crude protein, and 3100 kcal). The HG birds were separated from LG birds for the first 3 weeks (at which time LG chickens were provided crushed feed pellets) to increase early survival of the LG and after which both lines were placed together and raised in 4.4 m x 3.9 m floor pens. Continuous light was provided for the first two days followed by a maintenance of a 14 hr light /10 hr dark cycle (14L:10D). Infrared gas heaters provided supplemental heat and ambient temperature was progressively decreased from 32° C at hatching until 22° C was reached at 22 days. At 1, 3 5, 7, 9 and 11 weeks of age, eight fed cockerels from each genetic line were randomly selected, weighed and bled into heparinized syringes prior to cervical

dislocation and excision and weighing of abdominal adipose tissue. Abdominal adipose tissue samples were immediately snap frozen in liquid nitrogen and stored at -75° C until further processing. All animal procedures were performed under the strict supervision of a French government veterinarian and in accordance with protocols approved by the French Agricultural Agency, the Scientific Research Agency, and the Institutional Animal Care and Use Committees at INRA, Nouzilly, France. These procedures were also in compliance with the United States Department of Agriculture guidelines on the use of agricultural animals in research and approved by the University of Delaware Agricultural Animal Care and Use Committee.

4.2.2 Transcriptional Analysis

4.2.2.1 RNA extraction

Abdominal fat aliquots from forty-eight individuals (4 HG and 4 LG per age at 1, 3, 5, 7, 9 and 11 wk of age) were homogenized and total cellular RNA was extracted from using guanidine thiocyanate and CsCl gradient purification [21], followed by DNase I treatment. Quality was analyzed with an RNA 6000 Nano Assay kit and the Model 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA). All samples were determined to have an RNA integrity number (RIN) greater than 9.0.

4.2.2.2 Microarray analysis

The Del-Mar 14K Chicken Integrated Systems Microarrays (Geo Platform # GPL1731), described earlier [22], were used for transcriptional profiling of four abdominal fat samples from each the HG and LG across 11 weeks of juvenile development (48 total individuals). Methods used for microarray preparation including: labeling, hybridization, and image acquisition were described earlier (see Chapter 2). Briefly, twenty-four Del-Mar 14K Chicken Integrated Systems Microarrays were hybridized with 48 labeled samples using a balanced block design, where half of the birds from each genotype and age were labeled with Alexa Flour 647 (red dye) and the other half with Alexa Flour 555 (green dye; see Figure 4.1 for experimental design details). These hybridized microarrays were scanned with a GenePix 4000B scanner using GenePix Pro 4.1 software (Molecular Devices, Union City, CA) at wavelengths of 635 nm (Alexa 647-labeling) and 532 nm (Alexa 555-labeling) producing a combined TIFF image file for each slide. Laser power was set at 100% with the photomultiplier tube (PMT) setting adjusted for each scan producing a PMT count near unity. All slides were manually checked for quality and all spots with inadequacies in signal, background or morphology were eliminated. The image analysis results were merged with Excel files (in GPR format) containing clone identification, spot location on slide, and most current gene name/function (based on BLAST score).

Age (wk)	Red (F635)	Green (F532)	Red (F635)	Green (F532)	Red (F635)	Green (F532)	Red (F635)	Green (F532)
	Slide 51		Slide 50		Slide 49		Slide 48	
1	LG1	HG1	HG2	LG2	LG3	HG3	HG4	LG4
	Slide 47		Slide 46		Slide 45		Slide 26	
3	LG5	HG5	HG6	LG6	LG7	HG7	HG8	LG8
	Slide 43		Slide 42		Slide 41		Slide 40	
5	LG9	HG9	HG10	LG10	LG11	HG11	HG12	LG12
	Slide 39		Slide 38		Slide 37		Slide 36	
7	LG13	HG13	HG14	LG14	LG15	HG15	HG16	LG16
	Slide 35		Slide 34		Slide 33		Slide 32	
9	LG17	HG17	HG18	LG18	LG19	HG19	HG20	LG20
	Slide 31		Slide 30		Slide 29		Slide 28	
11	LG21	HG21	HG22	LG22	LG23	HG23	HG24	LG24

Figure 4.1 Experimental design for hybridization of 48 abdominal fat samples from HG and LG cockerels. Twenty-four microarrays were hybridized to 48 abdominal fat RNA samples (4 birds/genotype x 2 genotypes x 6 ages).

4.2.2.3 Statistical analysis of microarray data

The GPR files were used to determine differentially expression in abdominal fat of HG and LG chickens. Log2 transformed median intensity values (for each dye) were normalized using a global LOWESS transformation (without background subtraction) to remove dye bias within microarray [23]. A two-way ANOVA was used to determine main effects of age (A) and genotype (G; differences in genotype for each age were not analyzed), and the interaction of age and genotype (A x G). The Benjamini-Hochberg procedure [24] was used to control the experiment wise false discovery rate (FDR) associated with multiple testing. Expression values at 7 wk of 25 genes were retrieved from the microarray analysis for comparison across methods. For these 25 genes, a Student's T-test was used to determine significant differences

between genotypes. The MIAME-compliant microarray data were deposited in the NCBI Gene Expression Omnibus as Series GSE45825.

4.2.2.4 RNA-Sequencing analysis

Extracted RNA (described above) from adipose tissue of eight (4 HG and 4 LG) 7 wk old individuals was used for preparation of indexed (bar-coded) sequencing libraries. Libraries were made from 2 µg of total adipose RNA with the Illumina TruSeq® Stranded mRNA library preparation kit following standard Illumina protocols. All eight libraries were pooled and paired-end sequenced (101-bp reads) on an Illumina HiSeq 2000 Sequencing System (http://www.illumina.com/systems/hiseq_2000.ilmn, Illumina, InC., San Diego, CA) at Delaware Biotechnology Institute, University of Delaware.

4.2.2.5 RNA-Sequencing data and statistical analysis

Sequences were trimmed for quality using a combination of custom perl scripts and Btrim64 [25]. Boxplot graphing of pre-and-post trimming reads confirmed the absence of outlier samples based on read count. After trimming, reads were mapped to the chicken genome (Ensembl chicken 2.0 – WASHUC2) using Tophat (version 1.3.3), followed by assembly and quantitation using Cufflinks (1.3.0). The resulting gtf files were merged with cuffmerge, and differential expression was assessed using

Cuffdiff. P-values were adjusted [24] to correct for FDR associated with multiple testing.

4.2.2.6 Quantitative RT-PCR analysis

Candidate DE genes from both the time course microarray analysis (1-11 wk) and RNA-Seq analysis (7 wk) were selected for verification of expression by quantitative RT-PCR (qRT-PCR) analysis. Superscript III reverse transcriptase (Invitrogen) and an oligo(dT) primer were used to prepare cDNA from 1 µg of RNA. Primers were designed using Primer Express v2.0 software (Applied Biosystems, Foster City, CA). Detailed information for each primer pair including gene name, gene symbol, primer sequences (forward and reverse), GenBank accession number and amplicon size are provided in the Appendix.

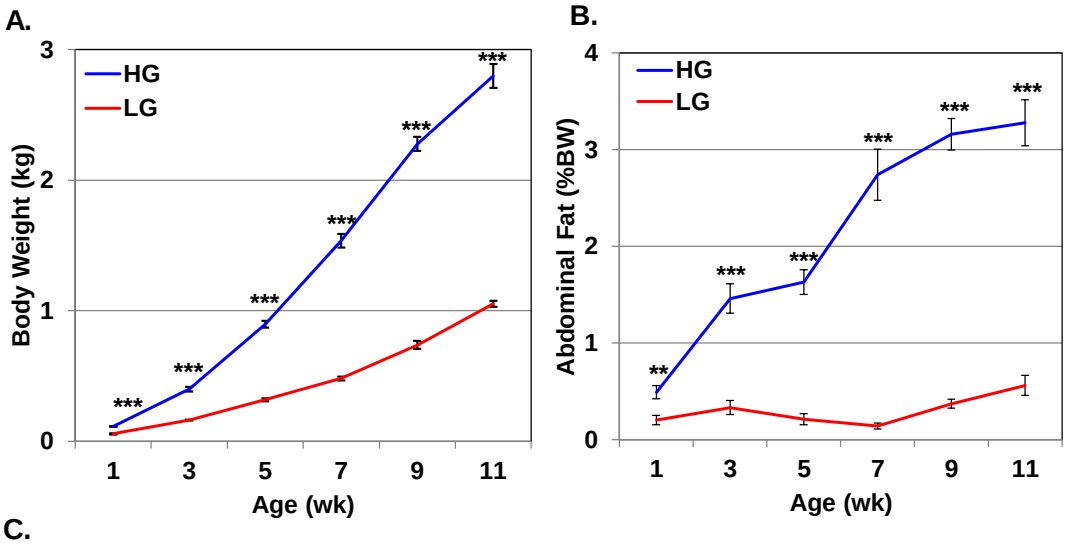
The qRT-PCR assay was performed in an ABI Prism Sequence Detection System 7900HT using 50 ng of cDNA, Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 400 nM of each primer (forward and reverse; Sigma-Aldrich, St. Louis, MO) in duplicate wells. Disassociation curves were analyzed to confirm specific amplification and verify absence of primer dimers. Resulting PCR products were analyzed using agarose gel electrophoresis to compare approximate product size to expected amplicon size. To verify gene expression from the microarray and RNA-Seq analyses, the cycle time (Ct) for each sample was normalized to the corresponding sample geometric mean of two housekeeping genes:

cytochrome c oxidase subunit VIIa polypeptide 2 like (*COX7A2L*) and ribosomal protein L14 (*RPL14*). These housekeeping genes were selected based on invariability in the microarray and RNA-Seq analyses and were also determined to be the most stably expressed genes assessed by qRT-PCR analysis using RefFinder (http://www.leonxie.com/referencegene.php). The $2^{-(\Delta\Delta Ct)}$ formula was used to calculate relative transcript abundance [26]. The statistical analysis was performed using a general linear model procedure in SAS v9.3 and differences between genotypes at each age were determined using Tukey's multiple comparisons test. For genes only analyzed at 7 wk, a Student's T-test was used to analyze differential expression. The significance level for all tests was set at $P \leq 0.05$ unless otherwise noted.

4.3 Results

4.3.1 Phenotypic measurements

Body weight (BW) and abdominal fat relative BW (%BW) in juvenile HG and LG chickens are presented in Figure 4.2. HG cockerels were 2.7 fold larger ($P \leq 0.001$; Figure 4.2-A) and 8-fold fatter ($P \leq 0.001$; Figure 4.2-B) than LG on average (1 through 11 wk). The greatest difference in BW and %BW was observed at 7 wk where there was a 3.2 and 19.6 fold difference between the lines, respectively (Fi



C.

Phenotype fold-difference (HG/LG)						
Age (wk)	1	3	5	7	9	11
Body weight (BW)	2.0	2.5	2.8	3.2	3.1	2.7
Abdominal Fat (%BW)	2.5	4.4	7.8	19.6	8.5	5.9

Figure 4.2 **Phenotypic measurements of juvenile HG and LG chickens.** Data points represent the means ± SE of 8 individual HG or LG chickens from 1 to 11 wks of age. Significant differences between genotypes at each age were determined using a one way ANOVA and Tukey's multiple comparisons procedure at a significance level of $P \leq 0.01$ (**) and $P \leq 0.001$ (***). A.) Average body weight (kg) of 8 individual birds for each the HG (blue line) and LG (red line) chickens plotted by age (1-11wk). B.) Average abdominal fat (as a percent of bodyweight) of 8 individual birds for each the HG (blue line) and LG (red line) chickens plotted by age (1-11wk). C.) Fold change differences (HG/LG) in body weight (kg) and abdominal fat (%BW) from 1-11 wk. They grey box highlights that the largest fold change values for both measurements were seen at 7 wk.

4.3.2 Analysis of abdominal fat gene expression

Differentially expressed (DE) genes were defined as those having a significant false discovery rate adjusted *P*-value (FDR≤0.05 for microarray or FDR≤0.10 for RNA-Seq). For the microarray analysis, main effects of genotype (312 DE genes) and age (2,918 DE genes), and the age by genotype interaction (718 DE genes) were analyzed. For the RNA-Seq experiment (7 wk) the main effect of genotype (223 DE genes with FDR≤0.05 and 280 with FDR≤0.1) was observed. All fold change values are given as (+) HG/LG expression values for genes expressed higher in HG, or (–) LG/HG for genes expressed higher in LG chickens. The RNA-Seq analysis was also used to identify the top 900 highest expressed (HE; high growth expression value plus LG expression value) genes in abdominal fat at 7 wk.

4.3.3 Ingenuity Pathway Analysis (IPA) of gene interactions and functional pathways

Significant genes from the microarray analysis (main effect of genotype combined with the age by genotype interaction list; 905 unique genes) were annotated using the GeneBase tool on our website (http://cogburn.dbi.udel.edu/), which provides protein IDs (from GenBank or Swiss-Prot) derived from BLASTX analysis of microarray cDNA probes. Lists of DE genes [from the microarray (FDR≤0.05; 905 genes as described above) and RNA-Seq analyses (FDR≤0.1; 280 genes)] and the HE genes at 7 wk (900 genes) containing the protein ID and fold change ratio for each

gene were submitted to the IPA knowledgebase (http://www.ingenuity.com/) for functional annotation and mapping to canonical metabolic and regulatory pathways. "Analysis ready" genes were mapped by IPA for the time course (1-11 wk) microarray analysis (680 DE genes), the RNA-Seq DE gene analysis (7 wk; 245 DE genes) and the RNA-Seq HE genes analysis (7 wk; 747 DE genes).

The summary of the overrepresented DE (1-11 wk) "Top Biological Functions" are shown in Table 4.1 compared across analyses (DE and HE genes identified by RNA-Seq analysis at 7wk). There are several categories that stand out including those that are involved in the regulation of adiposity ("Lipid Metabolism", "Carbohydrate Metabolism", "Mitochondrial Dysfunction", "Triacylglycerol Biosynthesis" and "Cholesterol Biosynthesis I"), growth ("Connective Tissue Disorders", "Skeletal and Muscular Disorders", "Cellular Growth and Proliferation", "Organismal Development", "Connective Tissue Development", "Embryonic Development" and "Cardiovascular System Development"), hemostasis ("Hematological Disease", "Hematological System Development and Function", "Acute Phase Response Signaling", "Coagulation System" and "Complement System") and oxidative stress ("NRF2-mediated Oxidative Stress Response" and "Superoxide Radicals Degradation"). All of these categories are overrepresented in the microarray analysis results and in the RNA-Seq HE gene analysis, while four of these categories ("Mitochondrial Dysfunction", "EIF2 Signaling", "NRF2-mediated Oxidative Stress Response" and "Superoxide Radicals Degradation") are not significantly differentially regulated at 7wk by RNA-Seq analysis. Alternatively, the

RNA-Seq DE gene analysis at 7 wk was enriched with hemostasis categories ("Acute Phase Response Signaling", "Coagulation System" and "Complement System") and "LXR/RXR Activation" genes.

Table 4.1 Top biological functions of differentially expressed and highest expressed genes in HG and LG chickens

Diseases and Disorders	**M DE Genes**	**R DE Genes**	**R HE Genes**
Connective Tissue Disorders	92	40	140
Skeletal and Muscular Disorders	145	43	86
Inflammatory Disease	90	44	158
Hematological Disease	93	20	146
Reproductive System Disease	114	62	193
Molecular and Cellular Functions			
Lipid Metabolism	117	50	164
Small Molecule Biochemistry	158	57	179
Molecular Transport	155	71	176
Cellular Growth and Proliferation	218	92	317
Carbohydrate Metabolism	76	22	102
Physiological System Development and Function			
Hematological System Development and Function	101	66	168
Organismal Development	133	62	214
Connective Tissue Development	65	31	135
Embryonic Development	40	33	99
Cardiovascular System Development	103	51	167
Top Canonical Pathways			
Mitochondrial Dysfunction	20/186	NS	21/186
EIF2 Signaling	20/200	NS	65/200
NRF2-mediated Oxidative Stress Response	19/192	NS	17/192
Superoxide Radicals Degradation	3/8	NS	3/8
Triacylglycerol Biosynthesis	6/46	3/46	2/46
Acute Phase Response Signaling	11/179	19/179	18/179
LXR/RXR Activation	10/136	15/136	11/136
Coagulation System	2/38	8/38	4/38
Complement System	5/35	4/35	7/35
Cholesterol Biosynthesis I	1/40	2/40	3/40

Ingenuity® Pathway Analysis (IPA) Software was used to analyze functional categories of differentially expressed (DE) genes identified by microarray (M) analysis of abdominal fat from 1-11 wk of age, DE genes identified by RNA-Seq (R DE) analysis (7 wk) and highest expressed (R HE) genes identified by RNA-Seq analysis. Functional groups of genes were determined to be significantly present in the analysis ($P \leq 0.05$) except those marked as NS (not significant). For Top Canonical Pathways the ratio, X/Y, given is the number of genes present in the dataset (X) out of the number of genes known (by the IPA knowledgebase) to be involved in that process (Y).

The IPA generated interaction network presented in Figure 4.3-A shows the interaction of several DE regulators of transcription [i.e., nuclear receptors or transcriptional regulators; proliferator-activated receptor delta (*PPARD*), proliferator-activated receptor gamma (*PPARG*), CCAAT/enhancer binding protein (C/EBP), alpha (*CEBPA*), F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase (*FBXW7*), AE binding protein 1 (*AEBP1*) and Snf2-related CREBBP activator protein (*SRCAP*)] with each other and with numerous of their targets. Among these targets are transcripts for metabolic enzymes up-regulated in HG [diacylglycerol O-acyltransferase 2 (*DGAT2*), fatty acid synthase (*FASN*), farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*) and stearoyl-CoA desaturase (delta-9-desaturase; *SCD*)] or LG chickens [acyl-CoA synthetase family member 2 (*ACSF2*) and hydroxysteroid (11-beta) dehydrogenase 1 (*HSD11B1*)]. Also in this enriched network are regulators of gluconeogenesis including an inhibitor of the pyruvate dehydrogenase complex— pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*; higher in LG chickens) and phosphoenolpyruvate carboxykinase 1 (*PCK1*; higher in HG chickens). Further, the adipocyte specific fatty acid binding protein (*FABP4*) was higher in HG chickens while the transporter for very low density lipoprotein (*VLDLR*) was up-regulated in LG chickens.

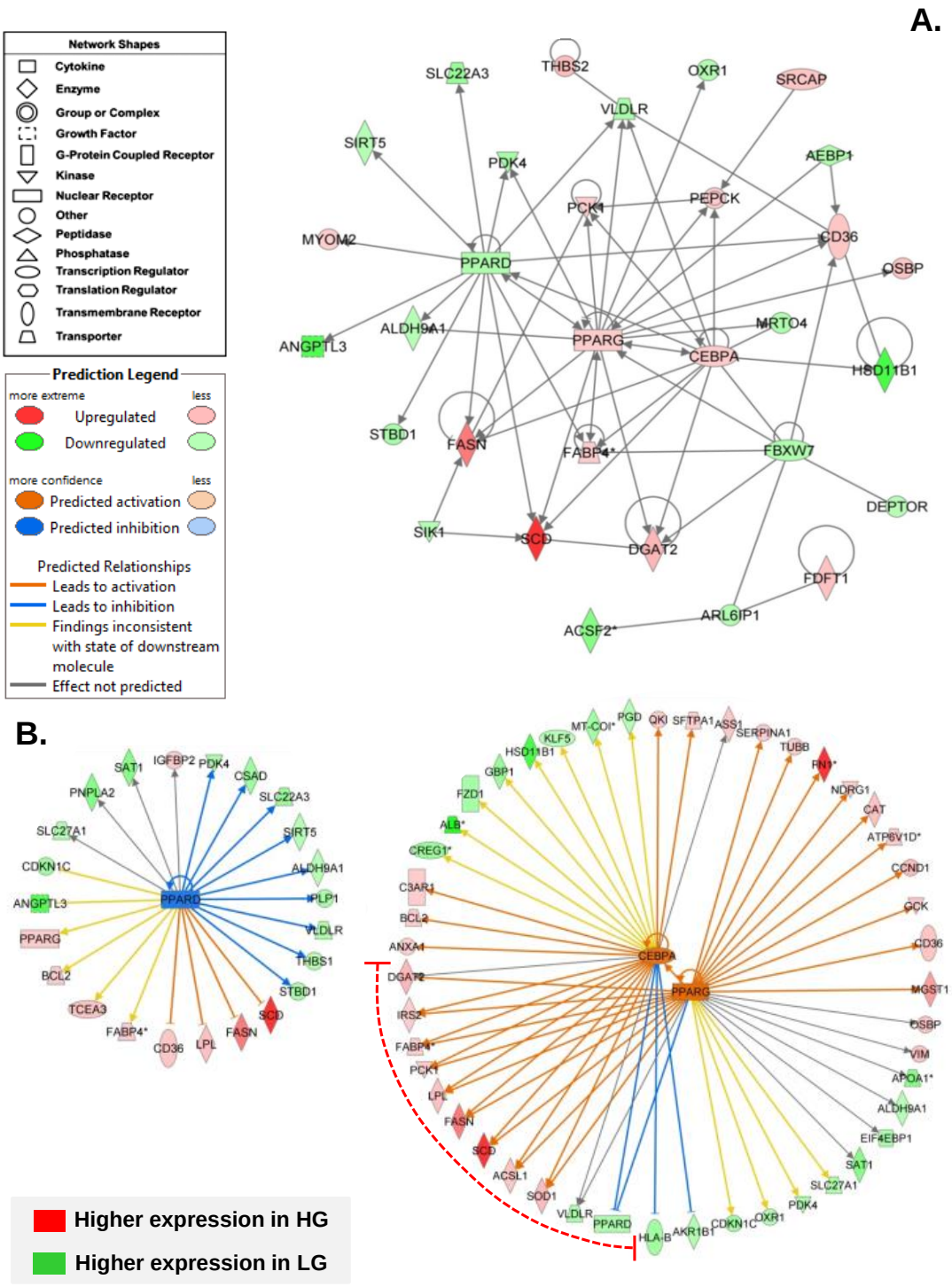

A.
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
SLC22A3
THBS2
OXR1
SRCAP
VLDLR
SIRT5
PDK4
AEBP1
PCK1
PEPCK
MYOM2
CD36
PPARD
OSBP
ANGPTL3
ALDH9A1
PPARG
MRTO4
CEBPA
H6D11B1
STBD1
FASN
FABP4*
FBXW7
DEPTOR
SIK1
SCD
DGAT2
FDFT1
ARL6IP1
ACSF2*
B.
SAT1
IGFBP2
PDK4
CSAD
PNPLA2
SLC22A3
SLC27A1
SIRT5
CDKN1C
ALDH9A1
ANGPTL3
PPARD
PLP1
PPARG
VLDLR
THBS1
BCL2
STBD1
TCEA3
SCD
FABP4*
CD36
LPL
FASN
MT-CO1*
PGD
QKI
SFTPA1
ASS1
SERPINA1
KLF5
TUBB
HSD11B1
GBP1
FN1*
FZD1
NDRG1
ALB*
CAT
CREG1*
ATP6V1D*
C3AR1
CCND1
BCL2
GCK
ANXA1
CEBPA
CD36
DGAT2
PPARG
MGST1
IRS2
OSBP
FABP4*
VIM
PCK1
APOA1*
LPL
ALDH9A1
FASN
EIF4EBP1
SCD
SAT1
ACSL1
SLC27A1
SOD1
PDK4
VLDLR
PPARD
HLA-B
AKR1B1
CDKN1C
OXR1
Higher expression in HG
Higher expression in LG

Figure 4.3 Major differentially expressed gene interactions in abdominal fat of HG and LG chickens throughout juvenile development. Functional gene interactions networks were identified by Ingenuity Pathway Analysis (IPA) software of a combined list with genotype and age by genotype interaction effects from the microarray analysis. A.) This network shows direct gene interactions leading to fatness (higher in HG) or leanness (higher in LG) mediated by three major transcriptional regulators (PPARD, PPARG and CEBPA). B.) IPA predicted the up-regulation of PPARD in LG chickens and the up-regulation of PPARG and CEBPA in HG chickens based on the literature and the observed values in our microarray analysis. All DE genes that are regulated by PPARD, PPARG and CEBPA are shown. PPARG and CEBPA synergistically affect 11 genes which are highlighted by a red dotted line. Red gene symbols indicate higher expression in the HG and green gene symbols indicate higher expression in the LG.

A more in depth depiction of the PPARD, PPARG and CEBPA DE targets in the abdominal fat of HG and LG chickens is shown in Figure 4.3-B. IPA predicted (based on literature and our observed values) PPARD to be up-regulated in our LG chickens (blue color) and both PPARG and CEBPA to be higher in the HG chickens (orange color) which agrees with our observed values for the transcripts for these transcription factors (see Figure 4.3-A and section on quantitative RT-PCR verification below). PPARD directly activates (pointed blue arrow) nine genes which are higher expressed in LG chickens and blocks activation (blunted orange line) of four genes in HG chickens. There are ten genes individually up-regulated in HG chickens (pointed orange line) by PPARG and similarly, five genes individually up-regulated by CEBPA. A group of eleven genes (9 higher expressed in HG chickens) are acted on (activated or inhibited) synergistically by PPARG and CEBPA (highlighted by a red broken line around periphery of the targets).

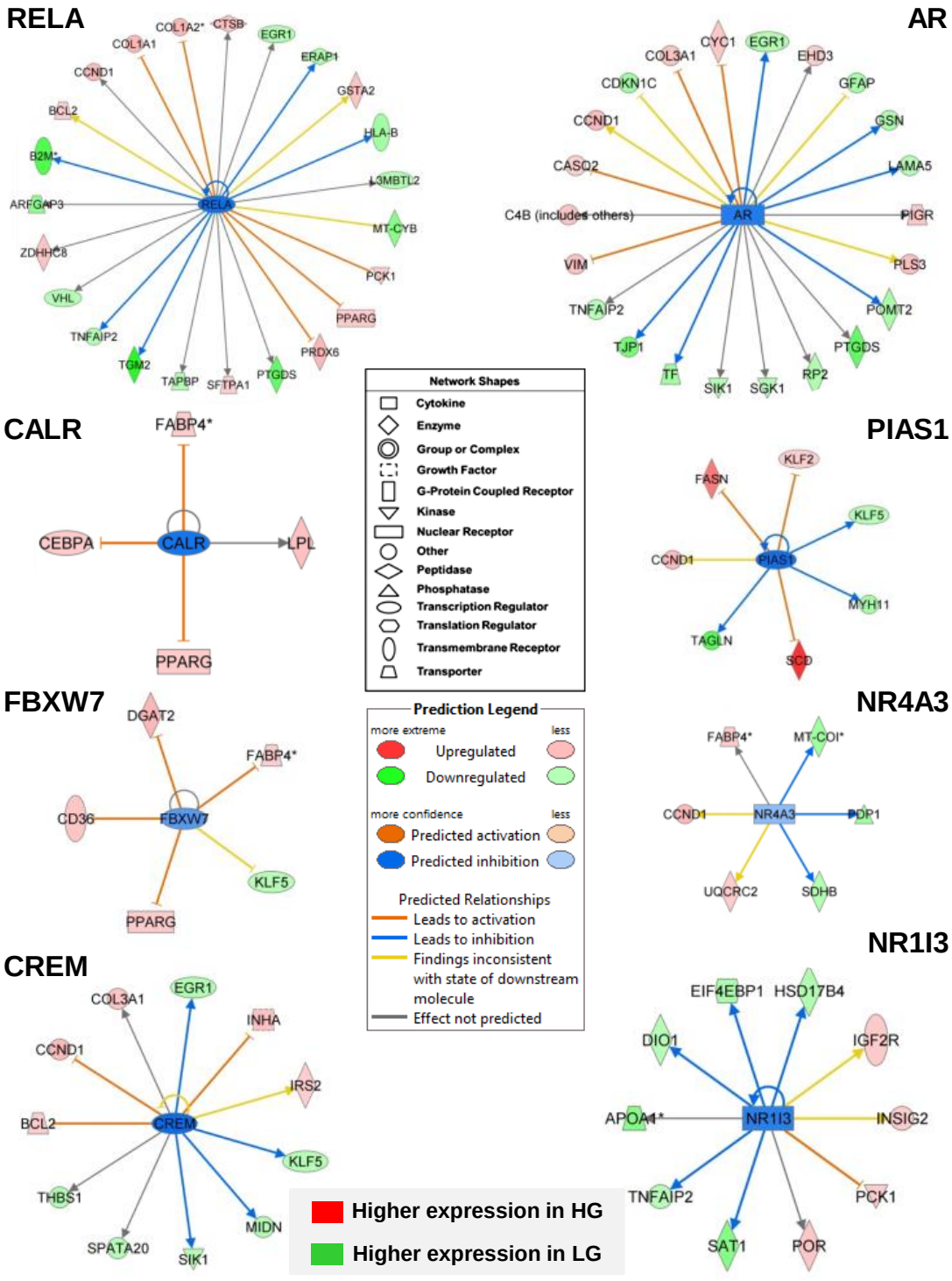

RELA
COL1A2*
CTSB
COL1A1
EGR1
ERAP1
CCND1
GSTA2
BCL2
HLA-B
B2M*
L3MBTL2
ARFGAP3
RELA
MT-CYB
ZDHHC8
PCK1
VHL
PPARG
TNFAIP2
PRDX6
TGM2
TAPBP
SFTPA1
PTGDS
AR
CYC1
EGR1
COL3A1
EHD3
CDKN1C
GFAP
CCND1
GSN
CASC2
LAMA5
C4B (includes others)
AR
PIGR
VIM
PLS3
TNFAIP2
POMT2
TJP1
PTGDS
TF
SIK1
SGK1
RP2
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
CALR
FABP4*
CEBPA
CALR
LPL
PPARG
PIAS1
KLF2
FASN
KLF5
CCND1
PIAS1
MYH11
TAGLN
SCD
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
FBXW7
DGAT2
FABP4*
CD36
FBXW7
KLF5
PPARG
NR4A3
FABP4*
MT-COI*
CCND1
NR4A3
PDP1
UQCRC2
SDHB
CREM
COL3A1
EGR1
INHA
CCND1
IRS2
BCL2
CREM
KLF5
THBS1
MIDN
SPATA20
SIK1
NR1I3
EIF4EBP1
HSD17B4
DIO1
IGF2R
APOA4*
NR1I3
INSIG2
TNFAIP2
PCK1
SAT1
POR
Higher expression in HG
Higher expression in LG

Figure 4.4 Transcriptional regulators of leanness in abdominal fat of LG chickens throughout juvenile development. Functional gene interactions were identified by Ingenuity Pathway Analysis (IPA) software of a combined list with genotype and age by genotype interaction effects from the microarray analysis. This figure presents eight transcriptional regulators predicted to be up-regulated in LG chickens (blue color) based on the literature and values in our microarray DE list. Red gene symbols indicate higher expression in the HG and green gene symbols indicate higher expression in the LG.

An additional eight regulators of transcription predicted by IPA to be up-regulated in LG chickens [v-rel reticuloendotheliosis viral oncogene homolog A (avian; RELA), calreticulin (CALR), FBXW7, cAMP responsive element modulator (CREM), androgen receptor (AR), protein inhibitor of activated STAT, 1 (PIAS1), nuclear receptor subfamily 4, group A, member 3 (NR4A3), nuclear receptor subfamily 1, group I, member 3 (NR1I3)] and their targets are shown in Figure 4.4. Eight transcriptional regulators [sterol regulatory element binding transcription factor 1 (SREBF1), sterol regulatory element binding transcription factor 2 (SREBF2), nuclear receptor coactivator 1 (NCOA1), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 (SMARCB1), CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), signal transducer and activator of transcription 5A (STAT5A), peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PPARGC1B), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A)] were predicted by IPA to be up-regulated in HG chickens (Figure 4.5).

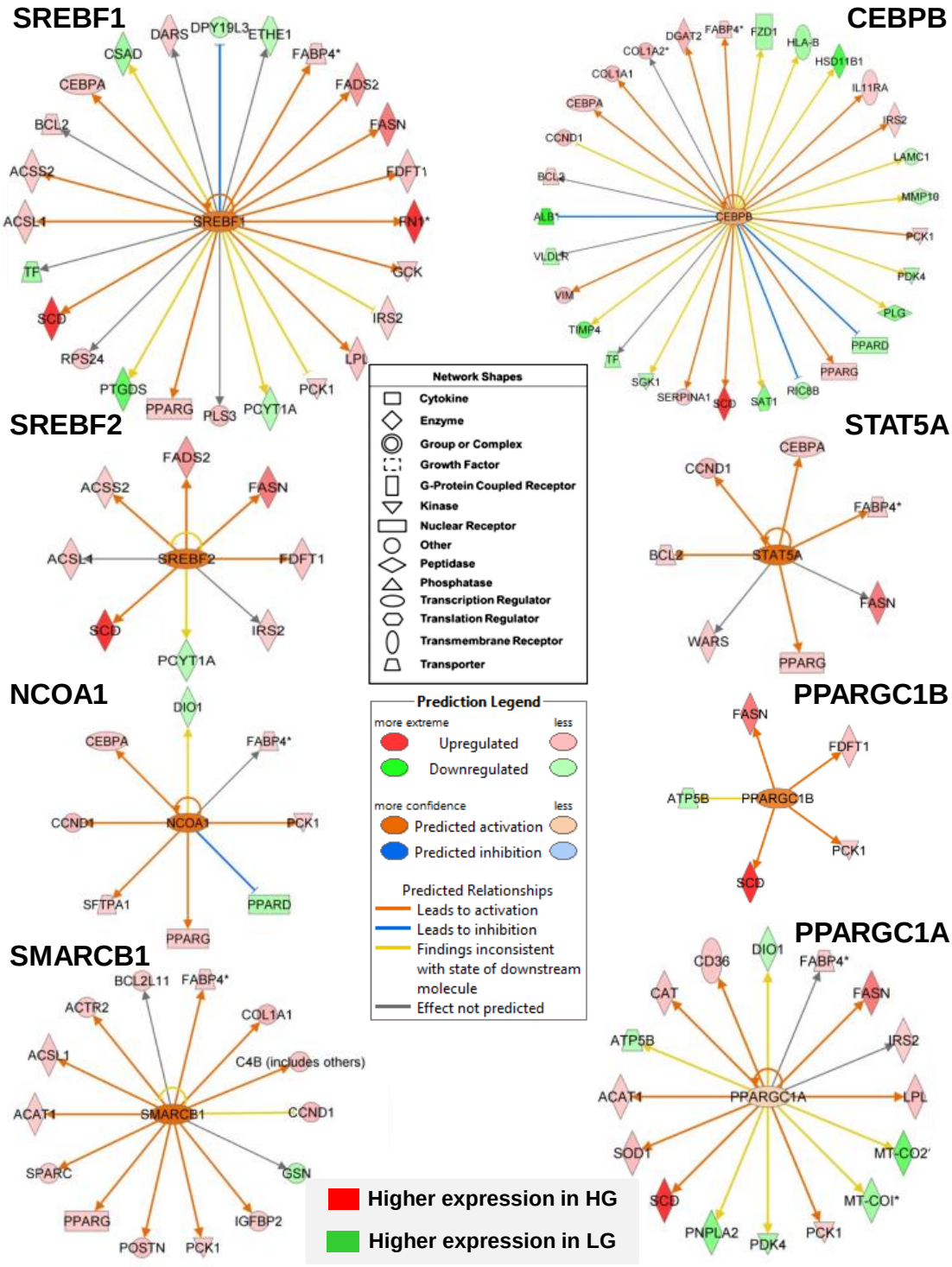

SREBF1
DARS
DPY19L3
ETHE1
CSAD
FABP4*
CEBPA
FADS2
BCL2
FASN
ACSS2
FDFT1
ACSL1
SREBF1
FN1*
TF
GCK
SCD
IRS2
RPS24
LPL
PTGDS
PPARG
PLS3
PCYT1A
PCK1
CEBPB
DGAT2
FABP4*
FZD1
HLA-B
COL1A2*
HSD11B1
COL1A1
IL11RA
CEBPA
IRS2
CCND1
LAMC1
BCL2
MMP10
ALB*
CEBPB
PCK1
VLDLR
PDK4
VIM
PLG
TIMP4
PPARD
TF
PPARG
SGK1
SERPINA1
SCD
SAT1
RIC8B
SREBF2
FADS2
ACSS2
FASN
ACSL1
SREBF2
FDFT1
SCD
IRS2
PCYT1A
STAT5A
CEBPA
CCND1
FABP4*
BCL2
STAT5A
FASN
WARS
PPARG
NCOA1
DIO1
CEBPA
FABP4*
CCND1
NCOA1
PCK1
SFTPA1
PPARD
PPARG
PPARGC1B
FASN
FDFT1
ATP5B
PPARGC1B
PCK1
SCD
SMARCB1
BCL2L11
FABP4*
ACTR2
COL1A1
ACSL1
C4B (includes others)
ACAT1
SMARCB1
CCND1
SPARC
GSN
PPARG
IGFBP2
POSTN
PCK1
PPARGC1A
CD36
DIO1
FABP4*
CAT
FASN
ATP5B
IRS2
ACAT1
PPARGC1A
LPL
SOD1
MT-CO2
SCD
MT-COI*
PNPLA2
PDK4
PCK1
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
Higher expression in HG
Higher expression in LG

Figure 4.5 Transcriptional regulators of fatness in abdominal fat of HG chickens throughout juvenile development. Functional gene interactions were identified by Ingenuity Pathway Analysis (IPA) software of a combined list with genotype and age by genotype interaction effects from the microarray analysis. This figure presents eight transcriptional regulators predicted to be up-regulated in HG chickens (orange color) based on the literature and values in our microarray DE list. Red gene symbols indicate higher expression in the HG and green gene symbols indicate higher expression in the LG.

Furthermore, six nuclear receptors [nuclear receptor subfamily 1, group H, member 3 (NR1H3; LXRA), nuclear receptor subfamily 4, group A, member 1 (NR4A1), nuclear receptor subfamily 1, group H, member 2 (NR1H2; LXRB), nuclear receptor subfamily 1, group I, member 2 (NR1I2), nuclear receptor subfamily 3, group C, member 2 (NR3C2), nuclear receptor interacting protein 1 (NRIP1)] were also predicted as higher expressed in HG chickens (Figure 4.6).

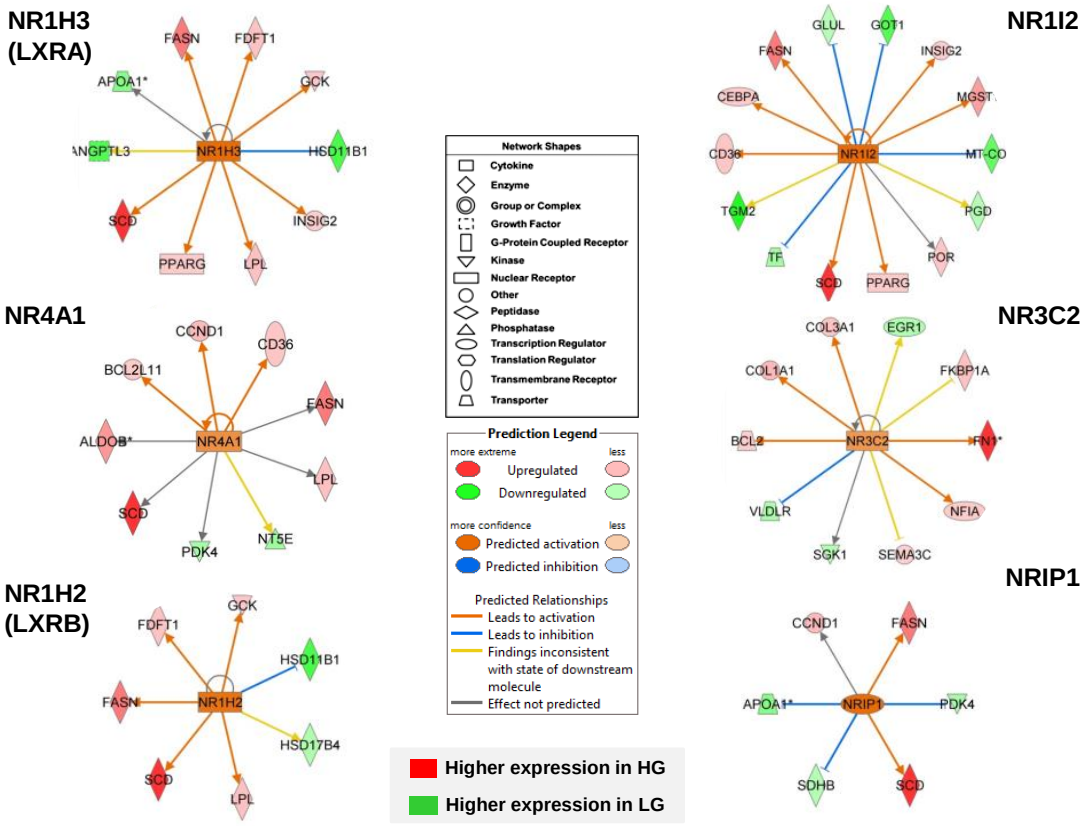


Figure 4.6 **Nuclear receptor regulators of fatness in abdominal fat of HG chickens throughout juvenile development.** Functional gene interactions were identified by Ingenuity Pathway Analysis (IPA) software of a combined list with genotype and age by genotype interaction effects from the microarray analysis. This figure presents six nuclear receptors predicted to be up-regulated in HG chickens (orange color) based on the literature and values in our microarray DE list. Red gene symbols indicate higher expression in the HG and green gene symbols indicate higher expression in the LG.

The potential of abdominal fat as a lipogenic tissue and as a local controller of abdominal fatness is reinforced by the abundance of HE transcriptional regulators of lipogenesis and adipogenesis (Figure 4.7-A).

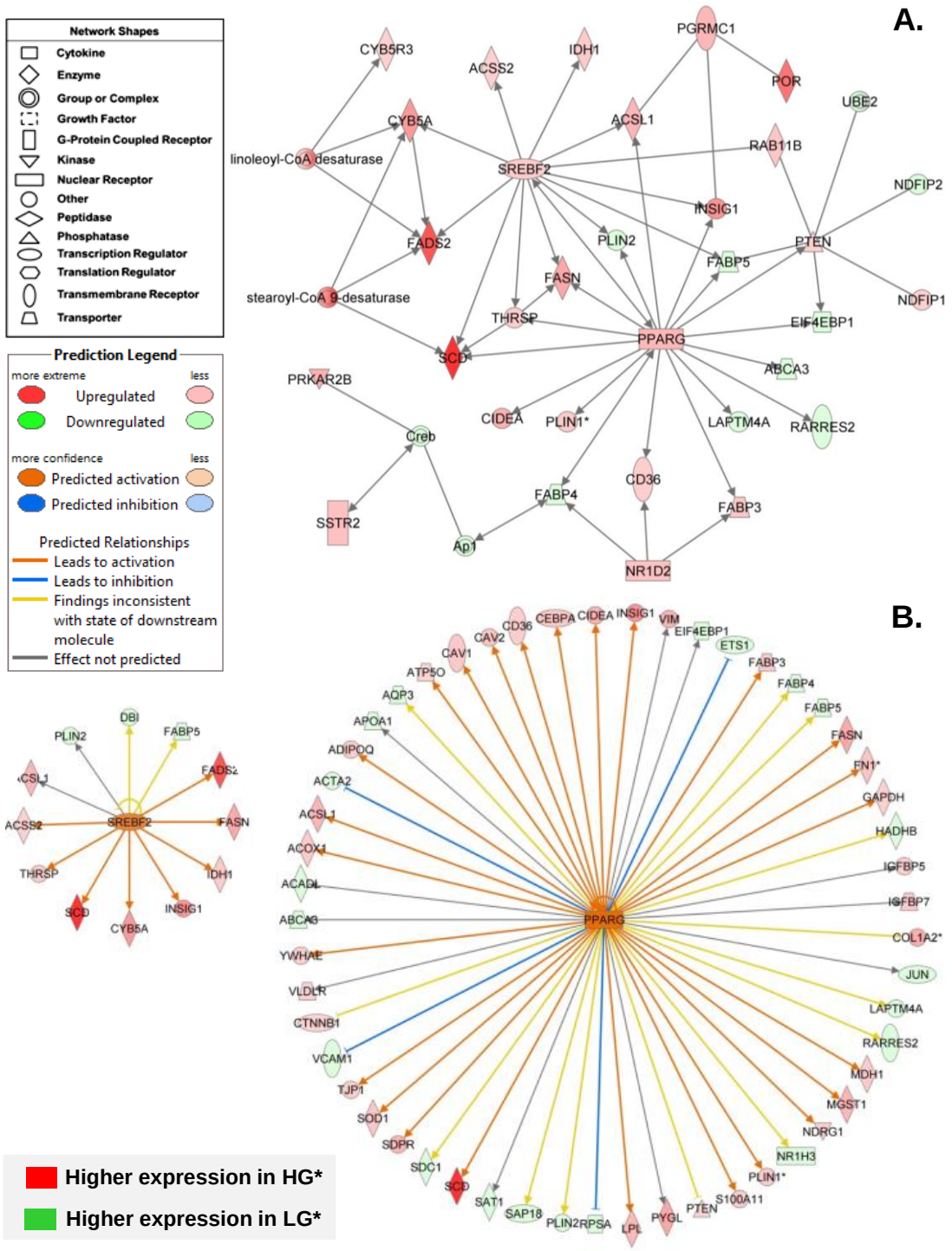

A.
Network Shapes
Cytokine
Enzyme
Group or Complex
Growth Factor
G-Protein Coupled Receptor
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Prediction Legend
more extreme
less
Upregulated
Downregulated
more confidence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent with state of downstream molecule
Effect not predicted
PGRMC1
CYB5R3
ACSS2
IDH1
POR
UBE2
CYB5A
ACSL1
RAB11B
linoleoyl-CoA desaturase
SREBF2
NDFIP2
INSIG1
FADS2
PLIN2
PTEN
FABP5
FASN
stearoyl-CoA 9-desaturase
NDFIP1
THRSP
EIF4EBP1
PPARG
SCD
ABCA3
PRKAR2B
CIDEA
PLIN1*
LAPTM4A
RARRES2
Creb
CD36
FABP4
FABP3
SSTR2
Ap1
NR1D2
B.
DBI
PLIN2
FABP5
FADS2
ACSL1
ACSS2
SREBF2
FASN
THRSP
IDH1
SCD
INSIG1
CYB5A
CD36
CEBPA
CIDEA
INSIG1
VIM
EIF4EBP1
CAV2
CAV1
ETS1
FABP3
ATP5O
FABP4
AQP3
FABP5
APOA1
FASN
ADIPOQ
FN1*
ACTA2
GAPDH
ACSL1
HADHB
ACOX1
IGFBP5
ACADL
IGFBP7
ABCA8
PPARG
COL1A2*
YWHAE
JUN
VLDLR
LAPTM4A
CTNNB1
RARRES2
VCAM1
MDH1
TJP1
MGST1
SOD1
NDRG1
SDPR
NR1H3
SDC1
PLIN1*
SCD
S100A11
SAT1
PTEN
SAP18
PLIN2RPSA
PYGL
LPL
Higher expression in HG*
Higher expression in LG*

Figure 4.7 Major highly expressed gene interactions in abdominal fat of HG and LG chickens at 7 weeks. Functional gene interactions networks were identified by Ingenuity Pathway Analysis (IPA) software of the highest expressed (HE; 900 genes) genes in abdominal fat at 7 wk from the RNA-Seq analysis (*no FDR cutoff). A.) This network shows direct gene interactions of many genes associated with lipogenesis including several transcriptional regulators (PPARG, SREBF2, THRSP, etc.). B.) All HE genes that are transcriptionally regulated by PPARG and SREBF2 are shown. Red gene symbols indicate higher expression in the HG and green gene symbols indicate higher expression in the LG.

The presence and interaction of HE transcription factors *SREBF2*, *PPARG*, and thyroid hormone responsive spot 14 (*THRSP*) result in the transcription or inhibition of transcription of many target genes. For example, some HE targets of *SREBF2* include *FADS2*, *FASN*, *SCD*, ATP citrate lyase (*ACLY*), acyl-CoA synthetase long-chain family member 1 (*ACSL1*), acyl-CoA synthetase short-chain family member 2 (*ACSS2*), cytochrome b5 type A (*CYB5A*), perilipin 2 (*PLIN2*), isocitrate dehydrogenase 1 (*IDH1*), insulin induced gene 1 (*INSIG1*), *PPARG* and *THRSP*. *PPARG* also targets *FASN*, *INSIG1*, *PLIN2*, *SCD* and *THRSP* and regulates transcription of fatty acid binding proteins 3 through 5 (*FABP3*, *FABP4* and *FABP5*) and several other HE genes [perilipin 1 (*PLIN1*), CD36 molecule (thrombospondin receptor), retinoic acid receptor responder 2 (*RARRES2*) and phosphatase and tensin homolog (*PTEN*)]. Other HE genes in abdominal fat of HG and LG chickens include progesterone receptor membrane component 1 (*PGRMC1*), P450 (cytochrome) oxidoreductase (*POR*) and somatostatin receptor 2 (*SSTR2*). A complete

representation of highly expressed targets of SREBF2 (13 HE genes) and PPARG (54 HE genes) is provided in Figure 4.7-B.

4.3.4 Verification of gene expression by quantitative RT-PCR

Based on biological function, candidate DE genes were selected from the microarray (1-11 wk) and RNA-Seq (7 wk) analyses for qRT-PCR verification. Several genes not identified by either analysis were also examined. Expression patterns for four transcriptional regulators which directly regulate adipogenesis and/or lipogenesis are shown in Figure 4.8-A. Three of these transcriptional regulators [*PPARG*, *CEBPA* and thyroid hormone responsive spot 14, alpha (*THRSPA*)] show very similar patterns of expression, being up-regulated in HG chickens at 1-7 wk (minus *PPARG* at 5 wk). Similarly, *SREBF1* was higher in HG chickens at all but 11 wk, with over a 4-fold increase at 1 wk (P=0.015). Four targets of these transcriptional regulators, that are necessary for lipogenesis, are shown in Figure 4.8-B. Interestingly, *SCD* was significantly increased in HG chickens throughout the experiment (1-11 wk: $P \leq 0.001$), being over ~110 and ~90 fold higher at 1 wk and 7 wk, respectively. Malic enzyme 1, NADP(+)-dependent, cytosolic (*ME1*) was up-regulated in the HG at 1, 5 and 7 wk, with its peak difference being at 5 wk (~2.5 fold increase in HG chickens). Both *FASN* and *DGAT2* were also significantly up-regulated in HG chickens ($P \leq 0.001$) at 1 and 9 wk with the greatest fold change observed at 7 wk (2.8 fold and 9.7 fold, respectively).

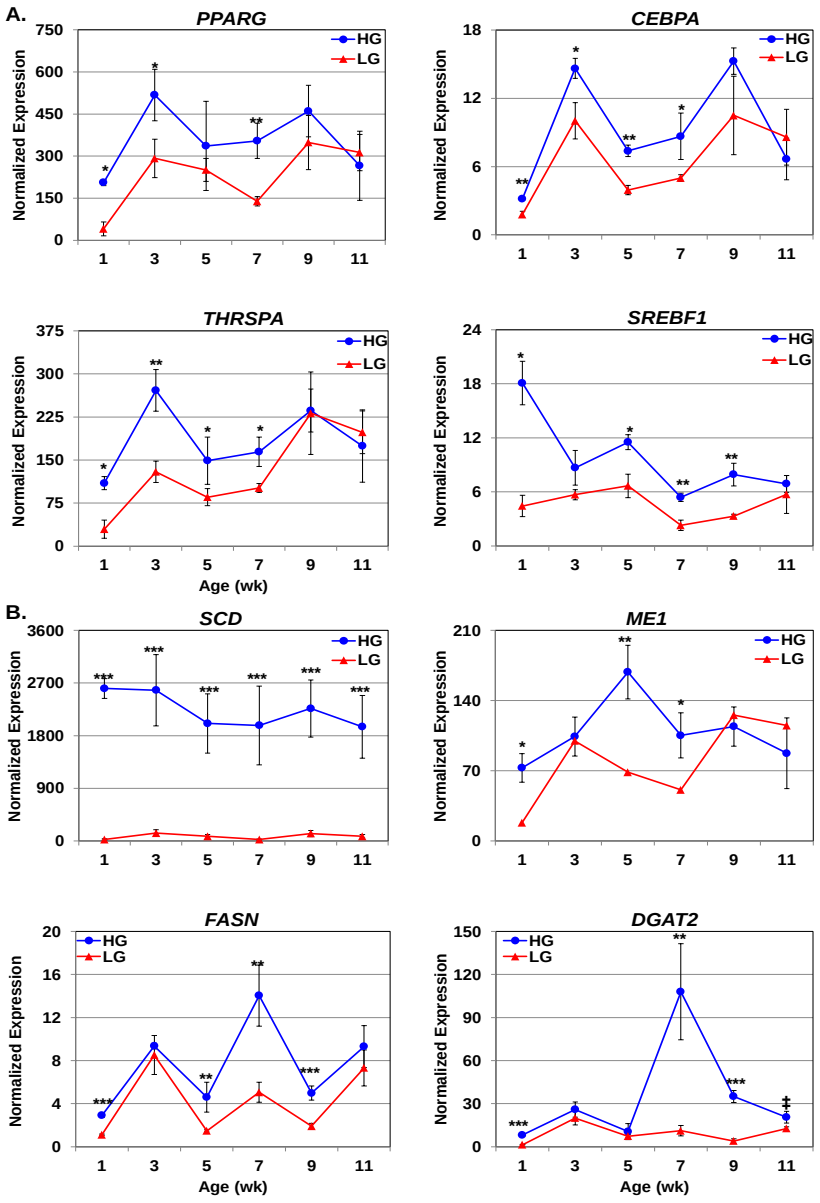


Figure 4.8 Verification of differentially expressed genes associated with fatness by qRT-PCR analysis. The abundance of eight genes associated with fatness [4 transcription factors (A.) and 4 metabolic enzymes (B.)] was determined by qRT-PCR analysis. Data points represent the mean ± SE of 4 birds/genotype. Significant differences between genotypes at each age were determined using a one way ANOVA and Tukey's multiple comparisons procedure at a significance level of $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***). ‡ denotes that the data point approaches significance ($P \leq 0.10$).

Transcript abundance was also examined by qRT-PCR for ten genes that appear to be associated with leanness (Figure 4.9). *PPARD* (see Figure 4.3) was up-regulated in LG chickens from 1-5 wk with an 8.6 fold increase at 1 wk. Similarly, both subunits of the mitochondrial trifunctional protein [hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, alpha subunit (*HADHA*) and hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, beta subunit (*HADHB*)] were up-regulated at 1-5 wk (4.8 and 5.6 fold, respectively at 1 wk). Carnitine palmitoyltransferase I (*CPT1A*), which mediates the transport of long chain fatty acids across the outer mitochondrial membrane, was also higher in the LG early (1-7 wk) with an average of a 2.4 fold increase across these weeks. Other mitochondrial genes [NADH-ubiquinone oxidoreductase chain 6 (*ND6*), cytochrome c oxidase subunit 3 (*COX3*), and malic enzyme 3, NADP(+)-dependent (*ME3*)] follow a similar trend with higher expression at 1-5 wk in LG chickens and large differences at 1 wk (31, 9.3 and 7.1 fold increases, respectively). Two mitochondrial enzymes involved in ketogenesis [HMG-CoA synthase 2 (*HMGCS2*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*HMGCL*)] also exhibit and early up-regulation in LG chickens. Interestingly, the Pr57 envelope protein (*envPr57*) is expressed higher in abdominal fat of LG chickens at all ages (1-11 wk).

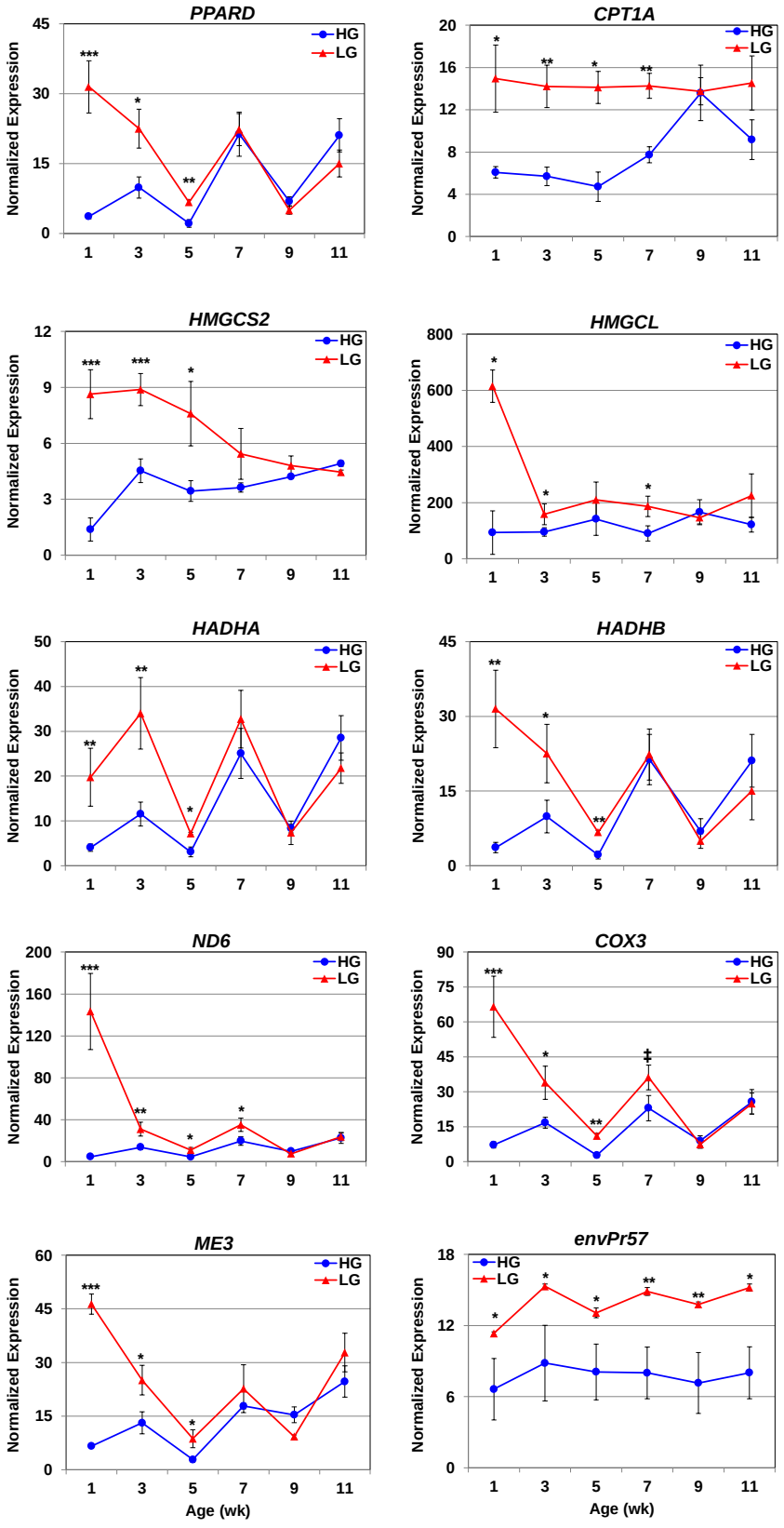

PPARD
CPT1A
HMGCS2
HMGCL
HADHA
HADHB
ND6
COX3
ME3
envPr57
HG
LG
Normalized Expression
Age (wk)

Figure 4.9 Verification of differential expression of genes associated with leanness by qRT-PCR analysis. The abundance of ten genes associated with leanness was determined by qRT-PCR analysis. Data points represent the mean ± SE of 4 birds/genotype. Significant differences between genotypes at each age were determined using a one way ANOVA and Tukey's multiple comparisons procedure at a significance level of $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***). ‡ denotes that the data point approaches significance ($P \leq 0.10$).

Gene expressions for growth hormone signaling genes (A.) and thyroid hormone converting enzymes (B.) are presented in Figure 4.10. The short chicken growth hormone (*scGH*) gene was expressed higher ($P \leq 0.01$) in HG chickens at 1 and 7 wk. The growth hormone receptor (*GHR*) was up-regulated in HG chickens from 1 to 7 wk with the largest differences observed at 1 and 7 wk (4.9 and 4.7 fold increase, respectively). Differences in the gene for thyroid hormone activating enzyme, deiodinase, iodothyronine, type I (*DIO1*), were seen at 3, 5 and 9 wk (up-regulated in LG chickens at all 3 ages) with the largest difference seen at 5 wk (7.7 fold higher in LG chickens). Conversely, the gene for thyroid hormone deactivating enzyme, deiodinase, iodothyronine, type III (*DIO3*), was only significantly different between the genotypes late in the experiment (9 and 11 wk) where it was expressed higher in HG chickens.

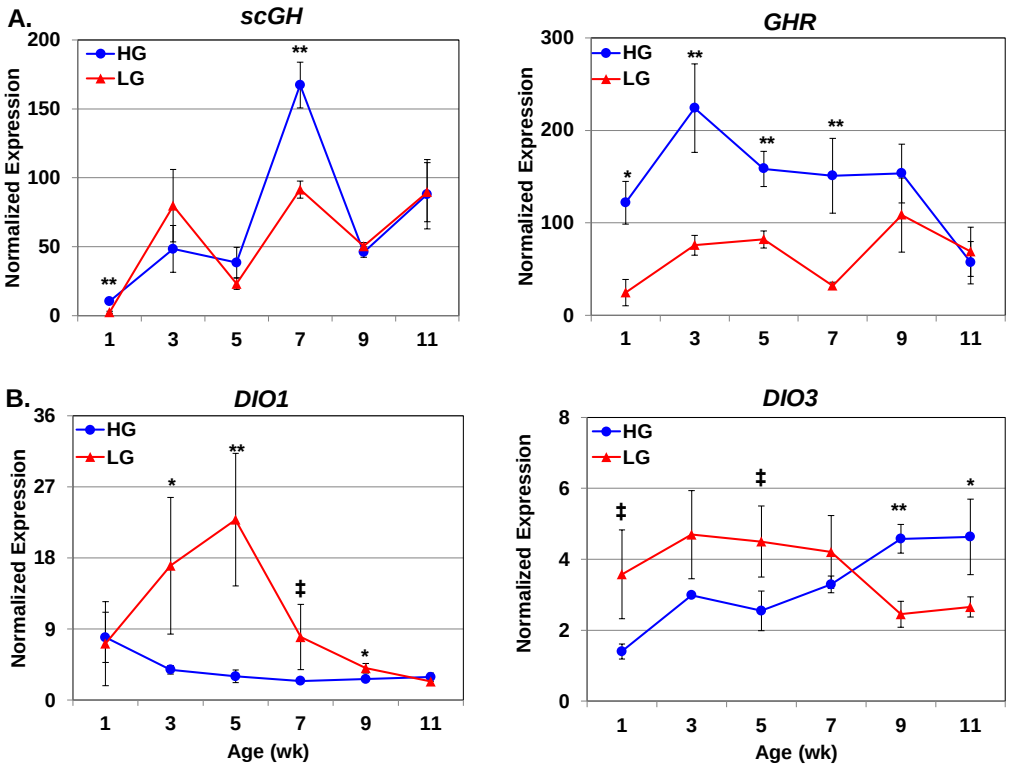


Figure 4.10 Verification of differential expression of genes associated with growth hormone and thyroid hormone signaling by qRT-PCR analysis. The abundance of two genes associated with growth hormone signaling (A.) and two genes associated with thyroid hormone metabolism (B.) was determined by qRT-PCR analysis. Data points represent the mean ± SE of 4 birds/genotype. Significant differences between genotypes at each age were determined using a one way ANOVA and Tukey's multiple comparisons procedure at a significance level of $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***). ‡ denotes that the data point approaches significance ($P \leq 0.10$).

Forty-seven genes analyzed at 7 wk by qRT-PCR and RNA-Seq analyses (including 25 genes also analyzed by microarray analysis—see section 4.2.2.3) are presented in Table 4.2. The twenty-five genes that were measured across all three analyses were selected to determine the correlation between microarray analysis and

the other two analyses used. Of these twenty-five genes, eleven genes were significantly different ($P \leq 0.05$) between abdominal fat of HG and LG chickens at 7 wk across all three analyses (*ALB*, *ALDOB*, *A2M*, *EX-FABP*, *FADS2*, *FGA*, *HSD17B7*, *PDK4*, *SCD*, *THBS2*, and *TTR*). Twelve genes were determined to significantly different ($P \leq 0.05$) by qRT-PCR but did not reach significance level in the RNA-Seq and/or microarray analyses (*AGTR1*, *ANXA1*, *CEBPA*, *F5*, *FASN*, *HPGDS*, *LDHA*, *LPIN1*, *LPL*, *ND6*, *PPARG*, and *PYGL*). Two genes were not significantly different by qRT-PCR or RNA-Seq analyses, but reached significance in the microarray analysis (*PLG* and *PGRMC1*). Expression fold change ratios of these twenty-five genes for qRT-PCR analysis vs. microarray analysis and RNA-Seq analysis vs. microarray analysis comparisons had significant ($P=0$) Spearman's rank coefficients (rho = 0.824615 and 0.854395, respectively). The additional twenty-two genes measured were similar across qRT-PCR and RNA-Seq analyses in both magnitude and direction of fold change as well as significance level, except for *GPD1*, *LCN15*, *PDE1C* and *SELP* which were not significant ($P \leq 0.05$) by RNA-Seq analysis but reached significance by qRT-PCR analysis. Expression fold change ratios for all forty-seven genes compared across RNA-Seq and qRT-PCR analyses produced a significant ($P=0$) Spearman's rank coefficient (rho = 0.928789).

Table 4.2 Comparison of gene expression across three methods in abdominal fat of HG and LG chickens at 7 weeks

	qRT-PCR Analysis		RNA-Seq Analysis		Microarray Analysis	
Gene Symbol	**Fold change**	**P-Value**	**Fold change**	**P-Value**	**Fold change**	**P-Value**
ALB	-345.12	1.38E-10	-197.9	3.65E-11	-1.48	0.01
ALDOB	2.75	0.0037	3.15	0.0001	1.4	0.0004
A2M	-2.27	0.0412	-57.75	2.17E-09	-5.81	0.0009
AGTR1	-2.08	0.0035	-2.23	0.162	-1.92	0.0579
ANXA1	3.4	0.0002	2.19	0.0997	2.52	0.1303
CEBPA*	1.73	0.0302	1.34	1	3.19	0.0055
F5	-3.21	0.016	-3.5	0.0056	-4.34	0.1336
EX-FABP	-41.12	1.22E-08	-31.62	0	-5.78	0.0069
FADS2*	4.9	0.0003	4.09	2.42E-09	2.15	0.0004
FASN*	2.78	0.0033	2.07	0.0833	1.35	0.0003
FGA	-1929.39	8.46E-11	-254.83	4.38E-12	-7.91	0.0002
HPGDS	-2.76	0.0003	-6.17	0.0589	-4.05	0.0033
HSD17B7	3.38	2.21E-05	4.14	0.0001	5.15	0.0401
LDHA	-2.74	0.015	-2.3	0.052	-1.58	0.0484
LPIN1	2.22	0.0278	1.53	0.6843	1.1	0.5185
LPL	3.69	0.0002	1.7	1	1.54	0.0007
ND6 **	-1.78	0.037	-1.98	0.1454	-1.28	0.0309
PPARG*	2.55	0.0047	1.68	0.2362	1.66	0.2626
PYGL	3.07	0.0068	2.14	0.0833	1.45	0.0052
PLG	-7.22	0.0652	-1945.09	0.2118	-3.52	0.0493
PGRMC1	1.36	0.1159	1.7	1	2.42	0.0205
PDK4	-4.95	0.0056	-6.22	2.22E-07	-1.25	0.0002
SCD*	89.12	0.002	138.31	0	3.53	4.52E-13
THBS2	3.57	0.0012	3.12	0.0006	5.18	2.33E-05
TTR	-20.01	9.86E-07	-26.81	0.0001	-1.73	0.0229
DHCR24	6.18	0.0001	5.07	2.79E-09	-	-
ACACA	1.85	0.056	1.87	0.0589	-	-
ACE	1.91	0.0269	2.61	0.0069	-	-
AGT	-24.69	1.73E-06	-48.36	0.0089	-	-
APOH	-24.98	1.48E-06	-84.89	0.0001	-	-
CDS2	1.99	0.0729	1.79	0.0994	-	-
F8	-1.49	0.0589	-2.23	0.0566	-	-
DGKQ	4	1.08E-05	3.52	0.0041	-	-
DPP7	2.73	0.0003	2.69	0.0086	-	-
FGB	-32.54	1.11E-06	-322.55	4.26E-08	-	-
FGG	-118.03	0.0016	-125.62	5.79E-09	-	-
FZD9	1.54	0.0201	2.14	0.0185	-	-
GPD1	3.25	6.74E-06	2.08	0.0655	-	-
GREM1	2.02	0.0007	3.72	0.0076	-	-
IGFALS	2.13	0.0287	2.71	0.0014	-	-
KLF5	-3.09	0.0076	-2.71	0.0307	-	-
LCN15	-2.27	0.0256	-2.02	0.0629	-	-
OSBP2	2.1	0.0561	2.63	0.0847	-	-
PDE1C	-1.84	0.0078	-2.47	0.083	-	-
SELE	-3.28	0.0139	-6.82	0.0089	-	-
SELP	-2.08	0.0089	-2.92	0.065	-	-
SERPINB2	-6.11	0.0035	-9.53	2.65E-05	-	-

Fold change values [(+) is higher expression in HG and (-) is higher in LG chickens)] provided across three independent transcriptional analysis methods at 7 wk. *Indicates that the data for this gene is a subset (7 wk) of the data presented in Figure

4.8. ** Indicates that the data for this gene is a subset (7 wk) of the data presented in Figure 4.9. *P*-value for qRT-PCR and microarray analysis results from a Student's T-test on expression values at 7 wk. *P*-value for RNA-Seq is FDR corrected.

4.4 Discussion

The HG and LG models are populations of broiler chickens that were divergently selected for either high (HG) or low (LG) body weight at 8 and 32 wks of age in order to study genetic changes resulting from large differences in growth rate [19]. These selection efforts resulted in a 2.7 fold increase in bodyweight in HG chickens on average from 1-11 wks (see Figure 4.2). Perhaps more remarkable is that across these same ages the HG chickens are on average eight times fatter (as a percentage of BW) than the LG. This difference in abdominal fatness is nearly three times larger than what was observed in our fat line and lean line chickens (2.5 fold difference from 3-11 wk), which were selected for differences in abdominal fat at a similar bodyweight and feed intake (see Chapter 2). This demonstrates that the selection for growth has a larger effect on abdominal fatness relative bodyweight than direct selection for abdominal fatness, suggesting that the HG and LG chickens also make a good model for studying fatness and leanness, which results incidentally alongside divergence in growth. In our previous analyses of fat line and lean line chickens (see Chapter 2 and 3) we determined that abdominal fat has the potential to be a highly metabolic tissue and that genes involved in both adipogenesis and

lipogenesis were amongst the highest expressed in this tissue. In the present study we used the Del-Mar 14K Integrated Systems microarray to identify changes in gene expression resulting from divergent selection for growth (and incidental fatness) in abdominal fat of HG and LG chickens across six juvenile ages (1-11 wk). The microarray analysis results were verified at 1-11 wk by qRT-PCR analysis and at 7 wk by both RNA-Seq analysis and qRT-PCR analysis. This study of abdominal fat serves to cross-experimentally validate our findings in fat line and lean line chickens, showing that increases in fatness (regardless of selection goal) are correlated with increased gene expression of adipogenic, lipogenic and lipolytic genes (including transcription factors) and decreases in fatness are correlated with increased expression of genes involved in hemostasis. Further, abdominal fat of our LG chickens appears to enhance energy production early in development (1-5 wk) which returns to levels similar to HG chickens by 7 wks. These findings are important in understanding the function of abdominal fat in chickens, which has the potential to be a major lipogenic tissue.

4.4.1 Processes in abdominal fat responsible for leanness in LG chickens

Selection for slow growth substantially inhibits the deposition and enhances the breakdown of abdominal fat in the domestic chicken (see Figure 4.2). Some of the most remarkable changes in gene expression in LG chickens (summarized in Table 4.3) appear early in development (1-5 wk; see Figure 4.9), and are discussed herein.

Table 4.3 Most interesting differentially expressed genes associated with leanness in abdominal fat

Biological Process	Gene Symbol	Gene Name	Analysis	FC
β-Oxidation	***CPT1A***	carnitine O-palmitoyltransferase 1	Q (1-11)	-1.83
	HSD17B4	hydroxysteroid (17-beta) dehydrogenase 4	M	-1.35
	HADHA	trifunctional protein, alpha subunit	Q (1-11)	-1.52
	HADHB	trifunctional protein, beta subunit	Q (1-11)	-1.41
	SLC27A1	solute carrier family 27, member 1	M	-1.37
Oxidative Phosphorylation	***ND1***	NADH dehydrogenase subunit 1 (Complex I)	M	-3.21
	ND2	NADH dehydrogenase subunit 2 (Complex I)	M	-1.93
	ND4	NADH dehydrogenase subunit 4 (Complex I)	M	-2.04
	ND5	NADH dehydrogenase subunit 5 (Complex I)	M	-1.53
	ND6	NADH dehydrogenase subunit 6 (Complex I)	Q (1-11)	-3.35
	SDHB	succinate dehydrogenase subunit B (Complex II)	M	-1.26
	SDHD	succinate dehydrogenase subunit D (Complex II)	M	-1.23
	CYTB	cytochrome b (Complex III)	M	-2.02
	COX1	cytochrome c oxidase subunit I (Complex IV)	M	-1.6
	COX2	cytochrome c oxidase subunit II (Complex IV)	M	-2.64
	COX3	cytochrome c oxidase subunit III (Complex IV)	Q (1-11)	-2.13
	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	M	-2.14
	ATP5B	ATP synthase, H+ transporting, mitochondrial F1, β	M	-1.23
	ATP5C1	ATP synthase, H+ transporting, mitochondrial F1, γ 1	M	-1.23
	ATP6	ATP synthase F0 subunit 6	M	-2.7
Hemostasis	***A2M***	alpha-2-macroglobulin	R	-57.75
	AGT	angiotensinogen	R	-48.36
	ALB	albumin	R	-197.9
	F2	coagulation factor II (thrombin)	R	-18.19
	F5	coagulation factor V (proaccelerin, labile factor)	R	-3.5
	F8	coagulation factor VIII, procoagulant component	R	-2.23
	FGA	fibrinogen alpha chain	R	-254.83
	FGB	fibrinogen beta chain	R	-322.55
	FGG	fibrinogen gamma chain	R	-125.62
	GC	group-specific component (vitamin D binding protein)	R	-218.12
	SERPINA1	serpin peptidase inhibitor, clade A, member 1	R	-147.33
	SERPINB2	serpin peptidase inhibitor, clade B, member 2	R	-9.53
	SERPINF1	serpin peptidase inhibitor, clade F, member 1	R	-3.54
	TTR	transthyretin	R	-26.81
Transcriptional Regulators	***AEBP1***	Adipocyte enhancer binding protein 1	M	-1.35
	AR	androgen receptor	M	IPA
	CALR	calreticulin	M	-2.35
	CREM	cAMP responsive element modulator	M	IPA
	FBXW7	F-box and WD repeat domain 7	M	IPA
	NR1I3	nuclear receptor subfamily 1, group I, member 3	M	IPA
	NR4A3	nuclear receptor subfamily 4, group A, member 3	M	IPA
	PIAS1	protein inhibitor of activated STAT, 1	M	IPA
	PPARD	peroxisome proliferator-activated receptor beta	Q (1-11)	-1.59
	RELA	v-rel reticuloendotheliosis viral oncogene homolog A	M	IPA
Miscellaneous	***ANGPTL3***	angiopoietin-like 3	M	-2.94
	DIO1	deiodinase, iodothyronine, type I	Q (1-11)	-2.71
	Env-Pr57	envelope protein, partial [Avian leukosis virus]	M	-52
	EX-FABP	extracellular fatty acid-binding protein precursor	Q (7)	-41.13
	HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase	Q (1-11)	-2.18
	HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2	Q (1-11)	-1.8
	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	M	-3.11

ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	M	-1.31
ME3	malic enzyme 3, NADP(+)-dependent, mitochondrial	Q (1-11)	-1.8
PDK4	pyruvate dehydrogenase kinase, isozyme 4	M	-1.5
PDP1	pyruvate dehyrogenase phosphatase catalytic subunit 1	M	-1.53

Fold change (FC) values (all genes higher in LG chickens) are provided for genes associated with leanness. Q(7) = gene measured by qRT-PCR at 7 wk; Q(1-11) = gene measured by qRT-PCR at 1 -11 wk; M = gene measured by microarray analysis; IPA = predicted up-regulation by Ingenuity® Pathway Analysis of microarray data. All genes present are significantly different at a significance level of $P \leq 0.05$ (qRT-PCR) or FDR≤0.05 (RNA-Seq and microarray). Significance not limited to the single analysis shown for each gene.

An increase in the breakdown of fatty acids in LG chickens can be traced from cellular up-take to cytosolic transport to the mitochondria for beta-oxidation and entrance into the citric acid (TCA) cycle (as acetyl-CoA) producing ATP and NADH to enter the electron transport chain for further production of ATP. The long-chain fatty acid transporter (*SLC27A1*) is up-regulated in LG chickens throughout juvenile development. This gene is very highly expressed in human adipose tissue and muscle and much lower in less metabolic tissues, and is barely detectable in liver [27]. The SLC27 family of proteins are responsible for the cellular up-take of long chain fatty acids for storage [28] or, more likely, for β-oxidation and entrance into the TCA cycle for energy production in LG chickens. Correspondingly *CPT1A*, the key enzyme in the carnitine-dependent transport of long chain fatty acids into the mitochondria (the rate limiting step of β-oxidation), is over expressed in abdominal fat of LG chickens. The over-expression of this gene is driven by increased energy requirements in chickens, where hepatic expression is up-regulated by fasting [29] and down-regulated after the embryo-hatch transition [30]. Furthermore, several single nucleotide polymorphisms

in *CPT1A* were identified in Yup'ik Eskimos which were associated with HDL-cholesterol and obesity phenotypes [31].

Once the long chain fatty acids are transported into the mitochondria, several steps of β-oxidation are catalyzed by the tri-functional protein, which produces acetyl-CoA for entrance into the TCA cycle. Both subunits of the tri-functional protein (*HADHA* and *HADHB*) were over expressed in LG chickens from 1-5 wk which corresponds with increases in gene expression of mitochondrial malic enzymes (*ME2* and *ME3*; suggesting an increase in TCA cycle activity) and several subunits of each of the enzyme complexes in the electron transport chain (see Figure 4.9 and Table 4.3). Though most long chain fatty acids undergo mitochondrial β-oxidation, several substrates including very-long-chain fatty acids, polyunsaturated fatty acids and several others must be shortened by peroxisomal β-oxidation in order to be transferred into the mitrochondria. The transcript for one of the major enzymes in peroxisomal β-oxidation, HSD17B4 (also known as multifunctional protein-2), was also up-regulated in abdominal fat of LG chickens. Deficiency of this enzyme in humans causes a severe developmental syndrome due to cellular build-up of long chain fatty acids and leads to death soon after birth; whereas murine knockout of *HSD17B4* still blocks the peroxisomal β-oxidation of very long chain fatty acids but does not result in neonatal death [32].

Ultimately, the TCA cycle is unable to handle the overload of acetyl-CoA produced by β-oxidation in abdominal fat of LG chickens, resulting in the over-

expression of *HMGCS2* and *HMGCL* (see Figure 4.9), the two main enzymes in mitochondrial ketone body synthesis. The mRNA expression of *HMGCS2* is greatly induced by fasting in the liver of suckling piglets [33] and 4-week old chickens [29]. In humans, both *HMGCS2* and *HMGCL* exhibit alternative splicing which may regulate which tissues these enzymes are active in [34]. Whether or not this complex regulation by alternative splicing exists among different tissues in chickens has yet to be determined. Taken together, these findings support that LG chickens increase cellular fatty acid up-take, peroxisomal β-oxidation, and transport to/into the mitochondria for β-oxidation and usage in the TCA cycle early (1-5 wk) driving their divergence in abdominal fatness from HG chickens.

The LG chickens appear to down-regulate the mitochondrial breakdown of pyruvate to acetyl-CoA through the up-regulation of *PDK4* which inhibits the pyruvate dehydrogenase (PDH) complex. PDH activity increases with increasing skeletal muscle stimulation (increasing contraction intensity) to a much greater extent in PDK4 knockout mice than in controls suggesting that PDK4 is essential for preventing the over-activation of the PDH complex [35]. Along with exercise, *PDK4* is over-expressed in rodents subjected to high fat diet or fasting [36] which is similar to what is seen in chickens where both fasting and acute insulin immunoneutralization (and fed) cause up-regulation when compared to a fed control [37]. Furthermore, PDK4 appears to be crucial pre-hatch for the acquisition of stored energy as its expression is quite high between day e16 and e20 but drops dramatically at hatch and remains low from day 1 through 9 post hatch [30].

Whether it is the reduced feeding seen in the LG chickens and other models selected for low bodyweight [38] or additional factors causing the near absolute depletion of abdominal fat has yet to be determined. A possible contributing factor may be the remarkably high expression of endogenous avian leucosis virus sequences in our LG chickens which was also reported in the brains of low weight selected chickens [39]. This study of high weight selected vs. low weight selected chickens only looked at gene expression in brain tissue and the authors concluded that these avian leucosis virus subgroup-E (ALVE) sequences could directly affect growth or may be closely linked to loci regulating growth. However, similar retroviral sequences (e.g., Genbank accessions: BG712672, BI065864, and AW355511) had the largest fold change differences in abdominal fat, liver and skeletal muscle (Cogburn LA; manuscript in preparation) in our LG chickens (1-11 wk), suggesting a similar mechanism in all groups selected for low body weight. Our findings support that these retroviral sequences do not only regulate growth but may also be involved in directly decreasing adiposity. Possible hypotheses for the mechanism of regulation include: 1.) The amount of resources required to transcribe the retroviral sequences greatly increases the energy need and feed intake is not sufficient to meet this need, or 2.) Cells in low growth lines perceive that they are infected resulting in an increase in the rate of lipolysis and β-oxidation. One study looked at knock-down of *FASN* and *ACACA* to inhibit the replication of rotavirus [40]. The authors found that knockdown of these genes involved in the biosynthesis of lipid, had a larger impact on reducing viral infectivity than on reducing viral RNA yield. This is in support of the second

hypothesis that LG chickens down-regulate genes involved in lipogenesis in an attempt to decrease infectivity from the retroviral sequences that are being transcribed. *EX-FABP*, expressed higher in LG chickens (especially at 7 wk; > 40 fold increase), has been identified as a stress lipocalin which selectively binds high affinity fatty acids and is regulated by inflammatory and anti-inflammatory agents in chickens [41]. This gene is homologous to the human lipocalin 2 which is known to be secreted by the host in response to *E.coli* produced ferric-siderophore complexes, and EX-FABP acts similarly during infection in chickens [42]. Whether *EX-FABP* is important for regulation of retroviral infection, or if it plays an alternative role in regulating adiposity is not known. Further investigation on the cause and effect of the large abundance of retroviral sequences in chickens selected for low body weight is needed.

Several transcriptional modulators (activators or inhibitors) are predicted by IPA to be associated with leanness (Figure 4.4) and/or are up-regulated in abdominal fat of LG chickens. Several of these transcriptional modulators (RELA, CALR, FBXW7, NR1I3, NR4A3 and PIAS1) inhibit transcription of genes involved in increasing adiposity (*SCD*, *FASN*, *DGAT2*, *FABP4*, *PCK1*, *LPL*, *PPARG*, *CEBPA*, etc.; discussed below) and activate genes associated with leanness (*COX1*, *HSD17B4*, *DIO1*, etc.). For example, PIAS1 suppresses LXR activation of fatty acid synthesis in murine hepatocytes [43] and NR1I3 is associated with hepatic ω-fatty acid oxidation in quercetin (a plant-derived flavonoid found in fruits, vegetables, leaves and grains) fed mice [44]. Gene expression of DIO1, the major enzyme responsible for the conversion of inactive T_4 to metabolically active T_3, and DIO3, which degrades active

T_3 and converts T_4 to inactive reverse T_3 (rT_3) [45] were analyzed (see Figure 4.9). Interestingly, *DIO1* appears to be over-expressed in LG chickens early (3 and 5 wk) while *DIO3* is higher in HG chickens at later ages (9 and 11 wk). This may indicate a higher level of the active thyroid hormone in abdominal fat of LG chickens throughout juvenile development which has been associated with leanness in chickens [46]. Nuclear receptor PPARD (see Figure 4.3) has similar activation and inhibition targets and directly up-regulates several additional genes associated with leanness (i.e., *ANGPTL3, PLP1* and *PDK4*). Selective adipose tissue knock-in of PPARD in $Lep^{db/db}$ mice has a profound impact on reduction of lipid accumulation and appears to be important in preventing obesity in mice [47]. It's early (1 to 5 wk) up-regulation in LG chickens may suggest that it is a major regulator of the breakdown of lipid for energy production (discussed above) in these animals. Furthermore, *AEBP1* may also be a significant regulator of leanness in LG chickens. An interesting study in Wistar rats demonstrated that feeding with a high fat sucrose diet (producing a fat phenotype) had no effect on adipose tissue expression of *AEBP1* but a dramatic increase in expression was observed when the high fat sucrose diet was combined with apple polyphenols (generating a lean phenotype) [48]. The anti-obesity effects of AEBP1 appear to be exerted through transcriptional repression of adipogenesis [49].

Hemostasis is the process that causes blood flow to halt at the site of vascular injury by generation of a clot, followed by dissolution of that clot once the injury is resolved. One of the major findings of our time course analysis (1-11 wk) of abdominal fat in divergently selected fat and lean chickens (see Chapter 2) was the up-

regulation of hemostatic genes, including many coagulation factors, in lean chickens. Our data suggested that coagulation and fibrinolysis enzymes may be involved in enhancing proteolytic processing of adipokines and other endocrine factors, which could contribute to the lean phenotype. It was therefore intriguing to us that in the present study, many of these hemostatic genes were not differentially expressed in the time course (1-11 wk) analysis of abdominal fat in HG and LG chickens. The time-course analysis may have missed these genes because we were taking a time-course average looking for differences that are important throughout juvenile development. However, hemostatic processes were amongst the top functional categories of both the RNA-Seq DE gene and HE gene analyses in abdominal fat at 7 wk (Table 4.1) suggesting that they are of critical importance at this age. The observed fold change differences for these genes in the RNA-Seq analysis were quite large (in many instances > 20 fold higher in LG) due to the high abundance in LG chickens and near absence in HG chickens. The significance of this finding specifically at 7 wk is unknown; however, it appears to correspond with the extreme depression in abdominal fat relative body weight in LG chickens at this age (see Figure 4.2). Remarkable up-regulation of several hemostatic genes in LG chickens were seen for those involved directly in coagulation (*FGA*, *FGB*, *FGG*, *F2*, *F5* and *F8*), serine protease inhibitors involved in coagulation or fibrinolysis (*SERPINA1*, *SERPINB2* and *SERPINF1*) and major plasma transporters (*A2M*, *ALB*, *GC*, and *TTR*). These plasma transporters may be of great importance for maintaining leanness. For example, *A2M* is known to inhibit proteases (including coagulation factors) and transport cytokines [50]. Further,

ALB, GC, and TTR are all part of the same family of proteins responsible for the transport of several metabolites associated with leanness including: steroids, fatty acids, vitamin D [51], thyroid hormone [46], and retinoids [52]. While the mechanisms by which these hemostatic genes regulate adiposity have yet to be defined in chickens, this study serves as a cross-model validation that they are associated with a lean phenotype and could potentially have a major impact on adipokine signaling due to their proteolytic activity and enhanced expression in abdominal fat.

4.4.2 Processes in abdominal fat responsible for fatness in HG chickens

Against the convention that abdominal fat has a minimal contribution as a lipogenic tissue [18] and similar to our finding in abdominal fat of fat line chickens (see Chapter 2 and 3), the HG over-express many genes involved in increasing adipoisty including those directly involved in adipogenesis and lipogenesis (see Figure 4.3, Figure 4.8; summarized in Table 4.4). The higher level control of adipogenesis and lipogenesis in abdominal fat of HG chickens is through the transcription factor activity and interaction of at least several major regulators including PPARG [53], CEPBA [54], SREBF1 [55] and THRSPA [56, 57] (see Figure 4.3 and 4.7). These transcriptional regulators are over-expressed throughout juvenile development (see Figure 4.8) and have a set of common lipogenic targets (also higher in HG chickens) including *DGAT2*, *FADS2*, *FASN* and *SCD* (discussed below). Furthermore, IPA predicts the up-regulation (in HG chickens) of an additional 13 transcriptional

regulators associated with fatness, including SREBF2 and CEBPB, which also directly increase the expression of lipogenic genes (see Figure 4.5 and 4.6). Microarray analysis of liver in mice with transgenic over-expression *SREBF1* or *SREBF2* determined that they have many common targets and both play a crucial role in regulating the synthesis of fatty acids and cholesterol [58]. *INSIG2*, also higher in HG chickens, regulates of fatty acid and cholesterol synthesis through the direct regulation of the SREBF chaperone and HMG-CoA reductase [59]. Two additional genes involved in cholesterol synthesis (*DHCR24* and *HSD17B7*) are commonly up-regulated by divergent selection for high body weight or high abdominal fatness (see Chapter 3). In human whole blood, *DHCR24* expression is positively correlated with weight loss after bariatric surgery [60]. Two other genes over-expressed in adipose of HG chickens, *ACACA* and *ACSS2*, were identified as predictors of weight loss (decrease in weight and hip circumference) in another group of patients who underwent bariatric surgery [61]. Acetyl CoA carboxylase (*ACACA* or *ACC1*) is critically important for the generation of malonyl CoA and the synthesis of long chain fatty acids. This was demonstrated in human hepatoma HepG2 cells where inhibition of ACC1 by soraphen A reduces *de novo* lipogenesis by attenuating the formation of malonyl CoA and consequently long chain fatty acids [62]. Overexpression of fatty acid elongases, *ELOVL5* and *ELOV6*, or desaturases, *FADS1* and *FADS2* was not successful in reversing the effect of soraphen A on the production of long chain fatty acids supporting the crucial role of ACC1.

Table 4.4 Most interesting differentially expressed genes associated with fatness in abdominal fat

Biological Process	Gene Symbol	Gene Name	Analysis	FC
Transcriptional Regulators	***CEBPA***	CCAAT/enhancer binding protein (C/EBP), alpha	Q (1-11)	1.4
	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	M	IPA
	NCOA1	nuclear receptor coactivator 1	M	IPA
	NR1H2	nuclear receptor subfamily 1, group H, member 2	M	IPA
	NR1H3	nuclear receptor subfamily 1, group H, member 3	M	IPA
	NR1I2	nuclear receptor subfamily 1, group I, member 2	M	IPA
	NR3C2	nuclear receptor subfamily 3, group C, member 2	M	IPA
	NR4A1	nuclear receptor subfamily 4, group A, member 1	M	IPA
	NRIP1	nuclear receptor interacting protein 1	M	IPA
	PPARG	peroxisome proliferator-activated receptor γ	Q (1-11)	1.55
	PPARGC1A	peroxisome proliferator-activated receptor γ, coactivator 1 α	M	IPA
	PPARGC1B	peroxisome proliferator-activated receptor γ, coactivator 1 β	M	IPA
	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	M	IPA
	SREBF1	sterol regulatory element binding transcription factor 1	Q (1-11)	2.08
	SREBF2	sterol regulatory element binding transcription factor 2	M	IPA
	STAT5A	signal transducer and activator of transcription 5A	M	IPA
	THRSPA	thyroid hormone responsive spot 14, alpha	Q (1-11)	1.43
Lipid Biosynthesis	***ACACA***	acetyl-CoA carboxylase alpha	R	1.87
	ACSL1	acyl-CoA synthetase long-chain family member 1	M	1.43
	ACSS2	acyl-CoA synthetase short-chain family member 2	M	1.29
	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	M	1.26
	DGAT2	diacylglycerol O-acyltransferase 2	Q (1-11)	3.69
	DHCR24	24-dehydrocholesterol reductase	R	5.07
	ELOVL5	ELOVL fatty acid elongase 5	M	1.41
	FADS2	fatty acid desaturase 2; delta-6 fatty acid desaturase	M	2.52
	FASN	fatty acid synthase	Q (1-11)	1.78
	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	R	4.14
	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	Q (1-11)	29.0
Miscellaneous	***ALDOB***	aldolase B, fructose-bisphosphate	M	2.58
	GCK	glucokinase (hexokinase 4)	M	1.4
	GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)	R	2.08
	GHR	growth hormone receptor	Q (1-11)	2.21
	HK1	hexokinase1	M	1.36
	IGF2R	insulin-like growth factor 2 receptor	M	1.31
	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	M	1.31
	INSIG2	insulin induced gene 2	M	1.24
	PGRMC1	progesterone receptor membrane component 1	M	2.02
	RETSAT	retinol saturase (all-trans-retinol 13,14-reductase)	M	1.73
	scGH	small chicken growth hormone	Q (1-11)	1.18

Fold change (FC) values (all genes higher in HG chickens) are provided for genes associated with fatness. Q(7) = gene measured by qRT-PCR at 7 wk; Q(1-11) = gene measured by qRT-PCR at 1 -11 wk; M = gene measured by microarray analysis; IPA = predicted up-regulation by Ingenuity® Pathway Analysis of microarray data. All genes present are significantly different at a significance level of $P \leq 0.05$ (qRT-PCR) or FDR≤0.05 (RNA-Seq and microarray).

Perhaps one of the most significant findings in abdominal fat of these animals is the expression pattern of *SCD*. SCD is the rate-limiting enzyme responsible for the conversion of saturated fatty acids to monounsaturated fatty acids. Mice with SCD deficiency exhibit a lean phenotype with decreased abdominal fat content compared to controls and *ob/ob* mice with deficiencies in SCD exhibit a large reduction in fatness [63]. At 7 wk, SCD is the highest expressed gene in the abdominal fat of HG chickens, while its expression is approximately 140 fold lower in the LG, and this difference is present throughout juvenile development (see Figure 4.8) where levels of SCD are nearly undetectable in LG chickens (1-11 wk). Another desaturase expressed very highly in HG chicken abdominal fat is *FADS2*, which is down-regulated in liver in response to fasting in both pigs [64] and chickens [29] as well as in the pre-hatch embryo [30]. The major multi-enzyme protein responsible for the synthesis of fatty acids, *FASN*, is also very highly expressed in HG chickens at several ages, with the peak difference observed at 7 wk, the age of maximal distinction in abdominal fat between the two genotypes. The essential role of *FASN* in fatty acid biosynthesis was highlighted in mouse embryos where *FASN* knockout mice died before birth and heterozygous knockout mice died at various stages of development [65]. An incredibly similar expression pattern (as *FASN*) was observed for *DGAT2*, which catalyzes the final (committed) step in triacylglycerol synthesis and is critical in the formation of adipose tissue [66, 67] where there is nearly a 10-fold increase in HG chickens at 7 wk. Overexpression of *DGAT2* in mammalian HEK293 cells significantly increases triglyceride synthesis while inhibition of *DGAT2* by compound 122 decreases

synthesis in a dose dependent manner [68]. Further, *DGAT2* is up-regulated in goose hepatocytes exposed to a mixture of long chain fatty acids [69]. Correspondingly, *ACSL1*, which generates fatty acyl-CoA for entry into the triacylglycerol synthesis pathway, and *AGPAT9*, which catalyzes the conversion of glycerol-3-phosphate to lysophosphatidic acid, the initial step of triacylglycerol synthesis [70] were both overexpressed in HG chickens.

Abdominal fat of HG chickens also over expresses *GPD1*, which serves as a link between carbohydrate metabolism and lipid metabolism by catalyzing the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate, the major component of glycerophospholipids. In fact, HG chickens up-regulate several genes involved in glycolysis including glucokinase (*GCK*) and hexokinase 1 (*HK1*), which phosphorylate glucose to produce glucose-6-phosphate, the first step in the glycolytic pathway, and *ALDOB*, which converts fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (substrate for *GPD1*). Taken together, HG chickens up-regulate genes required for production of acetyl-CoA for use in the biosynthesis of fatty acids, cholesterol and triglycerides, which are highly up-regulated processes in the abdominal fat of these animals.

Growth hormone (GH) signaling is an interesting topic in chickens, specifically in models divergently selected for differences in bodyweight. Dissimilar to the mammalian response to GH [71-73], exogenous GH sharply increases accumulation of abdominal fat of juvenile chickens with minimal effect on growth rate [74-78]. GH signaling has been examined in the HG and LG chickens used in the

present study (Cogburn LA, manuscript in preparation). Plasma levels of GH are increased in LG chickens throughout juvenile development (5 through 11 wks) which appears to be stymied by ineffective binding of GH to hepatic GHR in LG chickens. It is likely that the LG chicken GHR is also defective in adipose tissue, which could amplify the enhanced GH signaling in abdominal fat of HG chickens (see Figure 4.10). Insulin like growth factor signaling, which is stimulated by GH, is also up-regulated in HG chickens. For example, *IGF2R* and *IGFBP2* are both over expressed in HG adipose. The protein structure of IGF2R and IGFBP2 are similar [79] suggesting a common goal for their up-regulation in HG chickens, which may include an involvement in the development of juvenile adiposity [80]. Further studies on GH signaling in chicken adipose tissue are needed to fully understand the implications of these findings.

4.5 Chapter Summary

Selection for increased or decreased bodyweight in chickens effectively amplifies or diminishes abdominal fatness, respectively. This is one of the first transcriptional studies to look at a metabolic tissue across juvenile development in chicken growth models to understand the genetic underpinnings of these incidental phenotypes. Throughout juvenile development, HG chickens over-express several transcription factors which regulate lipogenesis and adipogenesis. These transcription factors appear to be responsible for the up-regulation of several processes (i.e.,

biosynthesis of fatty acids, cholesterol and triglycerides) which increase abdominal fatness in HG chickens. The genes involved in these processes are not only differentially expressed but are also amongst the highest expressed in HG abdominal fat. Conversely to HG chickens, early on in juvenile development (1-5 wk) LG chickens transcriptionally up-regulate several energy producing processes which are likely responsible for their extreme leanness (i.e., peroxisomal β-oxidation, mitochondrial β-oxidation, ketogenesis and oxidative phosphorylation). These processes may be up-regulated as a defense mechanism to a perceived infection by avian leucosis retroviral sequences. Hemostasis also appears to have a critical role in the maintenance of extreme leanness in chickens. These findings validate that abdominal fat has a major contribution to the regulation of adiposity in divergently selected models which (directly or incidentally) result in large differences in abdominal fatness.

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Appendix

QRT-PCR PRIMER INFORMATION

Gene Symbol	Gene name	Forward (F) and reverse (R) primers	Accession	Size (bp)
A2M	alpha-2-macroglobulin	TGGAGGAGCCCTTAGAAGACTGT (F) TGTCATCTTCATTCATCTCCACTACTG (R)	XM_416476	92
ACACA	acetyl-CoA carboxylase α	AGGAGGGAAGGGAATTAGGAAA (F) GATCGGAGAGCCTGGGACTT (R)	NM_205505	82
ACE	AGT converting enzyme	CAGAACTGTGGGGGAAGAAA (F) TCTTCAGCTGTGGGTCACTG (R)	NM_001167732	75
ADIPOQ	adiponectin	AATGTCGTGTGCCAACTGGAT (F) TTCCAGGCAGCCCATTGT (R)	NM_206991	64
ADIPOR1	adiponectin receptor 1	GGCACCGGCCTCCAAT (F) TCGGTGTGTATTCGAAAGATGCT (R)	NM_001031027	63
AGT	angiotensinogen	TGGCAGCGAATGCTAAGAAA (F) AGATCGCCCTGTTTGAATCAA (R)	XM_419584	75
AGTR1	angiotensin II receptor, type 1	GGCCATAGTGCATCCAGTGA (F) CCAGATGACAATGCAGGTTACTCT (R)	NM_205157.3	75
AGTR2	angiotensin II receptor, type 2	CTGCTGTTGTTGTGGCCTTCT (F) AGGGCATCCAAAAACGTCAA (R)	XM_426266.3	66
ALB	albumin	CACTGCCATGGTTGACAAGTG (F) TGGCACCCTCTTCTCCAAAG (R)	NM_205261	67
ALDOB	aldolase B	GCGTGCAGTGTTGAAGATCAG (F) GGTGTTGGCGTTCTCTTGGA (R)	NM_001007977	67
ANGPTL4	angiopoietin-like 4	TGGCCGGGCAGCAA (F) AACAGTAGACTTTGAAGGGCTGAGA (R)	XM_001232283	71
ANXA1	annexin A1	GCTCAACGTCAGCAGATCAA (F) CTTCCAAGTGGCTTTTCAGC (R)	NM_206906	94
ANXA5	annexin A5	GAGAGACATCAGGCCATTTTCAG (F) CAACTCTGCCATCAGGATCTCTATT (R)	NM_001031538	74
APOH	apolipoprotein H	TGTGCCTGTTCAGTGTGTGA (F) TTGAAAAGAAGCCACGCTCT (R)	NM_001277994	71
AR	androgen receptor	TGTGCTGGCCATGACAACA (F) GCTGTCTCTCCCCAAGTTCATT (R)	NM_001040090	78
ATRN	attractin	TCTGCAGGCTGGCAAGGT (F) CCTCACGGGTCCAAAATGAC (R)	XM_420884	69
BCDO2	beta-carotene oxygenase 2	GCATTGTGGTCTCAGAATTTGG (F) GACATGAAGCGTGCAAAGACA (R)	XM_417929	72
BCMO1	beta-carotene monooxygenase	GCATCCAGAGCCCATAAAAGC (F) TGGGCCATTTCGGAGAAGTA (R)	NM_204635	78
BMP15	bone morphogenetic protein 15	TGATCTTGCACTCCTTTTGCA (F) ACCCATGCCAGAGGATTCAG (R)	NM_001006589	78
CDS2	CDP-TG-synthase 2	GGTACCGGTGCTTCACTTGT (F) CTCTGATGGCTCACAGTCCA (R)	XM_417669	77
CEBPA	CCAAT/enhanc	AGAACGAGCACTCCATCGAC (F)	NM_001031459	89

	er binding protein α	CTTGCTGTGCTGGAAGAGGT (R)		
CMKLR1	chemokine-like receptor 1	CGGTCAACGCCATTTGGT (F) GGGTAGGAAGATGTTGAAGAGGAA (R)	XR_140274	64
CNR1	cannabinoid receptor 1 (brain)	ACCTTCACGGTCCTAGAGAACCT (F) GCCTATGAAATGATAAGAGGGTCTACA (R)	NM_001038652	89
COX3	cytochrome c oxidase III	CAGGGTTCCACGGACTACAT (F) TGGTTTGGTGTGAAGTGGAA (R)	ENSGALT00000029087	91
COX7A2L	cytochrome c oxidase subunit VIIa, 2 like	GCCGGTGGATCGTAGGA (F) CGTGAAGCCGTTGAACTTGTAG (R)	NM_001277587	59
CPT1A	carnitine palmitoyltransferase 1A (liver)	AAGGGTACAGCAAAGAAGATCCA (F) CCACAGGTGTCCAACAATAGGAG (R)	NM_001012898	136
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	GCCTGTCCTGAGGAAATCCA (F) CGCGCGTTGCAGTTTCT (R)	NM_204979	72
DGAT2	diacylglycerol acyltransferase 2	AAAACCCACAACCTGCTGAC (F) TGTGCTGAAGTTGCAAAAGG (R)	XM_419374	93
DGKQ	diacylglycerol kinase, theta	CAGTGATGACCGCTTTGAGA (F) CTTGACCCATGTGAACAACG (R)	XM_424953	86
DPP7	dipeptidyl-peptidase 7	TGGGAAATCTCTTCCGTTTG (F) GCACTGCATAGTCAGCAAGG (R)	ENSGALG00000009105	92
DHCR24	24-dehydro-cholesterolreductase	GGCTGTGTCGGTGATCCATT (F) TCCCACTCTGAGAATCAGTGCTT (R)	NM_001031288	68
DIO1	deiodinase, iodothyronine type I	CTACTGTGAGGTTGGATTTGCTTTT (F) CTGAAGCACCTTGGGTAATGTG (R)	NM_001097614	79
DIO3	deiodinase, iodothyronine type III	CAGCCACGCTCTGTCAATAC (F) AGATCCCGAAGGAAGAGAGC (R)	NM_001122648	140
EDN1	endothelin 1	CCACCTGGATATCATCTGGATCA (F) CCGAGAAGGGCCTCCAA (R)	XM_418943	69
EDN2	endothelin 2	CGGTCCAGTATTACACATGAGCAT (F) GCTTAAGACAACCACAGGTTTTGG (R)	NM_001198655	77
ENG	endoglin	CCTGACGACGTGTCTAGCAGTCT (F) GACCTGGCGATGACGATTTC (R)	NM_001080887	85
envPr57	Pr57 env polyprotein precursor	AGAACCGAGCGGCTATTGAC (F) CCGGCAACGTCCTCACA (R)	JX570792	64
EP300	E1A binding protein p300	GCCAAGACCGCTTTGTTTACA (F) CACACAGTGCAGTGCCATCTG (R)	XM_001233887	72
EX-FABP	extracellular fatty acid-binding protein	GCCATGGCAATCTTCAGGAA (F) GCACGGCGACCATCTCAT (R)	NM_205422	64
F2	thrombin	GGCTGATGCTGGCAGGTT (F) GGCCCACGTTTCTTTCAGATT (R)	NM_204605	64
F5	coagulation factor V (proaccelerin)	GCAGGAAGCTACAGGTGGAC (F) AGCTCCATGTGTGTCTGCAC (R)	XM_001231900	73
F8	coagulation	GTGGCACTGTGACCTCAAGA (F)	XM_420193	75

	factor VIII	CCTTCCTTTCCACCATAGCA (R)		
F9	christmas factor	CAGGCATGACACACCACAGAA (F) ACAGGACTTTCCGTCTTCATGAA (R)	NM_204343	75
FAAH	fatty acid amide hydrolase	GGTGCGCGCCTTTCG (F) CGGTAAAGACAGAGAGAGGATGGA (R)	XM_422450	66
FADS2	fatty acid desaturase 2	CTCATCCCCGTGTATTTCCAA (F) CAGGTCCGCCCAGAACCT (R)	NM_001160428	68
FASN	fatty acid synthase	TTTCAATGATCCAAATCCAGATATTC (F) ACCGGTGTTGGTTTGCAAA (R)	NM_205155	74
FGA	fibrinogen alpha chain	TGTTGCTCTGCCTCAATTTAGC (F) CCTCCGCCCTCCTTTTCA (R)	NM_001271911	67
FGB	fibrinogen beta chain	CATCATCCAGCCAGATCCTT (F) GAATCAAAGTCCAGCCTCCA (R)	NM_001167683	83
FGFR2	fibroblast growth factor receptor 2	CAGGAATCCCAGTGGAGGAA (F) GGTGCAGTTGGCAGGTTTATC (R)	NM_205319	73
FGFR3	fibroblast growth factor receptor 3	AGGTATTCTTTGTGGTCGAGTCAAT (F) ATGCGCGTCATCCAAAGTC (R)	NM_205509	70
FGG	fibrinogen gamma chain	CACTGCTGACTATGCCGTGT (F) TCGCCACCAATAAAGTAGGC (R)	NM_204989	81
FZD9	frizzled family receptor 9	CGCGAGGACAAGGACTTTG (F) AAGGCGGTAGAGACGAAGCA (R)	NM_001276283	71
GCG	glucagon	TGCAATGGTTAATGAGCACTAAAAG (F) GATCCGGGAATTTGTCATTCTC (R)	NM_001190165	74
GCGR	glucagon receptor	TGGAGCACCAACCACAACAT (F) TCAGGATGGCCAGGAACAC (R)	NM_001101035	66
GHR	growth hormone receptor	CTCCTGAGTGACGATCATCTGAA (F) GGCACGTCCAGAATCATCATC (R)	NM_001001293	69
GLP1R	glucagon-like peptide 1 receptor	TGTCCAGCCAACAGCATCAG (F) CTGAAGGTAGCCTGGCAAGTG (R)	NM_001135551	76
GLUT1	glucose transporter, member 1	AGGTACAGATGCAAAGGTTTAGTCTC (F) ACAACTACAGTGCAGGTAATTAACTATA CAAA (R)	NM_205209	99
GLUT8	glucose transporter, member 8	CGGCAGAGGAGTCCCAGTAC (F) GCTTCTTGTTTTGCACTCTCAGGTA (R)	NM_204375	71
GPD1	glycerol-3-phosphate dehydrogenase 1	TCCCCGCTGGGCAGTA (F) TCTTCATATGGGCTCCATTGC (R)	XM_422110	61
GPRC5C	G protein-coupled receptor, family C, group 5, member C	GGCCACTCAGGCCTTCTTCT (F) CTGGCCCCACGATGAAGT (R)	XM_425386	73
GREM1	gremlin 1	GCCGCGCAGGATGGT (F) AGAAATCCCGTCAGAAGAAACACA (R)	NM_204978	62
GRM8	glutamate receptor, metabotropic 8	TCTTCTTGCCAATATCACCTTAGGT (F) GGATTGCTCCAATGCATAAGTG (R)	XM_425426	75
HADHA	trifunctional protein, alpha subunit	GAAGGTGATTGGGATGCACT (F) GCTGTGTCCTGGGATGTTTT (R)	NM_205056	91

HADHB	trifunctional protein, beta subunit	AGTATGCCCTCCGTTCACAC (F) ACTTTGAAGGGCACCACATC (R)	XM_420004	80
HIF1A	hypoxia inducible factor 1, alpha subunit	TCACTTTTTCAGGCAGTTGGAA (F) TTTTGCACGCCTTTACACGTT (R)	NM_204297	99
HK2	hexokinase 2	TCGTCCGCAACATCCTGAT (F) TTTCTATCTGGGACAGGAACTTTGT (R)	NM_204212	116
HMGCL	HMG CoA lyase	CATCTTGGTGGCACTTCAGA (F) AGCGACATTTCCTGATGCTC (R)	NM_001198715	95
HMGCS2	HMG CoA synthase 2	GACCGTCATCGACAAGTCCAA (F) CCCGACTCGCGGAAGAG (R)	XM_422225	63
HPGDS	hematopoietic prostaglandin D synthase	GACCGTCATCGACAAGTCCAA (F) CTCTGTCCCTGAGAGCCAAC (R)	NM_205011	71
HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	CTGGACTTCGTCTACCTCAATGC (F) CCTTCCAGAGGGCCTTGAA (R)	XM_422210	69
IGF1R	insulin-like growth factor 1 receptor	GGCCAAACGTTGACATTCG (F) AACCCTCAACGACCGTACAGTT (R)	NM_205032	71
IGFALS	IGF binding protein, acid labile subunit	CTCGACCTCTCCCATAACCA (F) GGCTGAGGTAGCGTAGGTTG (R)	XM_425222	79
IRS2	insulin receptor substrate 2	CATAATTTCAAGCCTATCACAGTACT (F) AGGTCCTGTCGCACACCAA (R)	XM_425588	81
JMJD1C	jumonji domain containing 1C	TGCCAAGAAAATATAGTCATCGTATT (F) TTGCAAGTCTAACACAGAGTCACAGT (R)	NM_001199546	90
KLF5	kruppel-like factor 5	TGCGACTGGCGATTTGC (F) CCCGGTGTGCTTCCTGTAA (R)	XM_417013	60
LCN15	lipocalin 15	GTTTCCAACTGCCCTGTGTT (F) CGTCTCCATGGTCATGTGTC (R)	ENSGALT00000014677	99
LDHA	lactate dehydrogenase A	GTGGCTGCAATCTGGACTCA (F) AGGATGGATGCCCAGTCTTTC (R)	NM_205284	65
LPIN1	lipin 1	GGAGCTCAGGCGCTGACA (F) GATGGCAATCGAAGGCAAAT (R)	XM_419957	70
LPL	lipoprotein lipase	TGCTGGTCCCACCTTTGAGTA (F) TGCAGGACATCCACAAAGTCA (R)	NM_205282	78
MC5R	melanocortin 5 receptor	GCTTCTCGGCATCTTCATTGT (F) AGGGCAGGAGATCATCAGGAT (R)	NM_001031015	69
ME1	malic enzyme 1	GCCAGCATTACGGTTTAGCATT (F) TGTCCCCGGTCATGGATAGT (R)	NM_204303	66
ME3	malic enzyme 3	AGGCTCTCCTGAGTGATCCA (F) ACCGCCTGCATAAACTCATC (R)	XM_003640570	97
NAMPT	nicotinamide phosphoribosyltransferase	GCGCCGAGTTCAACATCCT (F) TTGTATTGGGTGGATATTGCTTGT (R)	NM_001030728	74
NCOA3	nuclear receptor coactivator 3	GACCTCGGACCAATACACCAA (F) GCGCTTGACGGGTTTGATT (R)	XM_417385	89
ND6	NADH dehydrogenase subunit 6	GGGGTGGTTACTGTTGATGG (F) AACCCAACCCCACATGAATA (R)	ENSGALT00000029077	89
NPY	neuropeptide Y	ACTCGGCTCTGAGGCACTACA (F) TCAGTGTCTCTGGGCTTGATCTC (R)	NM_205473	74

NPY2R	neuropeptide Y receptor Y2	ACCACCGGCGTCAGAAAA (F) GCCAACTGACAGCGAACACA (R)	NM_001031128	65
OSBP2	oxysterol binding protein 2	GAGCACTTGCAATGACCTGA (F) GGCAGTTTCAGGTTCTCCAG (R)	XM_415293	81
PANK1	pantothenate kinase 1	CTGGATGAATTGGACTGTTTGATC (F) TCTGGTTGGCCATTGAAACC (R)	XM_421664	71
PCK1	phosphoenolpyruvate carboxykinase 1	TGATGACACGGATGGGAACA (F) CGAGTGAAGGCATTTCACAAAC (R)	NM_205471	70
PDE1C	phosphodiesterase 1C, calmodulin-dependent	CTGGGTTCAGACGTTCCAGT (F) CTGACCCAGTGCTCTTGACA (R)	XM_418850	78
PDK4	pyruvate dehydrogenase kinase 4	CAATACTTCTTGGATCGCTTTTACAT (F) AGAAGGGTGTGTTGGTTCATCA (R)	NM_001199909	70
PGR	progesterone receptor	GGAAGGGCAGCACAACTATTTATG (F) GCGACACGCTGGACAGTTC (R)	NM_205262	84
PGRMC1	progesterone receptor membrane component 1	GCTTTGGAATGTTGCTGCTAAA (F) AATTCAACGGTCAGGCCTATTG (R)	XM_001234321	72
PLG	plasminogen	CACAACACCGCCACCTGTAC (F) TTGCCTCGATAGTCTTCTCCTCTT (R)	XM_419618	72
PLIN2	perilipin 2	GGAAGACAAACAGCTTGAACACA (F) CCAAGGCTTTTTGACAGCTACA (R)	NM_001031420	111
POSTN	periostin, osteoblast specific factor	AAGGGCACGCGACCAA (F) TGCAGGTGCTGAAGTATTTCTTTT (R)	NM_001030541	79
PPARA	peroxisome proliferator-activated receptor α	CAAACCAACCATCCTGACGAT (F) GGAGGTCAGCCATTTTTTGGA (R)	NM_001001464	63
PPARD	peroxisome proliferator-activated receptor δ	GGCTTTGTGACCCGTGAGTT (F) TTGGGCTCCATGATCTCGTT (R)	NM_204728	64
PPARG	peroxisome proliferator-activated receptor γ	CACTGCAGGAACAGAACAAAGAA (F) TCCACAGAGCGAAACTGACATC (R)	NM_001001460	66
PRKAB2	protein kinase, AMP-activated, β 2	TGTGACCCGGCCCTACTG (F) GGAGAGCGCGTAGAGGTGAT (R)	NM_001044662	63
PRKAG1	protein kinase AMP activated γ 1	CCGCCTCCCCGTCATC (F) ATGCGTTTGTGGGTGAGGAT (R)	NM_001034827	63
PROC	protein C	TGTGGAAGCTCATCACCATAGG (F) AATATCGAGGCATGGCACACT (R)	NM_204441	69
PYGL	phosphorylase, glycogen, liver	CGTCCTCCATGTTTGATGTG (F) ATGTGCAGGCAGTTCATCAG (R)	NM_204392	73
RARRES2	retinoic acid receptor responder 2	CGCGTGGTGAAGGATGTG (F) CGACTGCTCCCTAAAGAGGAACT (R)	XM_003640642	71
RBP4	Retinol binding	CAAGGAGAACTTCGACAAGAACAG (F)	NM_205238	72

	protein 4	CAGCCCCTCAGGATCTTGATT (R)		
RETSAT	retinol saturase	CAGCCCGTCTGCCAAAGA (F) TGATGGACAACGTGCTTTTACC (R)	XM_424187.3	64
RPL14	ribosomal protein L14	CCTTCGGGAGGCATGCT (F) CCACTAGCGCCCTGTTCTG (R)	XM_418775	69
RXRG	retinoid x receptor, gamma	CCGAGCTGGCAGTCGAA (F) TTGGTTGAGCTCTCCGTGTTC (R)	NM_205294	63
SCD	stearoyl-CoA desaturase	CACCGTGTCCACCACAAGTTC (F) GAAGTAGCCCCGCATAGCATT (R)	NM_204890	65
scGH	short chicken GH	CCTCACCTGCACAGCTCTGA (F) CTGGATGAGAACCAGTGAAAACC (R)	AB066586	77
SELE	selectin E	ACCTGGGTTGGAACAAACAA (F) CTTTGCCATTTGGTTCACCT (R)	XM_422246	73
SELP	selectin P	GCTCTAATCTGCACGGGAAC (F) CACCCGGATAAATCCTTCCT (R)	XM_422207	74
SERPINB2	serpin peptidase inhibitor, clade B, member 2	AGCTGAAGGAGCCAGAAACA (F) AGGCACGGTGATTTGATAGG (R)	XM_418982	86
SERPIND1	serine peptidase inhibitor, clade D, member 1	TTATCGGAGTGTAGCGGACAAA (F) GCCATTGCAGTGGAAATACCA (R)	XM_001232766	77
SERPINE2	serine peptidase inhibitor, clade E, member 2	TTGCCATACCATGGAGAAATGA (F) GATAGCAGAGAGCGGTGTTGTG (R)	NM_001083920	75
SERPING1	serpin peptidase inhibitor, clade G, member 1	TCTTCATGGGACGCCTTAGTG (F) AAGAAACGGAGGCGAGGAA (R)	XM_003641376	67
SOD3	superoxide dismutase 3	CCAGTGATGGCTGATAATGAGACT (F) CTATTTTGGAGCTGGGCTTCA (R)	XM_420760	72
SREBF1	sterol regulatory element binding factor 1	GTCGGCGATCCTGAGGAA (F) CTCTTCTGCACGGCCATCTT (R)	NM_204126	105
SREBF2	sterol regulatory element binding factor 2	CATCGCCGCCTCCTAACA (F) CCCAATTCCTTTTGCAACATG (R)	XM_416222	67
SSTR2	somatostatin receptor 2	AGGCCCCCACCAATGC (F) GCGTTGCTGGTTAGGTCGAA (R)	NM_001030345	60
THBS2	thrombospondin 2	GTCGGCGATCCTGAGGAA (F) CTGGACAGCATTCACCTTCA (R)	NM_001001755	72
THRSPA	thyroid hormone responsive	CGGAGACTCCAAGGGTGATC (F) GGGACTTGGCACAGGAATAAAG (R)	NM_213577	64
TTR	transthyretin	CATGAATATGCTGATGTGGTGTTC (F) TGAGGAGAGCAGCGATGGTA (R)	NM_205335.2	72
TXNIP	thioredoxin interacting protein	GAGGAGCAGCCCGAGGAT (F) GCCAAACTTGTACTCGTATTTATTGC (R)	BM427637	71
WNT4	wingless-type member 4	TCTCTAACTGACCCTGCTTCTCTCT (F) TGCAGCGTCTGGGTCGTT (R)	NM_204783	65

Primers were designed with Primer Express 2.1 software (Applied Biosystems). Size refers to length of PCR amplicon.