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Novel Regulatory Mechanisms of Adipogenesis: Discovery of Vestigial-like 3 (Vgll3)

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Novel Regulatory Mechanisms of Adipogenesis: Discovery of Vestigial-like 3 (Vgll3)

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in Molecular Biology

by

Daniel Steven Halperin

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ABSTRACT OF THE DISSERTATION

Novel Regulatory Mechanisms of Adipogenesis: Discovery of Vestigial-like 3 (Vgll3)

by

Daniel Steven Halperin
Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2013
Professor Peter Tontonoz, Chair

The study of fat tissue or adipose over the last half century has dramatically altered the perception of the fat cell, also known as the adipocyte. Once only thought to store fat, the adipocyte is currently recognized to be a highly critical and important metabolically active cell. Adipogenesis, the process of adipocyte formation, has now become an intense subject of basic scientific research in large part due to the up-surge in obesity and metabolic disease in modern day society. PPARγ is the master regulator of adipogenesis and is also a validated target of anti-diabetic drug therapy. However, a renewed sense of urgency in discovering novel factors and mechanisms that regulate the expression and/or activity of PPARγ now exists due to recent

scrutiny in the clinical use of molecules that directly activate PPARγ. Therefore, this thesis attempts to address this need by investigating and uncovering entirely new modes of regulation in the differentiating adipocyte.

Part one of this thesis presents the construction of a transgenic mouse line that carries a reporter vector containing a cloned set of highly conserved non-coding genomic sequences endogenously found within and adjacent to the PPARγ locus in the mouse genome. This examination represents an effort to identify a genomic regulatory sequence that confers tissue selective expression of adipose-specific genes such as PPARγ. Identification of such an enhancer would undoubtedly shed new light on the regulation of gene expression in adipocytes and usher in a new paradigm in the study of adipose tissue.

Part two of this thesis presents the identification of Vestigial-like 3 (Vgll3) as an inhibitor of adipocyte differentiation. Vgll3 is a conserved transcriptional co-activator that is down-regulated during adipogenesis. This gene was initially observed to be differentially regulated between pre-adipocyte cell lines that exhibit contradictory potential to become lipid-laden adipocytes. When overexpressed in differentiating adipocytes *in vitro*, Vgll3 induces a potent block in PPARγ expression and adipocyte formation. Furthermore, ectopic expression of Vgll3 was observed to up-regulate the expression of genes previously determined to be inhibitors of adipogenesis and genes associated with other mesenchymal-derived cellular differentiation programs. These results point to Vgll3 as a gene whose expression must be carefully modulated during the formation of fully differentiated, mature adipose tissue.

The dissertation of Daniel Steven Halperin is approved.

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Dedicated in honor to those who came before me.

Consecrating and sacrificing their own lives, they bestowed upon me the opportunity to experience and pursue my own journey as a meaningful foundation for those yet to come.

מה רבו מעשיך ה״,
כלם בחכמה עשית
מלאה הארץ קנינך׃

תהלים קד

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Chapter 1

Introduction

Monumental shifts in human lifestyle and behavior over the last century have had an enormous impact on contemporary society. In particular, these changes have greatly effected where and how humans live, eat, drink and choose to recreate. Consequently, these adjustments have fostered the widespread escalation in the demand and quantity of daily caloric intake, reduced utilization of energy sources stored as fat and the sharp rise in the incidence of what is now called the "metabolic syndrome." Responding to these new circumstances will inevitably be an immense global challenge for human civilization as the number of different populations adopting a more sedentary way of life is expected to rise for the foreseeable future. Therefore, from the perspective of basic science, acquiring greater understanding of the fundamental biological processes that govern the use and storage of lipid molecules or "fat" is of critical importance in order to better understand the progression of the metabolic syndrome.

A significant portion of fat tissue, also called adipose tissue, is made up of lipid-storing cells called adipocytes. The fully developed adipocyte in mammals contains a single large lipid droplet encapsulated by a narrow ring of cytoplasm surrounded by a plasma membrane. Acting as an intracellular organelle, lipid droplets store neutral lipid in the form of triglyceride surrounded by a phospholipid monolayer that allows for contact with surface proteins and enzymes. As energy needs necessitate, fatty acids derived from these triglyceride stores can be mobilized and released into circulation to supply peripheral tissues. On the other hand, when energy supplies are abundant, specific enzymes expressed in adipocytes can mediate the process of synthesizing triglyceride, known as lipogenesis, for storage and use as future energy needs may require.

Adipose tissue is made up of loose connective tissue consisting of adipocytes, fibroblasts, macrophages and endothelial cells and was once thought to only function as a reservoir for lipid

molecules. However, adipose tissue is now thought to be an endocrine organ that plays a vital role in a variety of systemic processes. There are two overall types of adipose tissue that occur in mammals: brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is predominantly found in infants and hibernating animals, and it is thought to mainly function to produce heat from the burning of calories by uncoupling the process of oxidative phosphorylation carried out in mitochondria. On the other hand, WAT is found almost throughout all stages of development, and it serves to build up the storage of excess energy from dietary intake in the form of triglyceride concentrated in lipid droplets within the adipocyte.

The formation of mature, differentiated adipocyte cells occurs via a process called adipogenesis. Fully developed adipocytes arise from mesenchymal progenitor stem cells that descend from the mesodermal germ layer and can be stimulated to become committed pre-adipocytes that undergo adipocyte differentiation. This cellular differentiation process is chiefly orchestrated by the transcription factor Peroxisome Proliferator-Activated Receptor-γ (PPARγ), a transcription factor found in the nucleus that serves to regulate the expression of an assortment of downstream target genes that help drive this process forward. Expression of PPARγ and the presence of adipose tissue in general are so crucial for the development of life that mouse embryos unable to produce any PPARγ protein (and therefore any adipose tissue) are not viable[1]. The use of therapeutic small molecules that directly interact and promote the activity of PPARγ has been demonstrated to be an effective approach in treating type II diabetes. This practice however has elicited very problematic side effects that now limit the use of this strategy when treating patients. Therefore, there is a great need to uncover previously unidentified biological mechanisms that control the expression and/or activity of PPARγ in order to help provide for the development of improved therapeutic options.

Since the discovery of PPARγ as the master regulator of adipocyte differentiation, a number of key molecules that interconnect with PPARγ as either direct or indirect regulators of its expression and activity in adipose tissue have been discovered. Furthermore, a variety of target genes that are expressed when PPARγ is activated during the course of adipocyte differentiation have been identified. However, the current knowledge of these regulators and supporting factors likely represents only a small fraction of a very complex molecular and cellular puzzle. Thus, the world-wide explosion of metabolic syndrome is a compelling force that is driving the advancement of new knowledge and discovery of novel factors critical for the development and proper functioning of adipose tissue. In so doing, discoveries and innovations made at the basic level of adipose biology can serve to initiate the careful establishment of better clinical methods for use in the treatment of metabolic disease.

Adipose Tissue and the Metabolic Syndrome: A General Overview

The Metabolic Syndrome, also known as "syndrome X," is a collection of different risks factors that indicate a high likelihood for cardiovascular disease, type II diabetes and other metabolic disorders. The American Association of Endocrinology and the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults both have outlined the essential hallmarks of the metabolic syndrome as consisting of: increased triglycerides ($\geq$150 ng/dL), reduced HDL (< 40 mg/dL in men and < 50 mg/dL in women), high blood pressure (>130/85) and elevated fasting glucose levels (>110 mg/dL) [2, 3]. Using these criteria and census data from 2000, it has been estimated that as many as 47 million Americans are living with the metabolic syndrome[4]. The exact underlying cause of the metabolic syndrome is not known, however it has been suggested that the two most

significant factors contributing to the progression of the metabolic syndrome are insulin resistance and obesity.

Insulin resistance in white adipose tissue (WAT) has been implicated to play a substantial role in the development of the metabolic syndrome. Insulin resistance is classically defined by the gradual loss in sensitivity to the hormone insulin. Insulin is a hormone that facilitates the uptake of glucose into tissues and is produced by the β- cells of the pancreas. Decreased sensitivity for insulin in peripheral tissues results in an increased demand for insulin production by β- cells to compensate for increased levels of glucose in circulation (hyperglycemia). Clinical presentation of insulin resistance also consists of a compromised ability to tolerate glucose as indicated by an inability to properly respond to a glucose challenge[5]. Furthermore, most insulin resistant individuals are generally observed to be overweight, exhibit abdominal or visceral obesity, are relatively inactive on a daily basis and typically eat foods high in saturated fat.

WAT functions as a site for free fatty acid deposition and in turn helps to maintain metabolic homeostasis. For example, transgenic mice that possess almost no WAT have been observed to be highly insulin resistant with elevated circulating levels of glucose, free fatty acids, triglycerides and insulin in mice and humans[6-8]. In addition, transgenic lipoatrophic mice lacking adipose tissues exhibited normal levels of circulating glucose and insulin as well as demonstrated improved insulin sensitivity only after the surgical introduction of adipose cells[9]. WAT that has become insulin resistant typically becomes enlarged (hypertrophic) and secretes an abnormally high level of free fatty acids[10]. This is caused in large part by the inability of insulin to inhibit lipolysis of triglycerides in adipose that is no longer sensitive to insulin. High levels of free fatty acids can instigate the liver to produce abnormal levels of glucose, triglyceride and very low-density lipoprotein (VLDL). Excess free fatty acids in muscle tissue

prevent the appropriate absorbing of glucose as mediated by insulin[5]. Additionally, insulin resistant adipose tissue can release inflammatory cytokines. These pro-inflammatory factors are thought to provide support for the development of insulin resistance by at least in part stimulating pathways that drive the lipolysis of triglycerides stores in adipocytes, thereby promoting the release of free fatty acids into circulation[10]. Ultimately, an increased amount of consumed dietary fat, higher circulating levels of free fatty acids and the heightened inflammatory state of adipose tissue are all considered to be important factors contributing to the development of insulin resistance[11]

Obesity is undoubtedly the major reason for the expected rise in the numbers of patients with disorders linked to the metabolic syndrome. Individuals who are obese can expect deleterious impact to their finances, overall quality of life and length of life[5]. It has even been suggested that life expectancy in developed countries could decrease as a result of the coming global obesity epidemic[12]. Some originally defined obesity by calculating body mass index (BMI), a figure derived by dividing one's weight (kg) by the square of one's height (m^2), and determining this figure to be greater than 30 kg/m^2. However, it is so-called "central obesity" defined as an excess of abdominal or visceral adiposity as measured by waist circumference (and other proposed new indices) that is now considered the strongest indicator for increased risk for diseases of the metabolic syndrome such as type II diabetes and cardiovascular disease[13,14]. The increased likelihood of hazardous disease that surplus visceral fat is associated with strongly suggests there must be key differences between adipose tissues found in distinct anatomical locations or "depots". Recently published reports demonstrating remarkable biological and physiological dissimilarities between adipose depots now provide support to this hypothesis[15-17].

Physiological differences between the adipose tissue of obese versus non-obese subjects highlight how metabolic syndrome could be mediated and how it might someday be prevented and/or therapeutically treated. One intriguing investigation measured the level of ^{14}C integration in human subjects exposed to atmospheric nuclear emissions during the mid-20th century[18]. This study showed that the total number of human adipocytes appears to be set in childhood and adolescence, and this number of adipocytes, once established, appears to remain relatively the same throughout adulthood. However, the number of adipocytes as set in childhood and adolescence by obese individuals appears to be dramatically higher when compared to their lean counterparts. Thus, based on the model from this work, the overall re-modeling of adipose tissue in human adults might occur mostly by modulating the physical size of adipocytes. In fact, other studies have shown that obese, adult individuals are observed to have adipocytes that are considerably larger compared to adipocytes from lean, adult subjects[5, 11]. Consistent with such a model, the presence of enlarged human adipocytes has been observed to be inversely correlated with insulin sensitivity and normal functioning adipose tissue[5].

Adipose tissue is now defined as an important organ in the endocrine system. There are now numerous peptides known to be secreted form adipose tissue that most likely serve to regulate systemic energy balance, in particular, the hormones leptin and adiponectin[5]. Leptin regulates feeding behavior by communicating with the central and sympathetic nervous system to control appetite and promoting the utilization of energy stores. In addition, leptin has also been shown to increase hepatic lipid oxidation and stimulate lipolysis in muscle and adipose tissues as well[19]. Hypertrophic, enlarged adipocytes, as observed in obese individuals, are known to express, produce and release substantially more leptin RNA and protein[20]. Thus, continual, high levels of leptin results in a failure to properly respond to leptin signaling and

ultimately facilitates metabolic imbalance. Adiponectin is another important adipose-secreted hormone whose expression becomes dysregulated in obese adipose tissue. In fact, the level of adiponectin is abnormally decreased in obese adipose tissue. Adiponectin normally functions to inhibit hepatic glucose production and enhance glucose uptake in muscle. Furthermore, adiponectin has been reported to display anti-inflammatory properties[11]. Strikingly, in one insulin-resistant mouse model, adiponectin administration was observed to reverse insulin resistance and hyperglycemia[21].

An important hallmark of obese adipose tissue is inflammation. Two characteristics that exemplify the inflamed state of obese adipose tissue are the augmented production and release of cytokines and the escalating level of infiltration of inflammatory macrophages into adipose tissue. One of the initial cytokines secreted by adipose tissue to be identified was tumor necrosis factor-α (TNF-α). TNF-α enhances the production of other pro-inflammatory cytokines and inhibits the level of secreted adiponectin in obese adipose tissue[22]. It has also been shown to promote insulin resistance through inhibition of insulin signaling mediated by insulin receptor substrate-1 (IRS-1)[23]. IL-6 is another pro-inflammatory cytokine that is released at an increased level by obese adipose tissue. A direct correlative association between IL-6 levels and insulin resistance and the development of diabetes has been established[22]. One study has even reported that IL-6 deficient mice themselves become obese when fed a high-fat diet[24]. IL-6 also signals to the central nervous system to limit dietary intake and increase utilization of stored energy similar to the action of leptin. It has therefore been proposed that the development of IL-6 resistance may possibly occur during progression of the obese state[25].

One of the major sources of TNF-α and IL-6 secreted from adipose tissue appears to be newly recruited inflammatory macrophages. Currently, the functional role of adipose tissue

macrophages (ATMs) remains unknown. However, an elevated number of ATMs is highly correlated with insulin resistance and obesity[26]. Investigations using knock-out mice have demonstrated that C-C chemokine receptor-2 (CCR2) and its ligand CCL2 (C-C chemokine ligand-2) / monocyte chemoattractant protein-1 (MCP-1) are necessary for stimulating the migration of pro-inflammatory ATMs[27, 28]. Both of these knock-out lines, exhibit lower number of ATMs, down-regulated levels of pro-inflammatory gene expression in adipose tissue and improved insulin sensitivity on a high-fat diet. Alternatively, overexpression of MCP-1 increases the number of ATMs and promotes insulin resistance. Interestingly, CCR2- and MCP-1-deficient mice still maintain a substantial amount of resident ATMs, even though pro-inflammatory gene expression was decreased. This finding was further supported by the observation that diet-induced obesity helps to drive a phenotypic switch in ATMs, thereby promoting the conversion of ATMs to a more pro-inflammatory state[29].

Adipose Tissue Developmental Lineage

Adipose tissues descend from the mesodermal germ layer that in turn gives rise to undifferentiated, pluripotent mesenchyme. It has been postulated that a common mesoderm/mesenchymal progenitor cell can give rise to bone, muscle, cartilage, white adipose, and brown adipose in response to appropriate developmental signals[30]. It was once presumed that the differentiation of a common adipose precursor cell was capable of giving rise to both WAT and BAT. This theory would seem to be supported by the ability of WAT to "transdifferentiate" into BAT under particular experimental settings[31]. In addition, recent work has identified so-called "beige" or "brite" adipocytes that seem to emerge from within WAT[32]. These cells express a distinctive gene expression pattern when compared to white and brown adipocytes but do seem to functionally respond to external stimuli in a more thermogenic, brown fat cell-like manner.

However, other published work has begun to establish that WAT and BAT may actually stem from dissimilar cellular precursors. First, one study has demonstrated that skeletal muscle progenitor cells that express the gene Myf5 can give rise to muscle cells or brown fat cells, however not white fat cells[33]. Second, other reports have now identified a variety of markers (Lin^-, $Sca1^+$, $CD34^+$, $CD24^+$, $a7^-$, $PDGFR\alpha^+$) unique for precursor pre-adipocyte cells that are capable of giving rise to adipocytes that make up WAT[34-36]. It has also been suggested that different depots may also have different precursors[37]. This may explain why differences in transcript expression of genes associated with development appear to exist between different depots of adipose tissue[38]. Although, future studies of adipocyte precursor cells will be required to fully validate and clarify the results of these published reports.

It has been demonstrated that adipocyte precursors derive from the stromal vascular fraction (SVF) component of adipose tissue. The SVF is a varied mixture of cells that are obtained by enzymatic dissociation and density separation, a technique that separates the mixture of cells that reside in the depot from the mature adipocytes[39]. This mixture of cells contains: mesenchymal stem cells (MSC), T regulatory cells, endothelial precursor cells, pre-adipocytes and macrophages[40]. Pluripotent stem cells found within adipose tissue can also be easily obtained from human subjects using suction-assisted lipectomy (i.e. liposuction) [41]. Although it remains unclear if MSCs obtained from the SVF are of the same lineage as these adipose-derived stem cells, both indirect and direct evidence now indicates that pluripotent precursor cells found in adipose tissue appear to originate from pericytes found in the microvasculature of the endothelial-based mural compartment of the SVF[39]. Additional data however will be required to more definitively identify the nature and characteristics of this putative stem cell niche of the elusive *bona fide* adipocyte progenitor cell.

MSCs are capable of being stimulated to become osteoblasts, chondrocytes, myocytes or adipocytes. However, pre-adipocytes have entered a later developmental stage that commits them to becoming fully mature adipocytes. Therefore, two fundamental stages of adipogenesis are typically described. The first stage has been termed adipocyte "determination" and is marked by the commitment of MSCs to the adipocyte lineage. This stage is most notably characterized by the G_1-growth arrest of cells that have lost their pluripotency as they become committed pre-adipocytes. With additional mitotic and adipogenic signals, growth arrested pre-adipocytes then go through at least one more round of cell division, termed "clonal expansion" as cells enter the second stage called terminal adipocyte differentiation. It is in this latter stage that the pre-adipocyte highly up-regulates expression and activity of PPARγ, expression of downstream PPARγ target genes and develops morphological features that are typical of the mature adipocyte.

The Master Regulator of Adipogenesis: PPARγ

The factor that is absolutely necessary and sufficient to orchestrate the process of producing mature, lipid storing adipocytes within adipose tissue is the transcription factor PPARγ[43, 44]. PPARγ is a member of the peroxisome proliferator-activated receptor family of type II nuclear hormone receptors. Each member of this family functions in multiple ways as transcription factors to regulate lipid metabolism and therefore ultimately metabolic homeostasis. PPARγ is expressed in two active isoforms due to alternative splicing, PPARγ1 and PPARγ2. The major physical difference between these two is the presence of thirty additional amino acids at the N terminal end of the PPARγ2 protein isoform. In addition, it is the PPARγ2 isoform that most specifically and potently drives adipogenesis forward and is expressed almost exclusively

in adipose tissue (with some expression detected in the large intestine as well). The PPARγ1 isoform appears to have weaker adipogenic capability than PPARγ2 and is more ubiquitously expressed[42]. In order to positively regulate adipogenic gene expression however, PPARγ must heterodimerize with its nuclear receptor partner, Retinoid X Receptor-α (RXRα). PPARγ/RXRα heterodimers directly regulate expression of genes in adipocytes known to be involved in lipid metabolism (i.e. LPL, SCD1), regulate glucose and lipid transport (i.e. GLUT4, CD36), secreted factors (i.e. adipsin, adiponectin) and important for other adipose-specific processes such as lipid droplet formation (i.e. perilipin). There are also many differentiation-dependent genes that are thought to be responsive to PPARγ/RXRα heterdimer activity but are not themselves direct targets.

The structure of the PPARγ protein product is one that embodies the form of most nuclear hormones receptors. This consists of an N-terminal domain, (important for the ligand-independent transactivation of transcription, called the AF1 domain), a centrally positioned DNA-binding domain (to recognize specific DNA sequences and interacting proteins) and a C-terminal ligand-binding domain (essential for dimerization and ligand binding). Fascinatingly, when the N terminus is deleted the transcriptional activity of PPARγ actually increases suggesting this domain may have some inhibitory function[43]. On the other hand, it has been shown that the N terminal region has a role in positively supporting PPARγ activity as well[45]. In fact, according to multiple reports, the N terminal domain is involved in mediating the interaction with transcriptional co-activators and co-repressors that have been shown to regulate PPARγ activity[45-47]. Additionally, it has also been reported that the N terminal region of PPARγ is an important site of post-translational modification as well. The MAP kinase family members,

ERK1 or ERK2, phosphorylate this region of the PPARγ protein and reduce its functional activity, thus potentially implicating this mechanism, at least in part, with regard to the inhibitory capacity of the N terminal domain[48, 49].

The DNA binding domain, composed of two highly conserved zinc finger domains, is a vital segment of the PPARγ protein that enables the recognition of specific binding sites on DNA. These binding sites have come to be known as peroxisome proliferator response elements (PPREs) and help to ensure the tissue-selective expression of adipogenic genes. PPREs contain imperfect direct repeats of a consensus sequence separated by one nucleotide (called a direct repeat-1, DR-1) that are most typified by the following recognition site: AGGTCANAGGTCA. PPREs are typically found within non-coding genomic enhancer regions that usually occur upstream of the loci of PPARγ target genes such as aP2, adiponectin and GLUT4. However, some PPREs may be located in proximate promoter regions as well.

The C-terminal end of PPARγ contains a domain that is crucial for activating transcription, termed the AF2 domain. While the exact identity of the endogenous ligand(s) for PPARγ is still unknown, the reasonably well-conserved AF2 domain is thought to recruit co-activator proteins in a ligand-dependent manner that go on to interact with the transcriptional machinery. This ligand-dependent interaction between co-activator proteins and PPARγ is known to be mediated by the presence of a LXXLL motif within many co-activators. Rosiglitazone, a synthetic PPARγ ligand, has been shown to induce a structural change in the AF2 domain allowing for greater contact with the LXXLL motif found within co-activating proteins[50, 51].

Several studies have reported that fatty acids or derivatives of fatty acids along with a variety of other molecules can act as PPARγ ligands to mediate its transcriptional regulatory activity. These molecules can consist of particular types of eicosanoids, oxidized fatty acids and lysophosphatidic acid[52-54]. Nonetheless, identification and isolation of the endogenous ligand that stimulates PPARγ to drive adipogenesis has not yet proved to be successful. In contrast, the synthetic ligands of the thiazolidinedione (TZD) class of anti-diabetic small molecules (i.e. rosiglitazone and pioglitazone) were positively determined to be direct agonists of PPARγ[55].

Adipose tissue as the critical site for TZD action has been supported by *in vivo* studies. Using lipodystrophic mice, it has been shown that the anti-diabetic effects of TZDs only occur in the presence of WAT[56]. In addition, insulin-resistant mice that have targeted deletion of PPARγ in adipose tissue exhibit an inability to respond to treatment by TZDs[57]. Ironically, the anti-diabetic effect of TZDs is thought to mainly occur by increasing adipose tissue mass[58]. Therefore, the prevailing notion holds that excess free fatty acids and lipids that are inappropriately shuttled and stored in the liver and muscle in diabetic patients are able to be properly deposited within the expanded mass of adipose tissue induced by TZDs thereby dramatically improving insulin sensitivity. Also, the ability of TZDs to elicit *de novo* adipogenic differentiation likely helps to re-establish proper levels of secreted factors like adiponectin that also help to normalize systemic metabolic homeostasis. Furthermore, it has been recently shown that the PPARγ-ligand rosiglitazone can block the ability of CDK5 to phosphorylate PPARγ[59]. CDK5-mediated phosphorylation of PPARγ is associated with obesity-induced dysregulation of PPARγ target gene expression, most notably adiponectin. Thus, the ability of TZDs to inhibit post-translational modifications made to PPARγ may contribute in part to their insulin sensitizing capability. Despite the robust anti-diabetic action of TZDs, clinical use of these

molecules in humans has been severely curtailed by the increased risk of cardiovascular side effects [60]. Thus, understanding how previously established and newly uncovered factors interact with and regulate the expression and activity of PPARγ in the context of adipogenesis is crucial to the development of potentially new ways to treat type II diabetes without substantial adverse, off-target effects.

Regulation of the Adipocyte Differentiation Program

PPARγ is a transcription factor that operates within a cascade of other regulatory events that serve to facilitate the production of fully differentiated adipocytes. Several extracellular factors have been shown to promote the early steps toward adipogenesis in mesenchymal stem cells and pre-adipocytes including: BMP2, BMP4, TGFβ, FGF1 and FGF2[61-64]. Not surprisingly, insulin signaling has also been shown to be essential for adipogenesis. For example, deletion of insulin receptor proteins (IRS-1 and IRS-3) *in vivo* results in early-onset severe lipoatrophy associated with marked hyperglycemia, hyperinsulinemia, and insulin resistance[65]. In addition, AKT1 and AKT2 are downstream mediators of insulin signaling that have been shown to be vital for the progression of the adipogenic differentiation program[66]. Alternatively, Wnt signaling has also emerged as a very potent inhibitory pathway of the adipocyte differentiation program. In fact, it has been demonstrated that suppression of Wnt signaling must occur for proper initiation of adipogenesis[67]. However, interestingly, there is evidence that indicates that Wnt signaling is able to inhibit adipocyte differentiation through both β-catenin-dependent and β-catenin-independent mechanisms[68]. One recent report has now published that inhibitory Wnt signaling can be counteracted by secreted frizzled-related protein 5 (SFRP5)[69]. Mice that are obese express a high level of SFRP5 in adipose tissue, and mice that

are deficient in SFRP5 are resistant to diet induced obesity. Although, surprisingly, the actual size of adipocytes, not the actual numbers of adipocyte cells, is what appears to be significantly altered in these SFRP5-deficient mice. Finally, factors that control cellular shape and structural integrity that make contact with the extracellular matrix (ECM) have also been reported to be crucial regulators of adipogenesis[70]. Differentiating pre-adipocyte cell lines have been demonstrated to express type I–VI collagens, laminin, and fibronectin and other ECM interacting molecules at dissimilar levels in comparison to undifferentiated cells[71, 72]. Numerous studies now point to a variety of factors associated with the ECM that can control the progression of adipocyte differentiation and are also found to be altered in expression level between obese and lean individuals[73]. These studies also suggest that processes associated with ECM remodeling are likely to play a role in the altered states of angiogenesis and fibrosis that have been observed in obese adipose tissue as well.

A number of genes that have been determined to regulate PPARγ expression and/or activity have been determined to act upstream of PPARγ as transcription factors or function as transcriptional co-activators or co-repressors. For example, C/EBPβ has been directly implicated as a promoter of adipose tissue formation given that C/EBPβ-deficient mice have reduced adiposity. Moreover, C/EBPβ and C/EBPδ double knockout mice demonstrate an even more significant decrease in adipose-tissue mass than when C/EBPβ is deleted alone[74]. Evidence from studies that describe the genome-wide profiling of C/EBPβ binding sites suggest that C/EBPβ associates with non-coding enhancer segments in committed pre-adipocytes and in the early stages of terminal differentiation[75, 76]. This data would seem to support the prevailing concept that C/EBPβ supports the remodeling of chromatin in this context consistent with its already established ability to up-regulate C/EBPα and PPARγ gene expression in differentiating pre-

adipocytes. C/EBPα is also a well-recognized regulator of adipocyte gene expression, including PPARγ, and published studies conducted *in vivo* clearly indicate a key role for this transcription factor in the development of adipose tissue[77]. For example, when the C/EBPα locus is replaced by C/EBPβ *in vivo*, reduced amounts of white-adipose tissue are observed[78]. Although, it has been clearly demonstrated that overexpression of C/EBPα in PPARγ-deficient fibroblasts cannot rescue adipogenesis[79]. Thus, it has become widely accepted that C/EBPα functions as part of a positive feedback loop with PPARγ but on its own is not sufficient to drive the adipogenic program. Other well-established pro-adipogenic factors consist of: KLF5, KLF15, STAT5a, KROX20, glucocorticoid receptor (GR) and thyroid receptor (TR)[80]. All of these proteins have been established as promoters of adipogenesis using loss-of-function, gain-of-function and/or knock out mouse studies.

Repressors of adipogenesis that have been identified include several members of the Gata (GATA2, GATA3), forkhead (FOXO1, FOXC2), kruppel-like factor (KLF2, KLF7) and interferon-regulatory factor (IRF3, IRF4) protein families[81, 82]. Many of these anti-adipogenic regulators are observed to be down-regulated during adipogenesis, and when using *in vitro* cell-based models to ectopically express these genes, are capable of potent inhibition of adipogenesis. Other studies have implicated at least some adipogenic inhibitors as promoters of other cellular differentiation programs, most notably those that derive from mesenchyme. For example, Pref-1, a member of the Notch family of epidermal growth factor-like repeat-containing proteins, is required to be down-regulated for proper adipogenic differentiation, strongly inhibits adipogenesis *in vitro* when overexpressed and causes reduced fat mass in a Pref-1 transgenic mouse line[83, 84]. Pref-1 has also now been shown to be capable of promoting the expression of Sox9, a critical factor in chondrogenic differentiation and can direct multipotent mesenchymal

cells toward this lineage[85]. Thus, many established pro-adipogenic factors likely promote adipogenesis by coordinating the down-regulation of anti-adipogenic proteins that control the expression of gene pathways associated with other cellular differentiation programs.

There are several co-factors that are now established to make direct contact with PPARγ and in turn modulate its transcriptional activity. Some of these factors have been found to act as generalized co-factors that can interact with a variety of nuclear hormone receptors as both co-activators (CBP/p300, SRC family and TRAP220) and co-repressors (SMRT, NCoR, RIP140)[86-90]. However, other factors have now been found that co-activate PPARγ in a much more selective manner in order to regulate particular sets of genes exclusively in adipose tissue. For example, PGC1α is a co-activator specifically induced in brown fat in response to cold exposure that interacts with PPARγ to selectively regulate genes associated with thermogenesis[91]. The most prominent brown fat-specific gene regulated by the interaction between PGC1α and PPARγ is uncoupling protein-1 (UCP1), a gene responsible for promoting the permeability of the inner mitochondrial membrane thereby decreasing the proton gradient established by oxidative phosphorylation used to generate ATP. In doing so, UCP1 decouples the respiratory chain allowing for energy to be dissipated in the form of heat. In addition, the transcriptional co-regulator PRDM16 has been determined to directly interact with PPARγ as a co-activator in order to selectively promote the development of brown adipocytes from MYF5 expressing precursor cells[33]. While deletion of PRDM16 was shown to cause such precursors to differentiate into muscle cells and result in a concomitant loss of brown fat cell characteristics, overexpression of PRDM16 in myoblasts pushed these cells toward brown fat differentiation. Finally, TLE3, a member of the groucho family of proteins first discovered in Drosophila, has been discovered to be a PPARγ target gene that functions as both a direct co-activator for PPARγ

and co-repressor for TCF to mediate the progression of white adipocyte differentiation[92]. This provides for an efficient mechanism to concurrently repress Wnt signaling, a feature that is already known to be required for proper initiation of adipogenesis, while also selectively up-regulating PPARγ target genes expressed in white adipose tissue. In this way, TLE3 may potentially function as a transcriptional co-regulator in white fat as PGC1α does similarly in brown fat. Furthermore, transgenic overexpression of TLE3 in adipose tissue improves insulin sensitivity in mice fed a high fat diet and seems to produce a phenotype similar to mice given low-dose amounts of TZDs. This would therefore seem to strongly suggest that TLE3 is an important mediator of the anti-diabetic effect of TZD treatment. Ultimately, future studies will be required to further clarify the mechanisms that govern the action of these transcriptional co-regulators in the context of adipogenesis so that potentially new ways to selectively modulate PPARγ activity may be uncovered.

Techniques and Strategies Used in the Study Adipogenesis

Since the discovery of PPARγ as the master regulator of adipogenesis, a variety of methods have been employed to identify factors that can control the formation of mature adipocytes. Many well-established adipogenic regulators have been identified and/or validated using immortalized cell lines, *in vitro*. The most common cell lines used to study adipogenesis *in vitro* are 3T3-L1 and 3T3-F442A cells. 3T3-L1 cells were originally isolated as a subclone of the Swiss 3T3 mammalian fibroblast cell line that demonstrated a high capacity for spontaneous lipid droplet formation when grown post-confluent[93]. 3T3-F442A cells were later obtained as an additional subclone with even greater susceptibility to become lipid-laden adipocytes[94]. It was later shown that this process could be accelerated by incubating confluent 3T3-L1 cells with a

differentiation cocktail consisting of insulin, dexamethasone and methylisobutylxanthine (IBMX) for 48 hours followed by exchange with standard growth media[95]. Dexamethasone, a glucocorticoid, is thought to bind to its nuclear hormone receptor (GR), regulate C/EBPδ expression and contribute to increasing cyclic AMP (cAMP) levels[96]. IBMX, a phosphodiesterase inhibitor, has also been demonstrated to elevate cAMP levels. It is generally thought that cAMP facilitates the early steps of adipogenesis by up-regulating expression of C/EBPβ and activating the cAMP-responsive element binding protein (CREB). CREB has been shown to be an important factor in regulating C/EBPβ, C/EBPα and PPARγ gene expression[97, 98]. Furthermore, forced constitutive expression of CREB can induce adipogenesis, while a dominant negative form can impair adipogenesis[99]. Other cell lines that exhibit adipogenic capacity include C310T1/2 (mesenchymal stem cell line), HIB-1B (brown adipocyte cell line) and primary mouse embryo fibroblasts (MEFs)[81]. Using these and other such cell lines, a variety of approaches such as microarray-based transcriptional profiling and high-throughput cDNA, chemical and siRNA screening have all been used to identify novel factors and small molecules capable of regulating adipocyte differentiation[92, 100-102]. Advances in sequencing platforms have also resulted in new genome-wide techniques to evaluate the remodeling of chromatin for use in the identification of transcriptionally active genomic regions, binding sites for established transcription factors, proteins that regulate chromatin states and histone modifications. Studies have now begun to show just how extensively these processes occur during the course of the adipogenic differentiation program[80, 103]. Furthermore, these approaches generate an enormous amount of data that can be subsequently mined to derive new candidates for analysis as novel regulators of adipocyte differentiation.

Gene knock-out and transgenic mouse lines have also been widely used to study the impact of loss-of-function and/or gain-of-function of selected genes on the development and function of adipose tissue *in vivo*. While whole body knock-out and transgenic mice can be used to study a particular gene *in vivo*, embryonic lethality or confounding, secondary effects as a result of altered expression levels in all tissues can occur. Thus, in order to produce a conditional, tissue-specific knock-out, the Cre-LoxP recombination system has been exploited in many studies analyzing adipose tissue *in vivo*. This is done by using transgenic mice expressing the Cre recombinase enzyme under the control of a -5.4 kilobase genomic fragment consisting of the enhancer/promoter region of PPARγ target genes aP2 or adiponectin and crossing this into another line carrying the floxed gene of interest[104, 105]. Making use of the aP2 or adiponectin enhancer/promoter is an ideal method for generating adipose-specific knock-out or transgenics because of the highly selective nature of aP2 and adiponectin gene expression in adipocytes. However, aP2 and adiponectin are only robustly expressed during the late stage of terminal adipocyte differentiation. Therefore, use of these promoter cassettes presents a limitation when attempting to model the effect of deletion or overexpression of candidate genes known to endogenously function during earlier stages of adipocyte differentiation or in adipocyte progenitors.

Conclusion

The development of adipose tissue is an important area of study from a basic and clinical perspective. Discovery of new paradigms and pathways of regulation from stem cell to mature adipocyte can make a dramatic impact on the nature of metabolic disease treatment while also uncovering new fundamental biological mechanisms. Future studies of adipocyte development will need to intensify their focus on the exact stem cell origins of adipocytes, the nature and role

of so-called “beige” adipocytes, the clinically relevant differences that exist between adipose depots, the discovery of the endogenous ligand for PPARγ and the identification of novel factors and mechanisms that up-regulate expression and/or activity of PPARγ. The knowledge and understanding that emanates from the investigation of these on-going problems in adipocyte research will inevitably be applied to the prevention, diagnosis and treatment of metabolic disease when reasoned to be appropriate.

This thesis describes two projects that attempt to discover novel factors and mechanisms that regulate the production of adipocytes. In the second chapter of this thesis, the generation of a transgenic mouse line expressing highly conserved non-coding genomic sequences found near the PPARγ locus is described. This project was carried out in an effort to uncover a novel enhancer that confers tissue-selective expression in adipose tissue. The discovery of such an enhancer could mark an important step in defining further how PPARγ is specifically regulated in adipose tissue. This project was met with significant challenges, however the tools and experience obtained in this study may be critical for future work carried out in this important endeavor. The third chapter of this thesis presents the discovery of an inhibitor of adipogenesis, Vestigial-like 3 (Vgll3). This gene was identified by transcriptional profiling using novel cell lines subcloned from the 3T3-F442A cell line. Interestingly, Vgll3 appears to share similar features as other known factors that repress adipogenesis, such as Pref-1. This thesis therefore characterizes Vgll3 not only in the context of adipogenesis, but identifies Vgll3 as a likely regulatory factor in other mesenchymal-based cellular differentiation programs as well.

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Chapter 2

Identification of an Adipose-specific Enhancer:

Generation of a LacZ Reporter Transgenic Mouse

Abstract

The exact mechanisms that control the tissue-specific production of the PPARγ2 isoform in adipose remain a mystery. Here we identify five highly conserved non-coding sequences both within and upstream of the PPARγ locus. We theorized that these sequences may contain a tissue-specific enhancer that selectively controls gene expression in adipose tissue. These conserved genomic regions were cloned into a LacZ reporter construct that subsequently was used to generate a transgenic mouse line. Measurement of the activity and gene expression of the product of the LacZ gene (β-galactosidase) in adipose tissue was then carried out. Any detectable presence of β-galactosidase activity or gene expression in mice determined to carry this construct was not observed in this study using an established transgenic mouse enhancer assay. However, these results cannot completely rule out the possibility that the presence of an enhancer capable of selectively directing gene expression in adipose tissue is located within the non-coding genomic sequences analyzed in this report. Discovery of such an enhancer element would constitute a major step toward greater understanding of the precise mechanisms that regulate expression of adipose-specific transcripts.

Introduction

A growing amount of evidence now strongly indicates that non-coding genomic DNA that has been conserved across multiple species likely contains many regulatory elements that serve to control gene expression[1, 2]. Identification and characterization of these elements can be an enormous undertaking given the amount of non-coding DNA found in a variety of genomes[1]. However, the effort to uncover such elements has taken on critical importance given their mounting association with human disease[3-5].

The most commonly studied regulatory elements found within genomic intervals of non-coding DNA are called enhancers. These distal-acting sequences function in *cis* to activate the expression of genes at a particular time and/or space. With the right balance of transcription factors, transcriptional co-activators and chromatin modifications, enhancers are able to regulate selective expression of genes under certain conditions or particular cellular contexts. This is thought to fundamentally occur via a mechanism that causes the looping of long stretches of DNA thereby activating the gene promoter by way of physical interaction with RNA polymerase II. Thus, enhancers can be found not only upstream of the transcriptional start site but also may be found within introns or far downstream of the genes they target[6].

Comparative analysis between the sequenced genomes of different species has been the most straightforward way to identify regions of conservation that may contain "extremely conserved" enhancer elements present in many species separated by great evolutionary distance[7]. These elements display a very high percentage (~80-100%) of sequence similarity across stretches of at least 200 base pairs. However, so-called "ultraconserved" non-coding regions made up of segments of genomic DNA ($\geq$ 200 base pairs) that are exactly identical between only

rodents and humans have also been identified[8]. Both of these types of conserved DNA fragments may in fact contain many types of regulatory elements such as enhancers, insulators, negative regulators and non-coding RNAs adding to the complexity of regulatory mechanisms that may govern the regulation of gene expression kilobases away from the start site of transcription. Furthermore, the discovery of non-coding genomic sequences provides no information with regards to when and where a putative enhancer may actually be utilized during development. Thus, assays that test the true validity of candidate enhancer sequences require an *in vivo* system that makes use of the developing organism.

Identification of a tissue-selective conserved enhancer that can regulate PPARγ expression in adipose currently remains elusive. It has been suggested that regions with significant genomic sequence conservation predominate nearby developmental genes[7]. Therefore, we reasoned that conserved non-coding regions in proximity to the exonic sequences that exclusively code for the PPARγ2 adipose-specific isoform may contain a previously unidentified enhancer that determines the selectivity for expression in adipose tissue. In an attempt to address this problem, five regions in proximity to the PPARγ locus displaying extreme genomic conservation were cloned into a LacZ reporter construct. Using this construct, a transgenic mouse line for use in performing a previously established transgenic mouse genomic enhancer assay was established[7, 9, 10]. Results obtained in this current study however did not identify the existence of a novel adipose-selective conserved enhancer.

Results

To locate conserved non-coding regions adjacent to the PPARγ locus, comparative sequence analysis using the genomes of multiple species was carried out within a region roughly ~100 kilobases up- and downstream of the PPARγ locus. Based on this analysis, 5 regions (CS1 – CS5) that exhibit "extreme conservation" of varying lengths within and upstream of the murine PPARγ locus were chosen for further molecular cloning (**Fig. 1**). We chose to order and position these fragments using a custom polylinker constructed from the multiple cloning site of the pBluescript KS (+) plasmid in order to mimic the native orientation of these fragments present in the human and mouse genomes (**Fig. 2**). Production of transgenic mouse lines was then carried out after successfully sub-cloning the complete conserved non-coding sequence into the *hsp68-LacZ* reporter vector under the control of minimal heat shock promoter (**Fig.3**).

Three founder mice positive for the reporter vector containing all five cloned conserved segments were produced. However, of these three founders, only one mouse was actually able to produce progeny (over the course of five months) suitable for use in a transgenic mouse genomic enhancer assay. Despite this, we selected four individual F_1 progeny that were genotyped as "positive" for the presence of the cloned genomic sequences to be tested in the enhancer assay and four other separate F_1 progeny genotyped as "negative" for use as negative controls. For use as a positive control, adipose tissue collected from an Agpat6 knockout mouse generated using a gene trap insertion containing a LacZ allele was obtained[11]. However, only the brown fat taken from this Agpat6 knockout mouse was observed to robustly stain for β-galactosidase activity consistent with what has been previously reported[11].

Next, we embarked on assessing the presence of LacZ reporter activity in adipose tissue of selected F_1 progeny. For these analyses, both brown adipose tissue (BAT) and white adipose tissue (WAT) were examined (subcutaneous and epididymal). Assessment of β-galactosidase activity by X-gal staining in both whole tissue and sliced tissue sections failed to detect the presence of any reporter activity in brown or white adipose tissue isolated from transgenic mice carrying cloned non-coding conserved sequences (**Fig. 4 and Fig. 5A**). As expected, all corresponding negative controls in all isolated adipose tissue samples were found to lack any notable signal indicating β-galactosidase activity (**Fig. 4 and Fig. 5B**). On the other hand, brown fat tissue collected from Agpat6 knockout mice was observed to stain strongly positive for β-galactosidase activity, however no intense staining in white fat was observed (**Fig. 4**). In order to further validate these results, real-time PCR was carried out to analyze for the presence of β-galactosidase transcript expression in subcutaneous white fat tissue (**Fig. 6**). Consistent with results obtained in staining experiments, very little to no β-galactosidase gene expression was observed in WAT taken from transgenic mice positive for the LacZ reporter containing conserved sequences. Conversely, expression of β-galactosidase transcript was clearly detected in WAT samples acquired from Agpat6 knockout mice. These results demonstrate that the presence of a conserved enhancer capable of directing tissue-selective gene expression in adipose could not yet be detected.

Discussion

It has been approximated by some that possibly up to 5% of non-coding DNA in the human genome is more conserved than would be projected given the predicted rate of evolution since the divergence from rodents[12]. In addition, several highly conserved elements that significantly align between the human and mouse genomes have been reported to have demonstrable association with rare alleles implicated in disease thus strongly suggesting a functional role for these genomic segments. It has also been thought that enhancers that are found within highly conserved segments are typically located near an assemblage of different transcriptional factor binding sites[13]. Indeed, we found binding sites for the transcription factors C/EBPα, C/EBPβ, PPARγ, PPARα, IRF1, IRF2, SRY, NKX2.5, SOX9 and CREB in distinct clusters located within the non-coding conserved sequence cloned into the LacZ reporter vector (**data not shown**). Therefore, the presence of extremely conserved genomic DNA located adjacent to the PPARγ locus, and our identification of consensus transcriptional factor binding sites as detailed above, provided initial support for our hypothesis that an adipose-specific enhancer might exist within the consolidated non-coding genomic segment analyzed and described in this report.

While the results of the transgenic mouse genomic enhancer assay only elicited negative results, this does not rule out the existence of an enhancer capable of directing adipose-specific expression located within the sequences cloned and analyzed in this study. First, of the three founders that were acquired, only one founder was able to mate and actually produce pups. This significantly reduced the number of replicates available for testing using the enhancer assay. Second, insertion of the linearized LacZ reporter construct into a transcriptionally silent region of the genome could not be excluded. Thus, any future studies using the LacZ reporter vector

containing the conserved sequences as described in this report will require more available replicate mice in order to minimize the risk of false negatives. Previous other studies have also reported negative results when analyzing non-coding conserved sequences *in vivo*[14, 15]. In particular, one intriguing study involved the deletion of four different "ultra-conserved" enhancers that were all determined to be positive in a mouse transgenic genomic enhancer assay, and each one was found to be located near genes that display striking phenotypes when inactivated[15]. Nevertheless, four separate enhancer knockout lines were observed to produce normal, viable mice as established by a variety of biological and physiological endpoints. Thus, conserved enhancers that regulate developmental genes are likely to have functionally redundant genomic counterparts.

Materials and Methods

Cloning of Conserved Non-Coding Sequences

All five sets of conserved sequences were amplified using the polymerase chain reaction (PCR) from a bacterial artificial chromosome (BAC) containing the region of chromosome (ch6) of the mouse genome that comprises the PPARγ locus. BAC clones were obtained from the "BACPAC Resource Center" located at the Children's Hospital Oakland Research Institute (CHORI). All sequences are based from the published mouse genome reference assembly (July 2007, NCBI37/mm9) reported by the UCSC Genome Browser. All selected conserved sequences were cloned into a 100 base pair (bp) custom polylinker consisting of specifically designed restriction enzyme digestion sites each separated by 5-10 base pairs and was placed into the multiple cloning site of the pBluescript KS (+) plasmid as indicated in Figure #2. The custom polylinker was cloned into pBluescript using *SacI* and *KpnI* restriction sites. Conserved sequence (CS) #1 was cloned at a length of 1,049 base pairs (bp) starting from ch6:115,361,228. CS1 was cloned into the custom polylinker using *NheI* and *XhoI* restriction sites. CS2 was cloned at a length of 712 bp starting from ch6:115,356,875. CS2 was cloned into the custom polylinker using *MluI* and *NheI* restriction sites. CS3 was cloned at a length of 992 bp starting from ch6: 115,339,651. CS3 was cloned into the custom polylinker using *SpeI* and *MluI* restriction sites. CS4 was cloned at a length of 358 bp starting from ch6:115,295,740. CS4 was cloned into the custom polylinker using *PstI* and *SpeI*. CS5 was cloned at a length of 454 bp starting from ch6:115,292,170. CS5 was cloned into the custom polylinker using *SmaI* and *PstI*.

LacZ Transgenic Reporter Mice

The LacZ reporter vector was linearized in preparation for tail-vein injection by *SalI*. Transgenic mice were generated at the UCLA transgenic core facility. Founder mice and subsequent progeny were identified and genotyped using two separate sets of real time PCR primers capable of generating DNA fragments specific for sequences only present within *hsp68-LacZ* reporter vector and not found within the endogenous mouse genome.

X-gal Staining and LacZ (β-galactosidase) mRNA Expression

A modified version of the X-gal staining protocol reported previously was used for assessing β-galactosidase (β-gal) activity in sliced tissue sections and whole tissues[16]. Briefly, adipose tissues were rinsed in 1X PBS before fixation, then placed in a fixation solution (1X PBS containing 2% formaldehyde, 0.2% glutaraldehyde) for 30 min. Tissues were then washed with wash buffer (2mM $MgCl_2$, 0.02% NP-40, 0.1M sodium phosphate buffer, pH7.4) 15 min for three times. Fat tissues were stained in a X-Gal solution (1X PBS containing 1mg/ml X-Gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide) overnight at room temperature on a rocker. Tissues were then washed in washed buffer (as above) for15 min for three times. Fat pads were stored in 70% Ethanol at 4^{o}C. Sliced tissue sections were prepared by the Translational Pathology Core Laboratory (TPCL) at UCLA. Real-time PCR primers specific for the β-gal transcript were used to detect the presence of β-gal gene expression in adipose tissues. Brown and white adipose tissue obtained from Agpat6 knockout mice was used as a positive control in X-gal staining (brown fat) and real-time PCR (white fat) experiments. Adipose tissue from mice determined to be negative for the consolidated non-coding genomic sequence cloned into the LacZ reporter vector was used as a negative control. Wildtype (WT) adipose tissue was also analyzed as a secondary negative control in real-time PCR experiments.

Figure Legends.

Figure 1. **Depiction of five conserved sequence (CS) regions at the murine PPARγ locus.** CS1, CS2 and CS3 are segments of conserved non-coding genomic DNA found in the intron located just upstream of the transcriptional start site (TSS) of the PPARγ2 isoform. CS4 and CS5 are found in the intergenic region immediately upstream of the TSS of the PPARγ1 isoform. The approximate distance of each cloned segment from the TSS of the PPARγ2 isoform is indicated in numbers of kilobases (kb) above each cloned genomic fragment. The chromosomal position on the "+" strand on chromosome 6 of each TSS in the PPARγ locus is also displayed.

Figure 2. Plasmid map of pBluescript KS (+) with custom polylinker.

A 100 base pair (bp) custom polylinker was created using restriction sites tailored for the cloning of each conserved sequence (CS). The custom polylinker was inserted into pBluescript at the SacI and KpnI restriction enzyme digestion sites. The orientation of each cloned fragment is depicted below the plasmid map and reflects their endogenous alignment in the mouse genome.

Figure 3. Plasmid map of the *hsp68-LacZ* reporter vector.

All conserved sequences were sub-cloned as one complete segment into the *hsp68-LacZ* reporter vector using the KpnI restriction sites. An "empty" reporter vector is under the control of a minimal heat shock promoter.

Figure 4. X-gal staining in brown adipose tissue

Sliced brown fat tissue sections stained for β-galactosidase activity. Agpat6-KO positive control (top), brown fat tissue sample from mouse positive for cloned non-coding sequences (middle)

and brown fat tissue sample from mouse negative for cloned non-coding sequences (bottom). All tissues are from male mice. Data is representative of at least two experiments.

Figure 5. X-gal staining in white adipose tissue

Whole white adipose tissue stained for β-galactosidase activity. Different adipose depots from one male and one female mouse that tested positive for cloned non-coding sequences (top) and different adipose depots from one male and one female that tested negative for cloned non-coding sequences (bottom). Data is representative of at least two experiments.

Figure 6. β-galactosidase gene expression in white adipose tissue (WAT)

Gene expression of the β-galactosidase transcript was assessed in subcutaneous (SC) white adipose tissue. Positive Control: SC WAT from Agpat6-KO mouse. "+": SC WAT from mouse positive for cloned non-coding sequences. "--": SC WAT from mouse negative for cloned non-coding sequences. WT: SC WAT from wildtype mouse.

Figure 1.

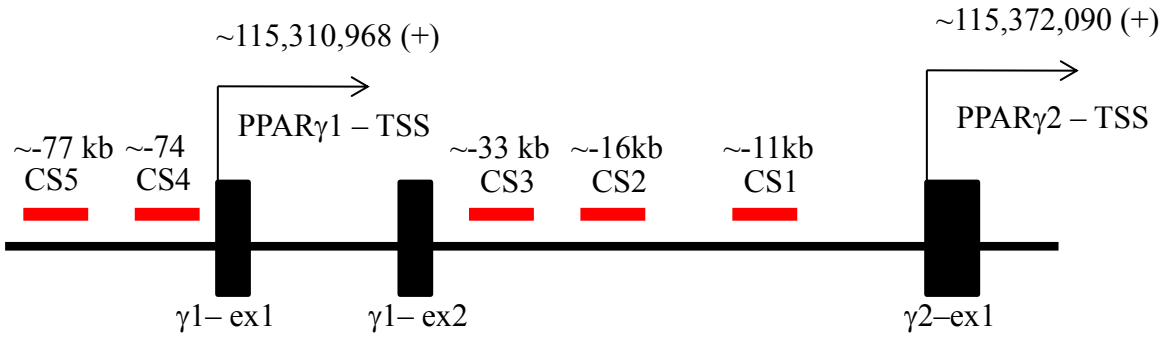
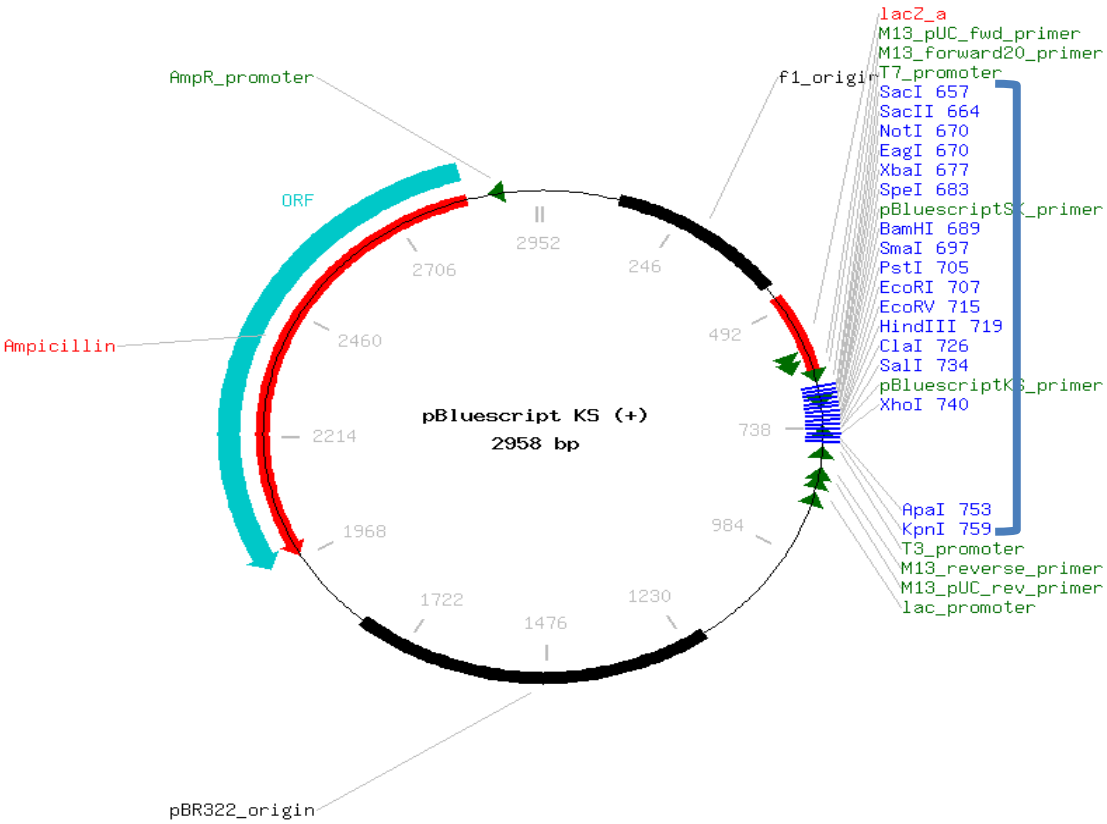

~115,310,968 (+)
~115,372,090 (+)
PPARγ1 – TSS
PPARγ2 – TSS
~-77 kb
CS5
~-74
CS4
~-33 kb
CS3
~-16kb
CS2
~-11kb
CS1
γ1– ex1
γ1– ex2
γ2–ex1

Figure 2.



Custom polylinker (100 bp):

SacI- KpnI – (SmaI **-CS5-***PstI*-**CS4-***SpeI*-**CS3-***MluI*-**CS2-***NheI*-**CS1-***XhoI) – KpnI*

Figure 3.

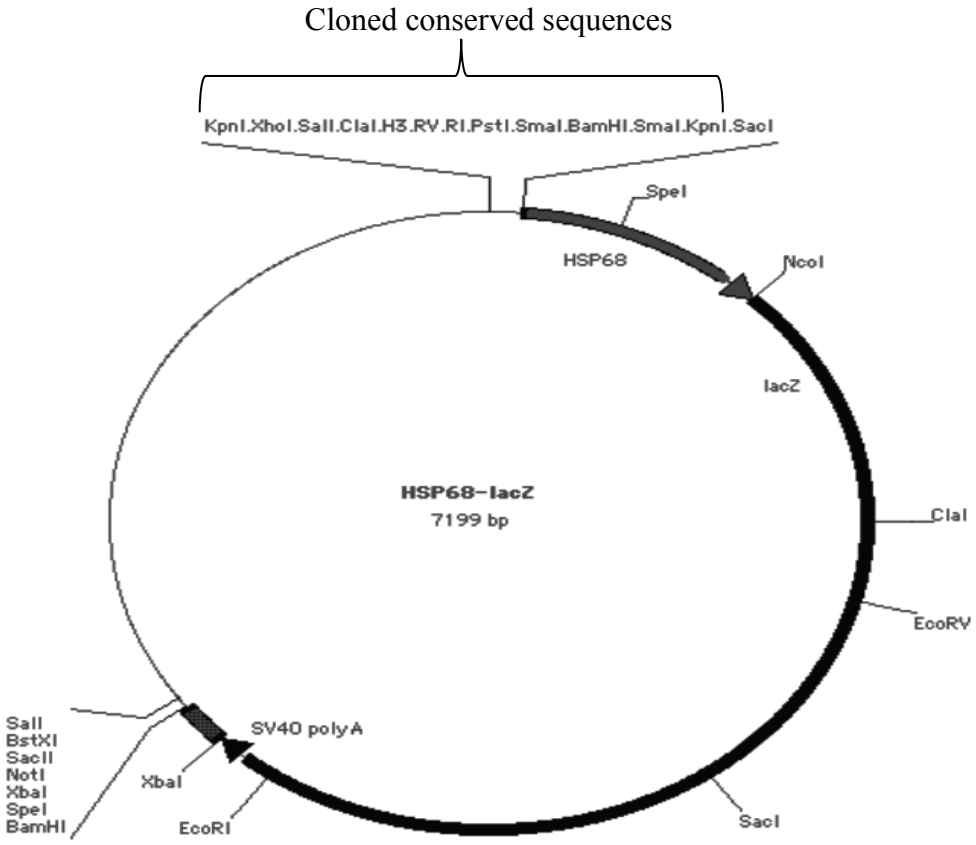
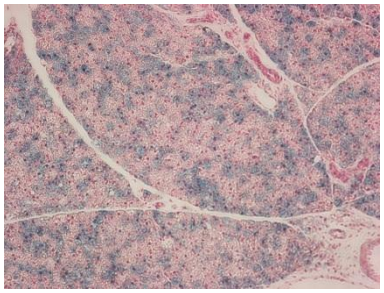
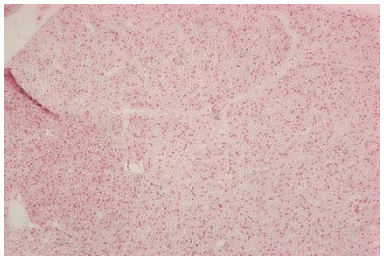

Cloned conserved sequences
KpnI.XhoI.SalI.ClaI.H3.RV.RI.PstI.SmaI.BamHI.SmaI.KpnI.SacI
SpeI
HSP68
NcoI
lacZ
HSP68-lacZ
7199 bp
ClaI
EcoRV
SacI
SV40 polyA
SalI
BstXI
SacII
NotI
XbaI
SpeI
BamHI
XbaI
EcoRI

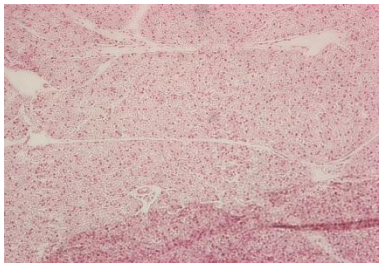
Figure 4.



BAT - LacZ Positive Control



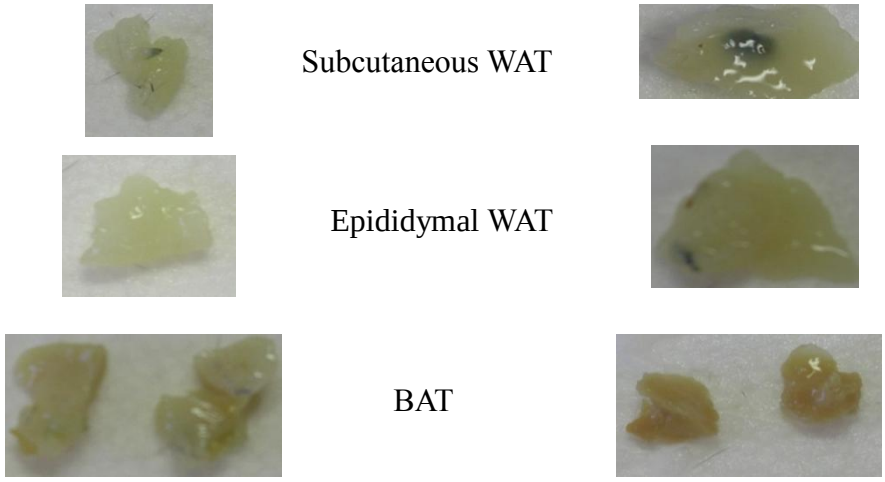
BAT – Positive for the LacZ Reporter Vector



BAT – LacZ Negative Control

Figure 5.

A. Whole Tissue Samples tested positive for LacZ reporter:



B. Whole Tissue Samples tested negative for LacZ reporter:

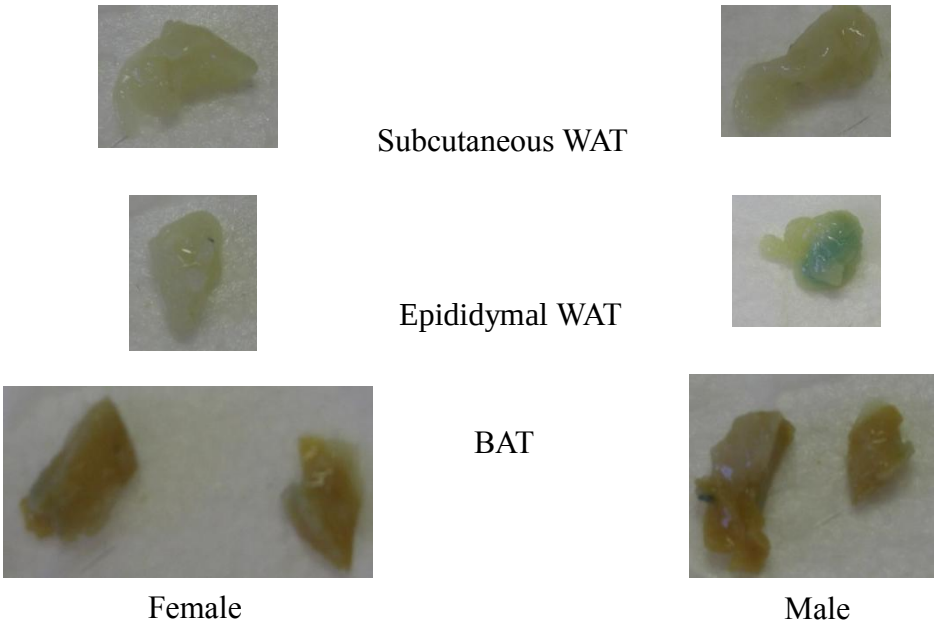
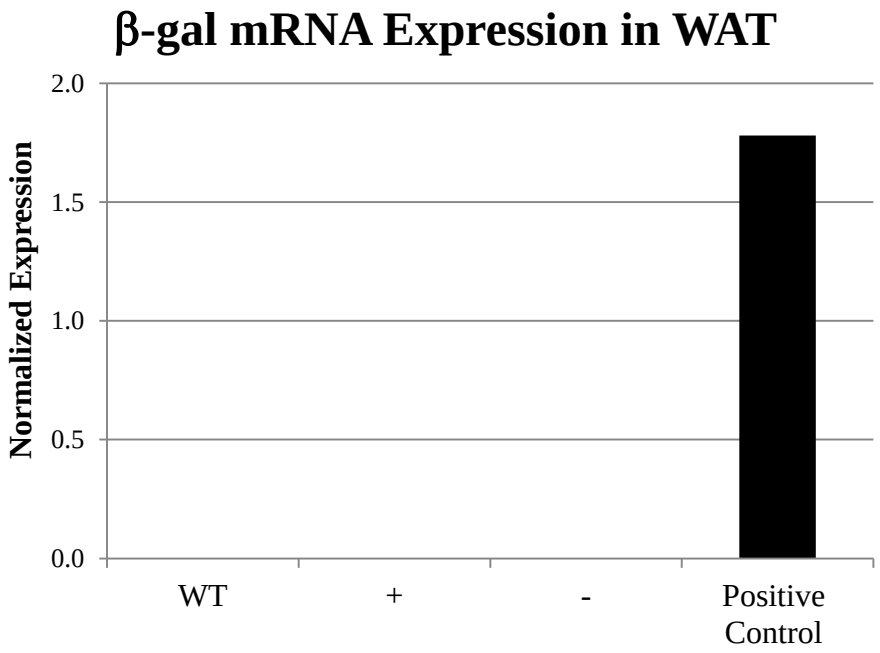


Figure 6.


β-gal mRNA Expression in WAT
Normalized Expression
2.0
1.5
1.0
0.5
0.0
WT
+
-
Positive Control

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Chapter 3

Discovery of Vestigial-like 3 (Vgll3) as an Inhibitor of Adipocyte Differentiation

Abstract

Adipose differentiation is a complex process controlled by a network of transcription factors and co-regulators. We compared the global gene expression patterns of adipogenic and non-adipogenic clones of 3T3-F442A pre-adipocytes and identified the transcriptional co-factor, Vestigial-like 3 (Vgll3), as an inhibitor of adipogenesis. Vgll3 expression is down-regulated during terminal adipocyte differentiation *in vitro* and negatively correlates with weight and total fat mass *in vivo.* Furthermore, enforced expression of Vgll3 expression inhibits the differentiation of pre-adipocytes in vitro, whereas shRNA-mediated knockdown of Vgll3 expression promotes differentiation. Interestingly, expression of Vgll3 promoted the expression of genes associated with bone and chondrocyte formation, suggesting that Vgll3 participates in the decision of mesenchymal cells to proceed down the adipocyte, bone or cartilage lineages. Ultimately, the elucidation of factors involved in specification of the adipocyte phenotype may aid in the identification of new strategies for the treatment of metabolic disease.

Introduction

The prevalence of metabolic disorders such as diabetes and obesity is increasing in developing and industrialized societies. Unraveling the intricate network of biological pathways and factors that govern adipogenesis, the process of development and differentiation of adipose tissue, is expected to provide insight into the physiology and pathophysiology underlying these disorders. A number of regulatory factors that participate in the positive and negative control of this process have been identified. Many established mediators of adipogenesis are involved transcriptional regulation, including PPARγ, the CEB/Ps, and members of the KLF family of transcription factors[1, 2]. More recently, transcriptional co-regulators such as PRDM16, PGC-1α and TLE3 have been found to be important for the fine-tuning of particular features of the adipogenic program[3-5]. However, it is likely that additional factors also contribute to this complex process that have yet to be identified and characterized.

The lipid-activated transcription factor PPARγ is the master regulator of adipocyte differentiation and is both necessary and sufficient for development of adipose tissue[6, 7]. Free fatty acids and eicosanoids are naturally-occurring ligands capable of activating PPARγ; however, a specific and biologically relevant endogenous ligand for PPARγ has yet to be identified. Clinically, PPARγ is the molecular target of the thiazolidinedione (TZD) class of therapeutic drugs that act as ligands capable of inducing PPARγ activity and the adipogenic program. TZDs are highly effective insulin sensitizers in humans, however their use has been limited by side effects and safety concerns[8]. The elucidation of novel factors that regulate the adipocyte differentiation program could provide the foundation for the potential identification of new therapeutic targets.

Since the discovery of PPARγ, a variety of approaches have been employed in an effort to uncover additional regulatory factors involved in the conversion of progenitor pre-adipocytes cells into fully mature, lipid-laden adipocytes. Such strategies have included transcriptional profiling of pre-adipocyte cell lines, analysis of knockout/transgenic mouse models, high-throughput cDNA screening and the identification of adipogenic small molecules[9-12]. Alternative strategies offer the potential of uncovering additional novel determinants of adipocyte differentiation not identified by prior approaches. For example, a recent study employed analysis of gene expression in clonal sublines derived from Swiss3T3 fibroblasts to identify zfp423 as a new regulator of adipogenesis[13].

In the current study, we compared global gene expression in clonal sublines of committed 3T3-F442A pre-adipocytes in an effort to uncover adipogenic modulators. We identified the mRNA encoding Vestigial-like 3 (Vgll3) as a transcript differentially regulated between adipogenic and non-adipogenic clones. Further analysis revealed that expression Vgll3 is down-regulated during adipocyte differentiation. Constitutive expression of Vgll3 in differentiating pre-adipocytes potently inhibits adipocyte differentiation and up-regulates the expression of osteogenic genes, whereas knockdown of Vgll3 promotes differentiation. These studies demonstrate that Vgll3 acts as a negative regulator of terminal adipocyte differentiation and support further investigation of Vgll3 as a regulator of mesenchymal-derived cellular differentiation programs.

Results

We isolated sublines of 3T3-F442A pre-adipocytes with varying capacities for adipogenic differentiation through clonal selection (**Fig. 1**). Two clonal sub-lines, designated as "B6" and "C2" were found to be highly divergent in their potential to become mature adipocytes. B6 displayed a very high propensity for adipocyte differentiation, whereas C2 exhibited almost no ability to differentiate (**Fig. 2A**). In accordance with this observation, expression levels of PPARγ2 and its target gene aP2 were up- and down- regulated in B6 and C2 sublines compared to the parental 3T3-F442A cell line, respectively (**Fig. 2B and 2C**). In an effort to uncover novel genes affecting adipogenesis, we performed global gene expression profiling in the B6 and C2 sublines using cDNA microarrays (**Fig. 3**). A large number of genes were differentially regulated between these two sublines. As expected, transcript levels of terminal adipocyte marker genes (Adiponectin, CD36) were highest in the B6 subline, whereas levels of expression of known anti-adipogenic genes (CHOP, KLF2) were highest in the C2 subline (**Fig. 2D and 2E**).

Many genes previously reported to be important mediators of adipocyte differentiation are known to function as transcription factors or transcriptional co-factors[1-6]. Therefore, we focused our subsequent analysis on established or putative transcriptional regulators of gene expression that also displayed at least a 2-fold differential expression signal ratio between the B6 and C2 sub-lines. Based on these criteria, Vestigial-like 3 (Vgll3) was identified as a candidate gene for further analysis. Upon further examination, we observed that endogenous Vgll3 gene expression was inversely correlated with the degree of mature adipocyte formation. In a time course of adipogenesis, Vgll3 expression steadily declined in the highly differentiated B6 cells at Day 6 (**Fig. 4A**). By contrast, there was a gradual escalation in Vgll3 expression in the anti-

adipogenic C2 cell line (**Fig. 4A**). Furthermore, Vgll3 expression levels were strongly reduced in adipogenically differentiating 3T3-L1 cells stimulated with PPARγ ligand (GW7845) (**Fig. 4B**).

We next analyzed the expression of Vgll3 in adipose tissue *in vivo*. Vgll3 transcript levels were correlated with clinical traits obtained from previous studies of expression quantitative trait loci (eQTL) in a mouse F2 population produced by the intercrossing of F1 mice [15, 16]. We found that expression of Vgll3 was inversely correlated with total body weight and adipose tissue mass (**Fig. 5**). Interestingly, this correlation was particularly strong in mesenteric and gonadal fat depots. However, correlation in abdominal retroperitoneal and subcutaneous depots did not reach statistical significance. These *in vivo* findings were consistent with our *in vitro* results, and suggested that down-regulation of Vgll3 gene expression could play a role in adipocyte development.

To explore this possibility, we ectopically expressed Vgll3 in committed pre-adipocyte cell lines using a retroviral vector. Multiple independent stable pools of control and Vgll3-expressing 3T3-F442A and 3T3-L1 cells were selected and analyzed for their differentiation capacity. In 3T3-F442A cells, enforced expression of Vgll3 resulted in a modest but reproducible decrease in lipid accumulation as assessed by Oil Red O staining at Day 7 of differentiation compared to vector controls (**Fig. 6D**). In 3T3-L1 cells, Vgll3 strongly inhibited lipid accumulation (**Fig. 6A and 6B**). Real-time qPCR analysis showed that the expression of adipocyte differentiation markers was also reduced in response to Vgll3 expression, confirming that Vgll3 was affecting differentiation *per se*. The expression of PPARγ2 and aP2 was reduced in cells constitutively expressing Vgll3 (**Fig. 6C and 6E**). Furthermore, Vgll3 overexpression was found to up-regulate the expression of Gata-2 and Pref-1, two genes expressed in adipocyte

precursors and previously reported to be strongly down-regulated during adipogenesis (**Fig. 7**) [17, 18].

To assess whether blocking endogenous Vgll3 expression had an impact on the progression of adipogenesis, retroviruses expressing inhibitory shRNAs were used to obtain multiple independent 3T3-L1 cell pools with reduced levels of Vgll3 transcripts. Vgll3 knockdown was validated by real-time qPCR (**Fig. 8A**) and the ability of the knockdown cells to differentiate was evaluated. In order to allow the detection of subtle changes in adipogenic capacity, these studies were carried out under conditions minimally required for adipogenic stimulation using an established differentiation cocktail (10% FBS, Dexamethasone (1 μM), IBMX (0.5 mM), Insulin 3.5 μg/mL). Remarkably, an increase in morphological adipocyte differentiation and lipid accumulation was observed in cell lines expressing Vgll3-specific shRNAs compared to control shRNA (**Fig. 8B**). Consistent with this result, the expression of the adipogenic markers PPARγ and aP2 were also increased (**Fig. 8A**). This data further supports the hypothesis that the decrease in Vgll3 expression during adipogenesis may be important for proper conversion of committed pre-adipocytes to mature adipocytes.

In an effort to investigate the mechanism for Vgll3 effects on adipogenesis, we tested the ability of Vgll3 to affect PPARγ transcriptional activity. We transfected PPARγ and RXR into 3T3-L1 pre-adipocytes combination with vector or Vgll3 expression plasmid and assayed the induction of the -5.4 kb aP2-luciferase reporter. In contrast to the recently identified PPARγ co-factor TLE3[5], Vgll3 had no effect on PPARγ activity in this assay (**Fig. 9**). This result suggested that Vgll3 was unlikely to be acting by directly inhibiting PPARγ transcriptional activity.

We next endeavored to determine the gene set acutely responsive to Vgll3 expression. We transduced 3T3-L1 cells expressing the coxsackie adenovirus receptor (CAR) with human Vgll3-expressing and LacZ-expressing adenoviral vectors. To identify differentially-regulated genes, we employed transcriptional profiling with cDNA arrays (data not shown). Interestingly, a number of genes previously reported to be associated with other mesenchymal differentiation programs were found to be up-regulated in response to potent Vgll3 activity. In particular, several genes associated with bone formation were induced in response to Vgll3 expression, including Adm, Opn, Mmp3, Thbs1, Col12a1, and Osr2. We confirmed that these and other genes linked with bone and chondrocyte differentiation were induced in differentiating 3T3-L1 cells stably expressing human Vgll3 by real-time PCR (**Fig. 10A)**. Furthermore, when 3T3-L1 cells expressing Vgll3 were cultured in osteogenic differentiation media for 21 days, an increase in alkaline phosphatase gene expression and von Kossa staining was observed (**Fig. 10B and Fig. 10C**). Together, these results indicate that inappropriate Vgll3 expression in adipogenically-differentiating pre-adipocytes leads to the expression of genes associated with bone and chondrocyte differentiation.

Discussion

The development of adipose tissue is a process that involves the coordinated action of genes that positively and negatively regulate this process. In this present study, we documented an inverse relationship between the degree of adipogenic capacity and expression of the putative transcriptional co-factor Vgll3. We also showed that body weight and adipose tissue mass was inversely correlated with Vgll3 expression in adipose tissue *in vivo*. Enforced expression of Vgll3 in pre-adipocytes inhibits adipocyte differentiation in association with the induction of bone and chondrocyte markers. Our results suggest that down-regulation of Vgll3 expression during adipogenesis may be important for the specification of adipocyte program and the suppression of genes associated with other mesenchymal cell fates.

Mammalian Vgll3 is related to the transcriptional co-factor *Vestigial* (*Vg*), originally described in *Drosophila melanogaster*. Drosophila *Vestigial* is involved in determining cell fate in the developing fly wing and muscle[19-21]. In mammals, there are four highly conserved "vestigial-like" genes. Previous reports have suggested that members of this protein family are associated with muscle development and function[22, 23]. For example, Vgll3 has been reported to be expressed in developing muscle tissues of the mouse embryo[24]. We showed that the suppression of adipogenesis by Vgll3 was accompanied by the induction of a panel of genes associated with other mesenchymal cell fates, including bone and cartilage. Thus, whether Vgll3 is a regulator of muscle, bone or chondrocyte development and differentiation are important questions that future studies will need to address.

We also found that stable overexpression of Vgll3 induced the expression of the well-characterized inhibitors of adipocyte differentiation Pref-1 and Gata-2. This observation

suggests that suppression of Vgll3 expression during differentiation may be important for the suppression of Pref-1 and Gata-2 expression. Previously published reports have suggested that Pref-1 and Gata-2 are positive regulators of other mesenchymal differentiation programs[25]. Furthermore, Sonic hedgehog (Shh) has been shown to up-regulate Gata-2 and genes associated with osteogenesis, similar to our observations with Vgll3[26]. Additional analysis of Vgll3 as a potential interacting player with Pref-1, Gata-2 and other known regulatory pathways that suppress formation of mature fat tissue is warranted.

In humans, the amount of intra-abdominal/visceral fat has been linked to the progression of the metabolic syndrome. More recently, it has been suggested that visceral mesenteric fat in particular may be causally associated with the development of insulin resistance and type 2-diabetes[27, 28]. Not surprisingly, specific adipose depots display stark differences in gene expression[29]. For example, genes associated with cell development have been reported to be differentially expressed between various fat depots[30]. In this current study, we found that Vgll3 expression is inversely correlated with mesenteric and gonadal adipose content. Interestingly, this result is reminiscent of previous observations of Tbx15. This developmental transcription factor is strongly differentially regulated between subcutaneous and visceral fat depots in rodents and humans and was shown to impair adipogenesis in 3T3-L1 cells when overexpressed[31]. For now, it remains an open question whether differences in Vgll3 expression exist between different fat depots in humans.

Materials and Methods

Reagents and Plasmids

GW7845 was kindly provided by T. Willson (GlaxoSmithKline). Insulin (#12585-014) was from Gibco® (Life Technologies). Dexamethasone (#D2915) and 3-isobutyl-1- methylxanthine (IBMX, #17018) were from Sigma-Aldrich. Human Vgll3 was obtained by using plasmid clone #30528902 obtained from Open Biosystems (Thermo Scientific) catalog #MHS1010-98050653. Primers used in order to obtain a full-length human Vgll3 PCR product (per NCBI Reference Sequence NM_016206) were 5′-GGGGACAAGTTT GTACAAAAAAGCAGGCTCTGCCACCATGAGTTGTGCGGAGGT-3′ (Forward) and 5′-TCAGTACCACGGTGATTCCT TACTCTTGTCTTGATGCTGTAGACCTGTA TCGAA-3′ (Reverse). This PCR product was then subsequently used as a template to produce an amplified Gateway®-adapted full length human Vgll3 PCR product using the forward primer described above and the primer 5′-GGGGACCACTTTGTACAAGAAA GCTGGGTCTCAGTACCACGGTGATTCCTTAC-3′ (Reverse). BP and LR recombination was employed to clone full length human Vgll3 into a Gateway®-adapted pCDNA-DEST47 mammalian expression vector (Invitrogen) and pBABEpuromycin retroviral expression vector [14]. LacZ and human Vgll3 adenoviral particles were generated using the pAd/CMV/V5-DEST Gateway®-adapted adenoviral vector (Invitrogen). All descriptions of Vgll3 mRNA overexpression refer to human Vgll3 unless specifically indicated otherwise.

Mammalian Cell Culture and Retro/Adeno-Virus Production

3T3-F442A and 3T3-L1 pre-adipocytes were maintained in DMEM supplemented with 10% calf serum. To prepare for adipocyte differentiation, 3T3-F442A and 3T3-L1 cells were grown to confluence in 6 well plate or 10 cm culture dish in DMEM supplemented with 10% fetal bovine serum (FBS). 3T3-L1 cells were stimulated to differentiate (1 day post confluent) by treating with dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/ml) for 2 days after confluence followed by either insulin (5 μg/ml) and GW7845 (10 nM or 20 nM when indicated) or insulin (5 μg/ml) alone. 3T3-F442A cells were stimulated to differentiate (1 day post confluent) by treating with insulin (5 μg/ml) with or without GW7845 (10 nM). Growth media was exchanged every 2 days during the time course of adipogenic differentiation. When treating 3T3-L1 with osteogenic media, cells were allowed to reach confluence and treated with 10% FBS, β-glycerophophate (1M) + Ascorbic Acid (50 μg/mL). Retrovirus was obtained by overnight transfection of Phoenix E cells with pBABEpuro-empty vector and pBABEpuro-hVgll3 using Fugene Transfection Reagent (Promega) followed by growth media exchange and harvesting of retrovirus 48 hours later. Adenovirus was amplified, purified and titered by Viraquest Inc.

shRNA Plasmids and endogenous Vgll3 knockdown

Vgll3 shRNA constructs were designed using BLOCK-IT RNAi designer tool (Invitrogen). Sense and antisense oligos were annealed and cloned into the pENTR/U6 plasmid (Invitrogen). Using LR recombination (Invitrogen), shRNA constructs were then sub-cloned into a Gateway®-adapted pBabe-Puromycin plasmid and transfected into Phoenix E cells. Oligos used in this study are: LacZ shRNA 5′ -CACCGGGCCAGCTGTATAGACATC TCGAAAGATGTCTATACAGCTGGCCC-3′, mVgll3 sh1 5′-CACCGAAAGAGCTG

AGCTGTCTCGCCCGAAGGCGAGACAG CTCAGCTC-3′, mVgll3 sh2 5′-CACCGG AACTTTAGCATCCAGATAACGAATTATCTGG ATGCTAAAGTTCC-3′. Only the sense strands are indicated here. Differentiation of 3T3-L1 pre-adipocytes expressing shRNAs was carried out using dexamethasone (1 μM), IBMX (0.5 mM), and insulin (3.5 μg/ml) for 48 hours followed by stimulation with insulin (3.5 μg/ml) only in 10% FBS.

Gene Expression and Microarrays

Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed using the iScript cDNA synthesis kit (Biorad). cDNA was quantified by real-time PCR using SYBR Green (Diagenode) and an ABI 7900 instrument. Gene expression levels were determined by using a standard curve. All data from genes analyzed were normalized to the housekeeping gene 36B4 and were performed in duplicate. Primers used for real time PCR are: (vgll3: fwd 5′-CCGGAACCCCTGGCAG-3′ rev 5′-CTTGTCCTGATGCTGAAGACC-3′, human Vgll3 fwd 5′-CTACAGTCACCTCTGCT ACCT-3′ rev 5′-CTTGTCTTGATGCTGTAGACC-3′, PPARγ fwd 5′-TGGTAATTTCTTGTGAAGTGC-3′ rev 5′- TGGTAATTTCTTGTGAAGTGC-3′, aP2 fwd 5′-CACCGCAGACGACAGGAAG-3′ rev 5′-GCACCTGCACCAGGGC-3′ , Adiponectin fwd 5′-CCGGAACCCCTGGCAG-3′ rev 5′-CTGAACGCTGAGCGATA CACA-3′, CD36 fwd 5′-GGCCAAGCTATTGCGACAT-3′ rev 5′-CAGATCCGAACACAGCGTAGA-3′, Chop fwd 5′-GCGACAGAGCCAGAATAACA-3′ rev 5′-GATGCACTTCCTTCTGGAACA-3′, Klf2 fwd 5′-CTAAAGGCGCATCTGCGTA-3′ rev 5′-TAGTGGCGGGTAAGCTCGT-3′ , 36B4 fwd 5′-ACTGGTCTAGGACCCGAGAAG-3′ rev 5′-TCCCACCTTGTCTCCAGTCT-3′, Ankrd1fwd 5′-GCTGGAGCCCAGATTGAA-3′ rev 5′-CTCCACGACATGCCCAGT-3′, Col1A1fwd 5′-CCGCTGGTCAAGATGGTC-3′ rev 5′-CTCCAGCCTTTCCAGGTTCT-3′, Col1A2 fwd 5′-

CGGAGAAGCTGGATCTGC-3′ rev 5′-CAGGAGGACCCATTACACCA-3′, Snai2 fwd 5′-TGCAAGATCTGTGGCAAG G-3′ rev 5′-CAGTGAGGGCAAGAGAAAGG -3′, Opn fwd 5′-CCCGGTGAAAGTGACTGATT-3′ rev 5′-TTCTTCAGAGGACACAGCATT-3′, Ptprv fwd 5′-AACACCACAGGCTGGACAC-3′ rev 5′-GGGCTTCACTGGTCACATTTA-3′ Sox9 fwd 5′-CAGCAAGACTCTGGGCAAG-3′ rev 5′-TCCACGAAGGGTCTCTTCTC-3′, Thbs1 fwd 5′-CCCCAACCTTCCCAACTC-3′ rev 5′-GGGTTGTAATGGAATGGAC AG-3′, MMP3 fwd 5′-TTGTTCTTTGATGCAGTCAGC-3′ rev 5′-GATTTGCGCCAAAAGTGC-3′ , Col12A1 fwd 5′-ACTGGGGAGGAGACCACTG-3′ rev 5′-TGGTCTGTATCTAATCCGATA-3′, Adm fwd 5′-TTCGCAGTTCCGAAAGAAGT-3′ rev 5′-GGTAGCTGCTGGATGCTTGT-3′, Gadd45β fwd 5′-CTGCCTCCTGGTCACGAA-3′ rev 5′-TTGCCTCTGCTCTCTTCACA-3′, Osr2 fwd 5′-CCAGGCAGACATCGGTTC-3′ rev 5′-GGGTGTGAGGGGGAAAAG-3′, Alp fwd 5′-AAACCCAGAACACAAGCATTC-3′ rev 5′-TCCACCAGCAAGAAGAAGCC-3′). Isolated sublines were prepared for microarray analysis by growing 3T3-F442A cells in 10% FBS to confluence and treating cells with Insulin (5 μg/mL) for 48 hours. For each cell subline, RNA was pooled from six biological replicates and analyzed using a whole mouse genome array from Illumina (MouseWG-6 BeadChip) carried out by the Southern California Genotyping Consortium at UCLA. Results were analyzed using GenomeStudio (Illumina). For adenovirus experiments, 3T3-L1 cells stably expressing the Coxsackie and Adenovirus Receptor (CAR) were grown in 10% FBS and Insulin (5 μg/mL). Upon reaching confluence, cells were treated with LacZ and hVgll3 expressing adenovirus overnight (12 hours) using an MOI of 50 in 10% FBS with Insulin (5 μg/mL) and GW7845 (20 nM). Cells were then exchanged with fresh 10% FBS containing Insulin (5 μg/mL) and GW7845 (20 nM) and RNA was harvested 48 hours later.

Three biological replicates were used to pool collected RNA and samples were processed by the UCLA Clinical Microarray Core using GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix). Results were analyzed using GenespringGX (Affymetrix). Vgll3 transcript expression in adipose tissue *in vivo* was correlated with quantifiable clinical traits using data obtained from studies as described previously[16, 17].

Luciferase Reporter Assay

3T3-L1 cells were grown in 10% FBS in 24 well culture plates until roughly 80% confluence. Cells were co-transfected with 100 ng of pGL3-aP2-luciferase[6], 100 ng of pCMX-PPARγ, 20 ng of pCMX-RXR, 100 ng of pCDNA-hVGll3, and 1 ng of Renila Luciferase control vector using Fugene Transfection Reagent (Promega). Cells were transiently transfected for 6 hours and then exchanged with fresh 10% FBS and allowed to incubate overnight. Media was then exchanged with 10% FBS with or without PPARγ ligand GW7845 (100nM). Cells were allowed to incubate for 48 hours and Luciferase activity was measured with the Dual-Luciferase® Reporter Assay System (Promega) and a GLOMAX® Luminometer (Promega). Firefly Luciferase activity was normalized to Renilla Luciferase.

Figure Legends

Figure 1. 3T3-F442A subline isolation strategy. FBS = Fetal Bovine Serum. CS = Calf Serum. Insulin was used at 5 μg/mL**.**

Figure 2 Isolation and characterization of isolated pre-adipocyte sub-lines. (A) Oil Red O Staining of 3T3-F442A parental, B6 and C2 sub-lines 8 Days after stimulation with 10% FBS containing Insulin (5 μg/mL) and GW7845 (10nM). (B) Gene Expression of PPARγ2 and aP2 by real time quantitative PCR (qPCR). Day 8. (C) PPARγ2 and aP2 expression in 3T3-F442A parental line, B6 and C2 isolated clonal sub-lines cultured in 10% FBS only. Day 0, 2, 4 and 6 post-confluent. (D) Expression of adipogenic genes in isolated sub-lines; 48 hours post-confluent incubated with Insulin (5 μg/mL). (E) Expression of anti-adipogenic genes in isolated sub-lines; 48 hours post-confluent incubated with Insulin (5 μg/mL). N=2.

Figure 3. Heat map depicting differentially regulated genes identified in isolated sublines of 3T3-F442A pre-adipocytes using whole mouse genome arrays. Cells were grown in 10% FBS with insulin (5 μg/mL). RNA was isolated 48 h post-confluence.

Figure 4. Vgll3 mRNA expression is down-regulated in highly differentiated pre-adipocytes undergoing adipogenesis. (A) Endogenous expression of murine Vgll3 mRNA during a time course of adipogenesis in 3T3-F442A parental, B6 and C2 isolated sub-lines. 10% FBS + Insulin (5 μg/mL). N=2. (B) Endogenous expression of murine Vgll3 in 3T3-L1 cells during a time course of adipogenesis. 10% FBS; DMI + GW7845 (20nM). N=2.

Figure 5. Correlation of *in vivo* Vgll3 mRNA expression with: (A) total weight, (B) total fat content, (C) retroperitoneal fat content, (D) gonadal fat content, (E) total fat content, (F)

mesenteric fat content, (G) retroperitoneal fat content and (H) subcutaneous fat content. (A-D) N=313[15]. (E-H) N =300[16].

Figure 6. Overexpression of Vgll3 inhibits adipocyte differentiation (A) Oil Red O Staining of 3T3-L1 overexpressing Vgll3 versus pBABE control at Day 8 of Adipocyte Differentiation. (B) Microscopic view of Oil Red O Staining of 3T3-L1 cells overexpressing Vgll3 versus pBABE control at Day 8 of Adipocyte Differentiation. (C) Expression of adipogenic gene markers in 3T3-L1 cells overexpressing Vgll3 versus pBABE control at Day 8 of adipocyte differentiation. N=2 (D) Microscopic view of Oil Red O Staining of 3T3-F442A cells overexpressing Vgll3 versus pBABE control at Day 7 of Adipocyte Differentiation. (E) Expression of adipogenic gene markers at Day 4 and Day 7 of adipocyte differentiation in 3T3-F442A cells overexpressing Vgll3 versus pBABE control. N=2.

Figure 7. Overexpression of Vgll3 during adipogenesis up-regulates previously established anti-adipogenic genes. (A) Expression of adipogenic inhibitory genes Pref-1 and Gata-2 was analyzed by qPCR during a time course of adipogenesis in 3T3-L1 pre-adipocytes. (B) PPARγ2 and Vgll3 expression were included as experimental controls. N=2.

Figure 8. Knockdown of endogenous Vgll3 in 3T3-L1 pre-adipocytes promotes adipocyte differentiation. (A) Expression of murine Vgll3, PPARγ2 and aP2 by qPCR in 3T3-L1 pre-adipocytes infected with retrovirus expressing two separate shRNAs targeting endogenous Vgll3 transcript. N=2. (B) Oil Red O staining of 3T3-L1 pre-adipocytes expressing shRNAs against endogenous Vgll3 and LacZ at Day 8 of adipocyte differentiation. Differentiation of 3T3-L1 pre-adipocytes expressing shRNAs was carried out using Dexamethasone (1 μM), IBMX (0.5 mM),

and Insulin (3.5 μg/ml) for 48 hours followed by stimulation with Insulin (3.5 μg/ml) only in 10% FBS.

Figure 9. Analysis of the activation of -5.4 kB aP2 promoter co-expressing PPARγ2/RXR and Vgll3 in undifferentiated 3T3-L1 pre-adipocytes in 10% FBS with insulin (5 μg/mL) with or without PPARγ ligand GW7845 (100 nM). N=4.

Figure 10. Overexpression of Vgll3 up-regulates genes associated with non-adipose tissue differentiation programs and promotes expression of markers of bone differentiation in 3T3-L1 cells grown under osteogenic conditions. (A) Expression of osteogenic- and chondrogenic-associated genes was analyzed in 3T3-L1 pre-adipocytes at Day 8 of adipocyte differentiation. N=2. (B) mRNA Expression of Alkaline Phosphatase in 3T3-L1 pre-adipocytes cultured for 21 days under osteogenic conditions. (C) Von Kossa Staining in 3T3-L1 pre-adipocytes cultured for 21 days under osteogenic conditions.

Figure 1

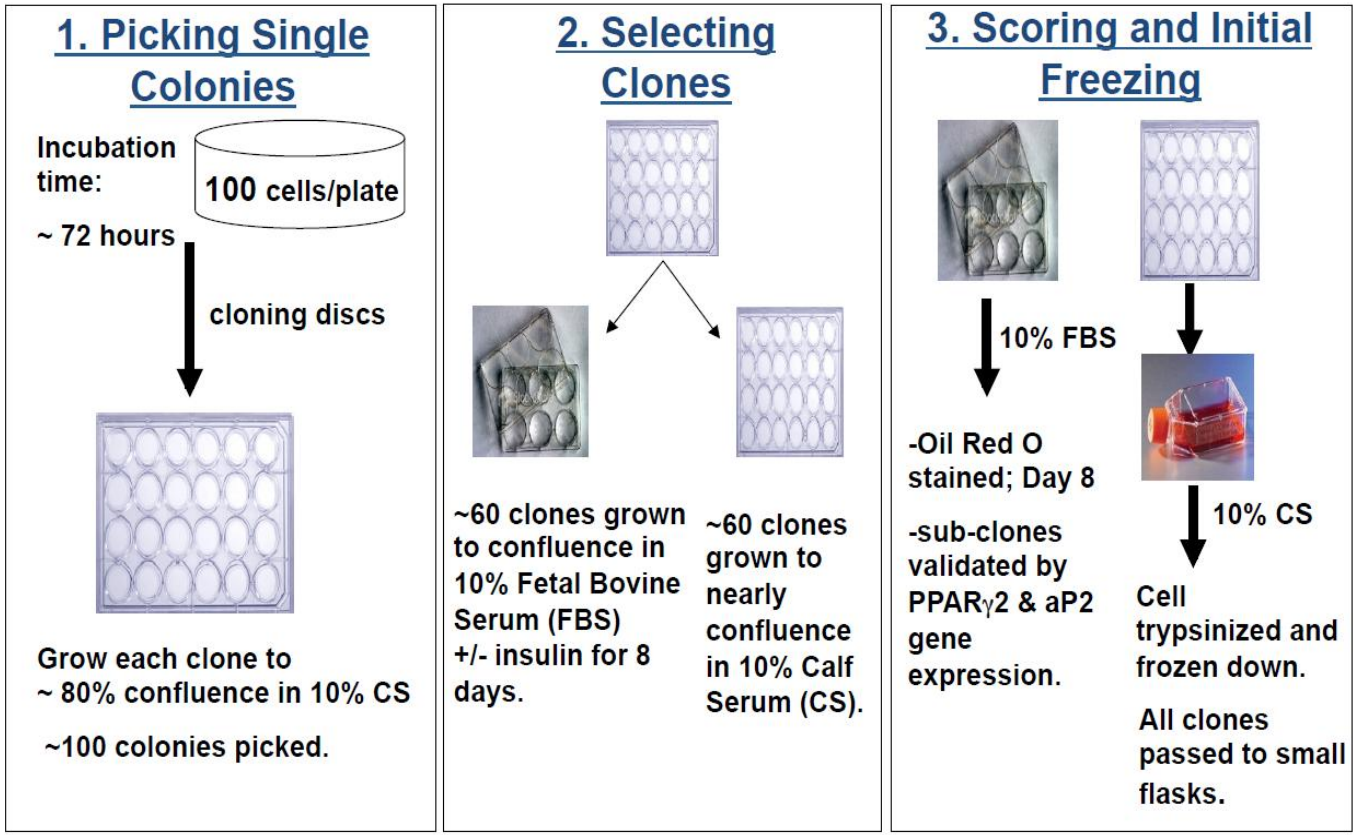
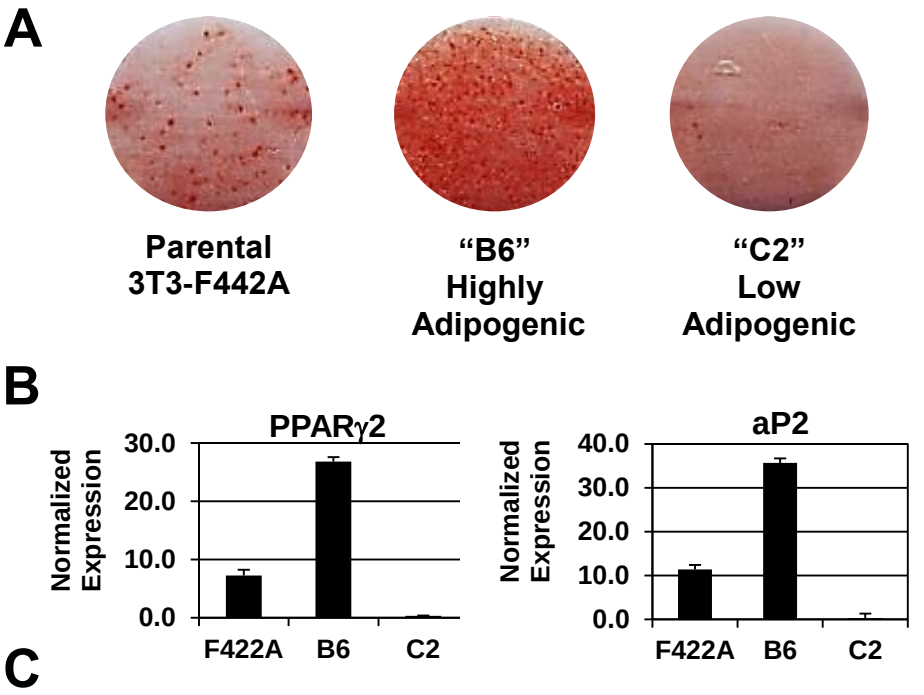

1. Picking Single Colonies
Incubation time:
~ 72 hours
100 cells/plate
cloning discs
Grow each clone to ~ 80% confluence in 10% CS
~100 colonies picked.
2. Selecting Clones
~60 clones grown to confluence in 10% Fetal Bovine Serum (FBS) +/- insulin for 8 days.
~60 clones grown to nearly confluence in 10% Calf Serum (CS).
3. Scoring and Initial Freezing
10% FBS
-Oil Red O stained; Day 8
-sub-clones validated by PPARγ2 & aP2 gene expression.
10% CS
Cell trypsinized and frozen down.
All clones passed to small flasks.

Figure 2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3T3-F442A
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Adipogenic
"C2"
Low
Adipogenic
B
PPARγ2
Normalized Expression
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20.0
10.0
0.0
F422A
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C2
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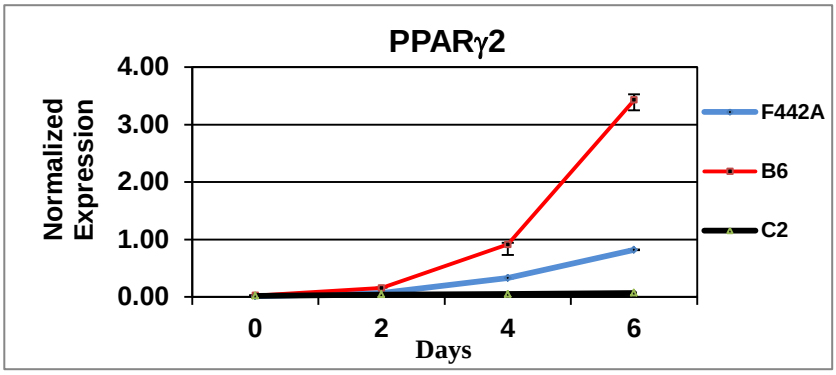
B6
C2
C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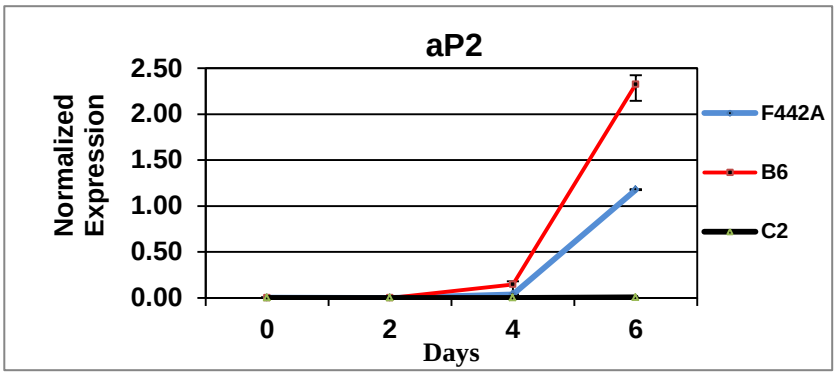
1.00
0.00
0
2
4
6
Days
F442A
B6
C2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Normalized Expression
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1.00
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0.00
0
2
4
6
Days
F442A
B6
C2

Figure 2 continued

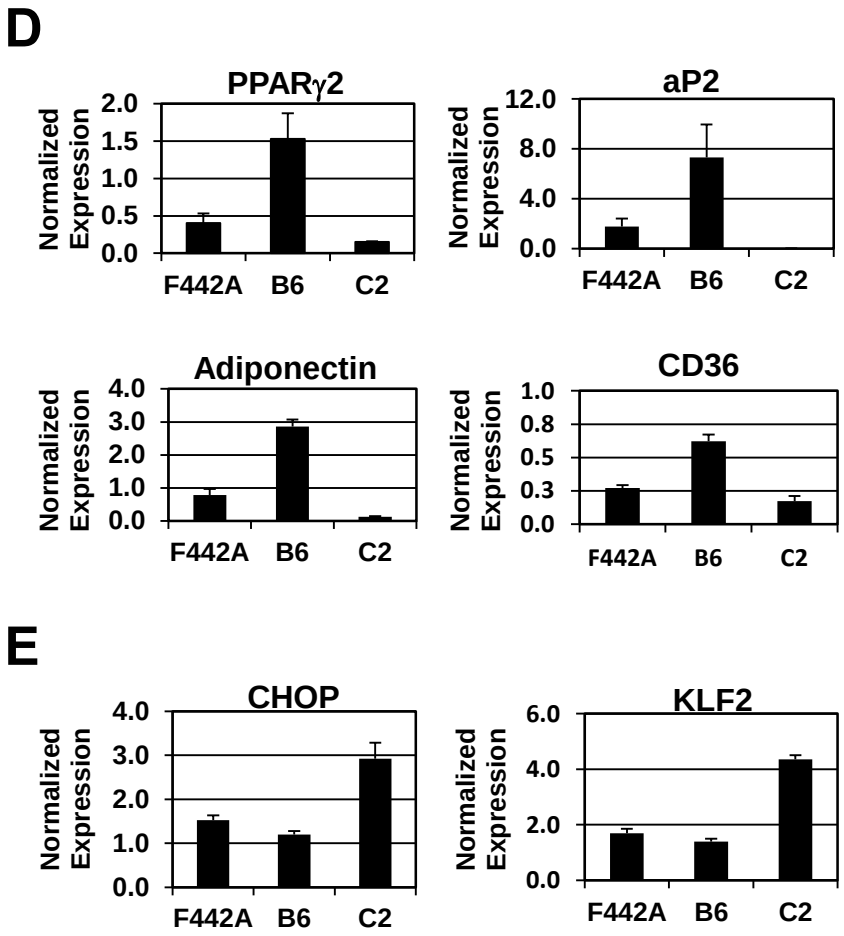


Figure 3

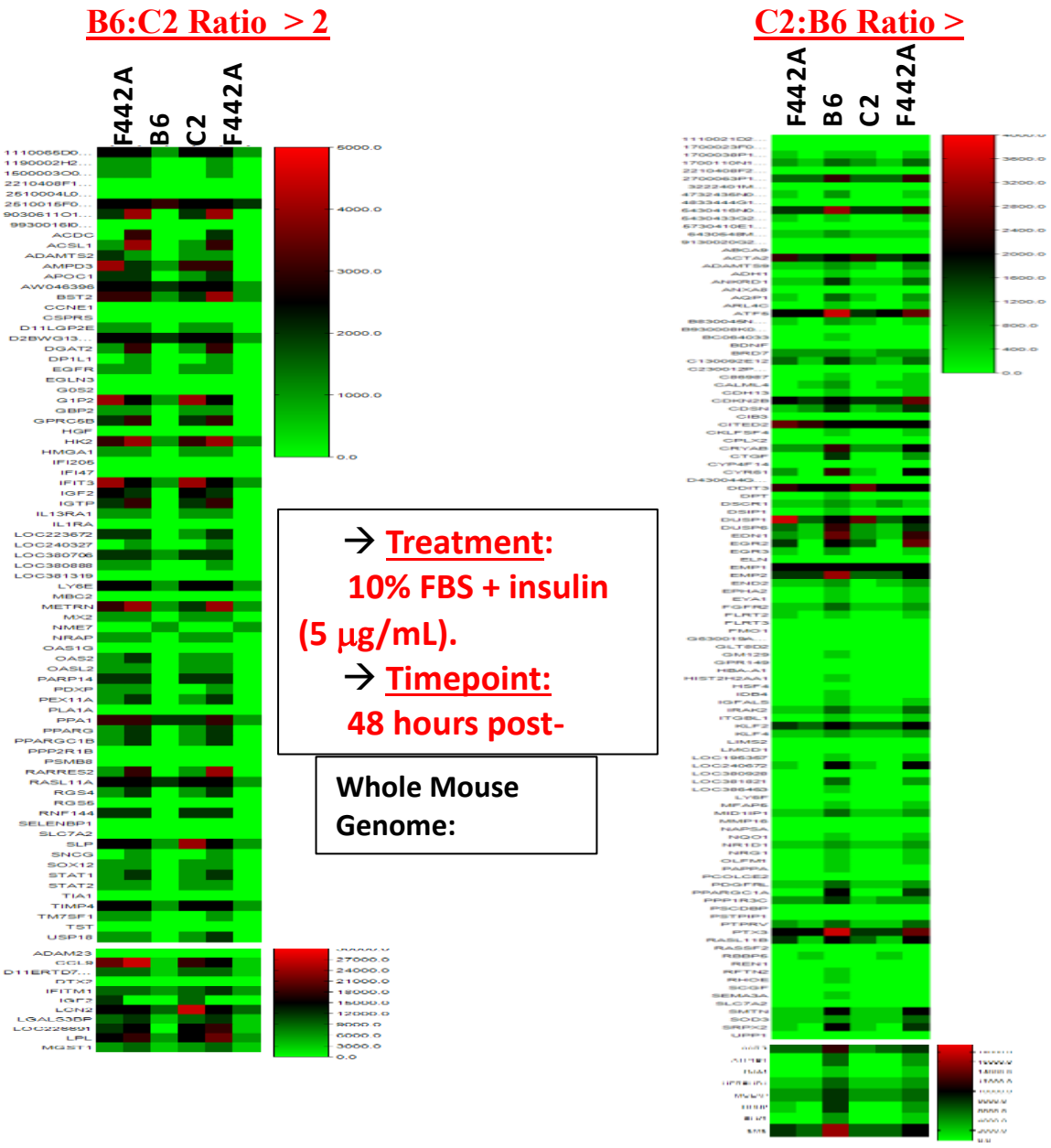
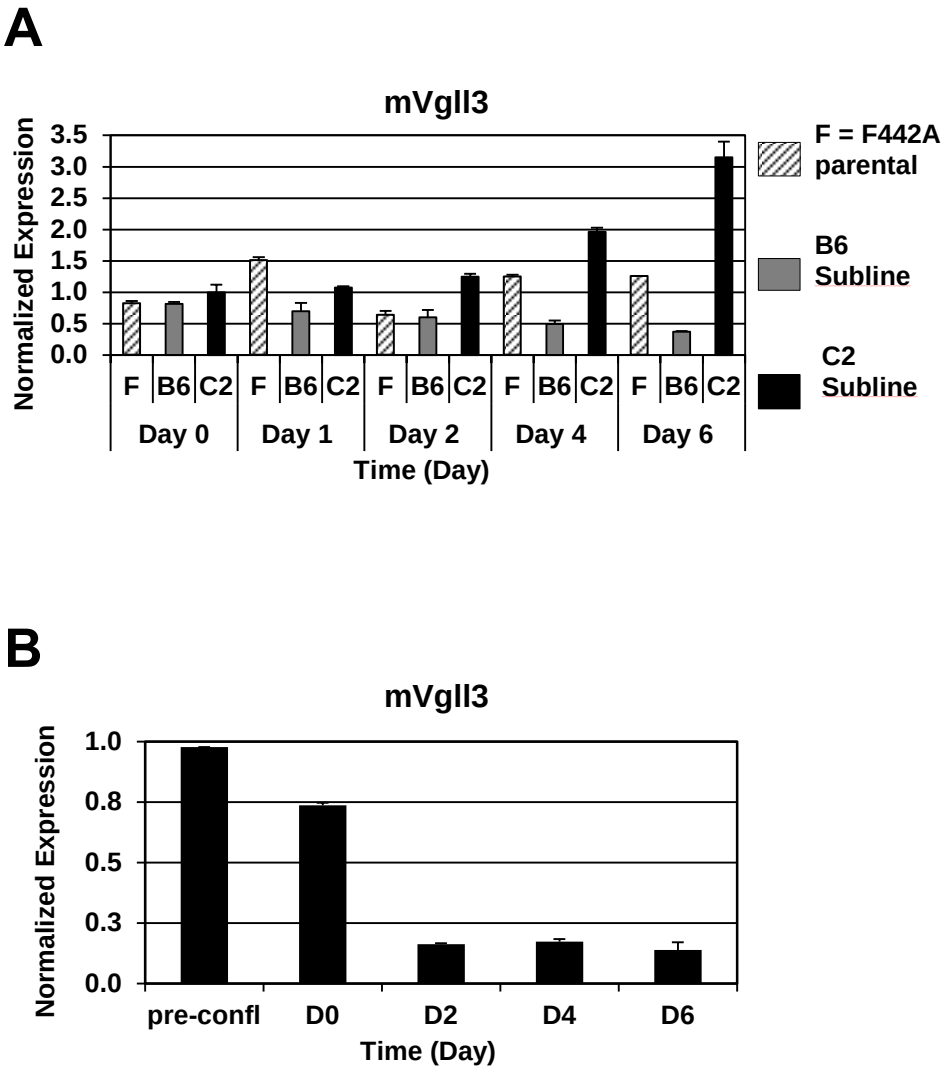

B6:C2 Ratio > 2
C2:B6 Ratio >
F442A
B6
C2
F442A
→ Treatment:
10% FBS + insulin
(5 μg/mL).
→ Timepoint:
48 hours post-
Whole Mouse
Genome:

Figure 4


A
mVgll3
Normalized Expression
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
F
B6
C2
Day 0
Day 1
Day 2
Day 4
Day 6
Time (Day)
F = F442A parental
B6 Subline
C2 Subline
B
mVgll3
Normalized Expression
1.0
0.8
0.5
0.3
0.0
pre-confl
D0
D2
D4
D6
Time (Day)

Figure 5

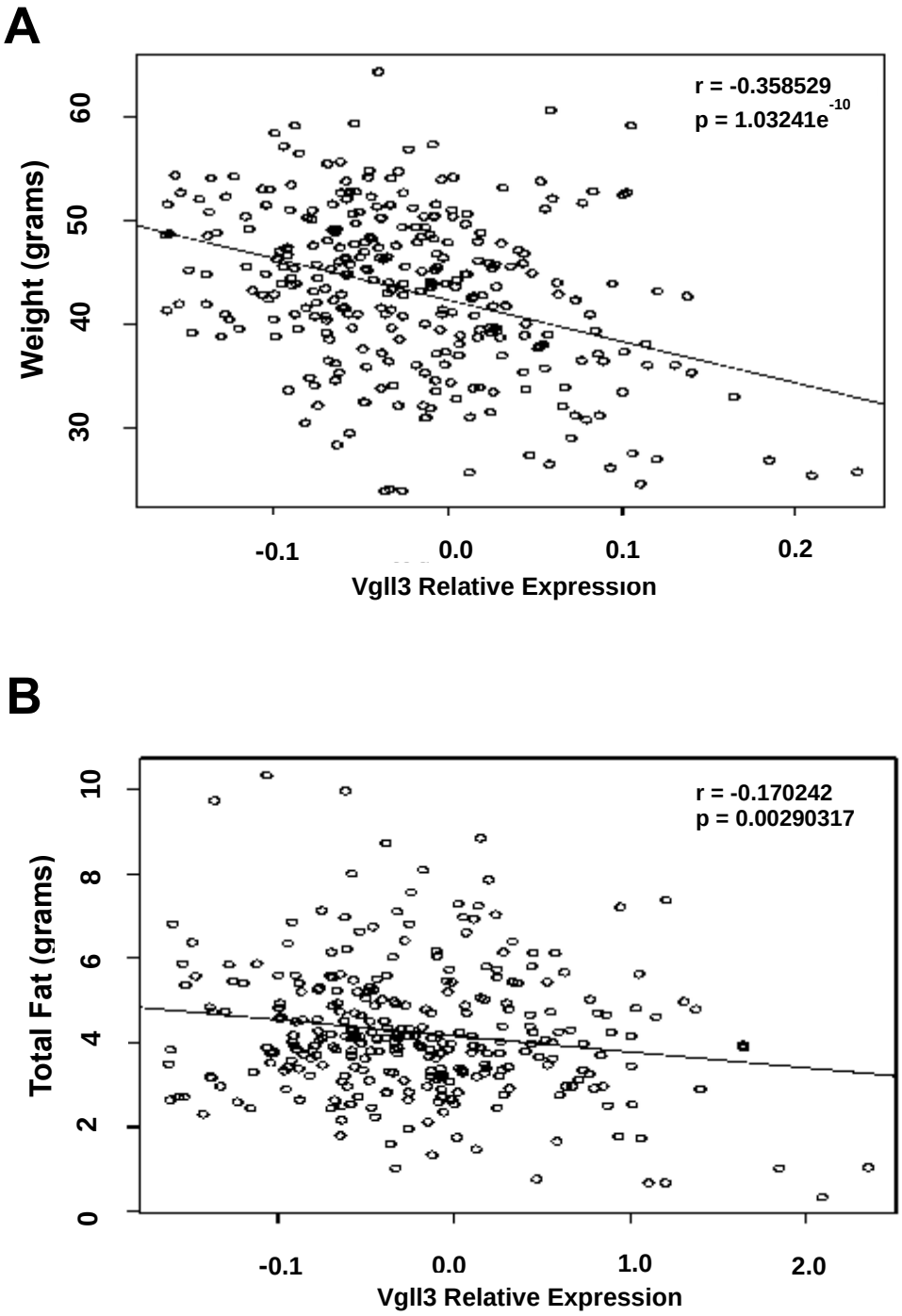
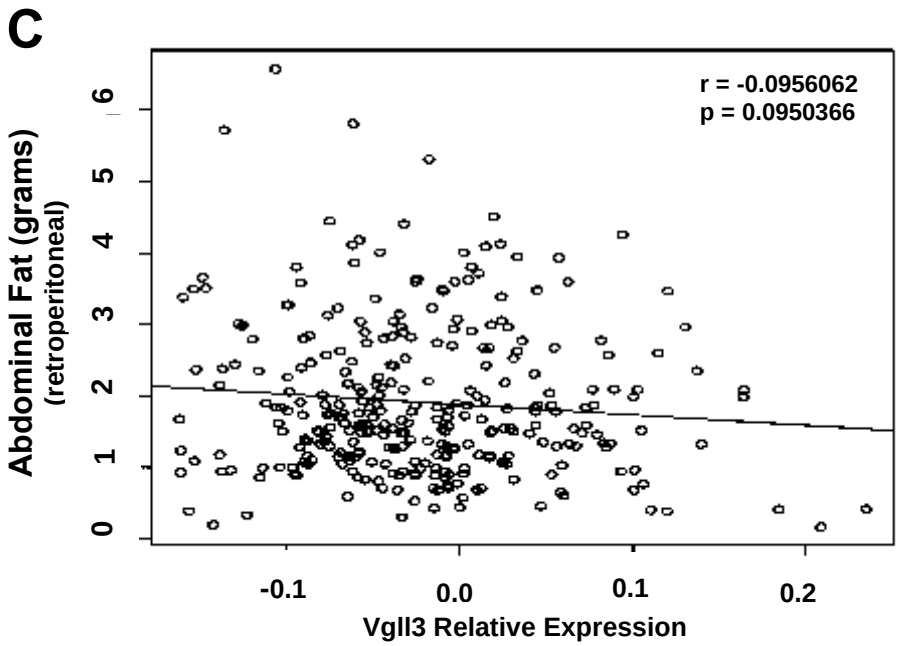

A
r = -0.358529
p = 1.03241e-10
Weight (grams)
30
40
50
60
-0.1
0.0
0.1
0.2
Vgll3 Relative Expression
B
r = -0.170242
p = 0.00290317
Total Fat (grams)
0
2
4
6
8
10
-0.1
0.0
1.0
2.0
Vgll3 Relative Expression

Figure 5 continued



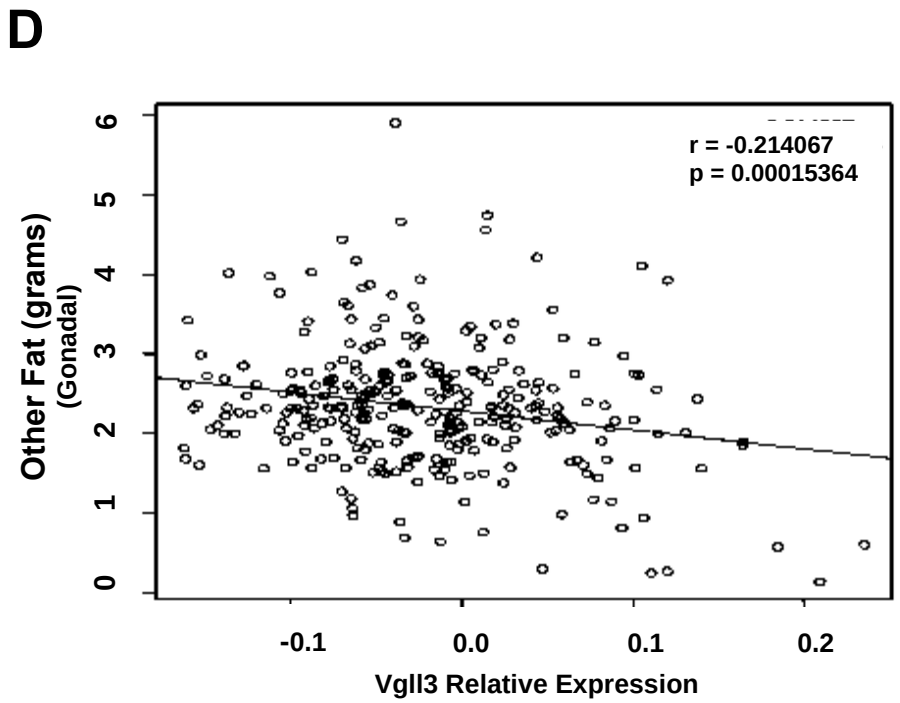


Figure 5 continued

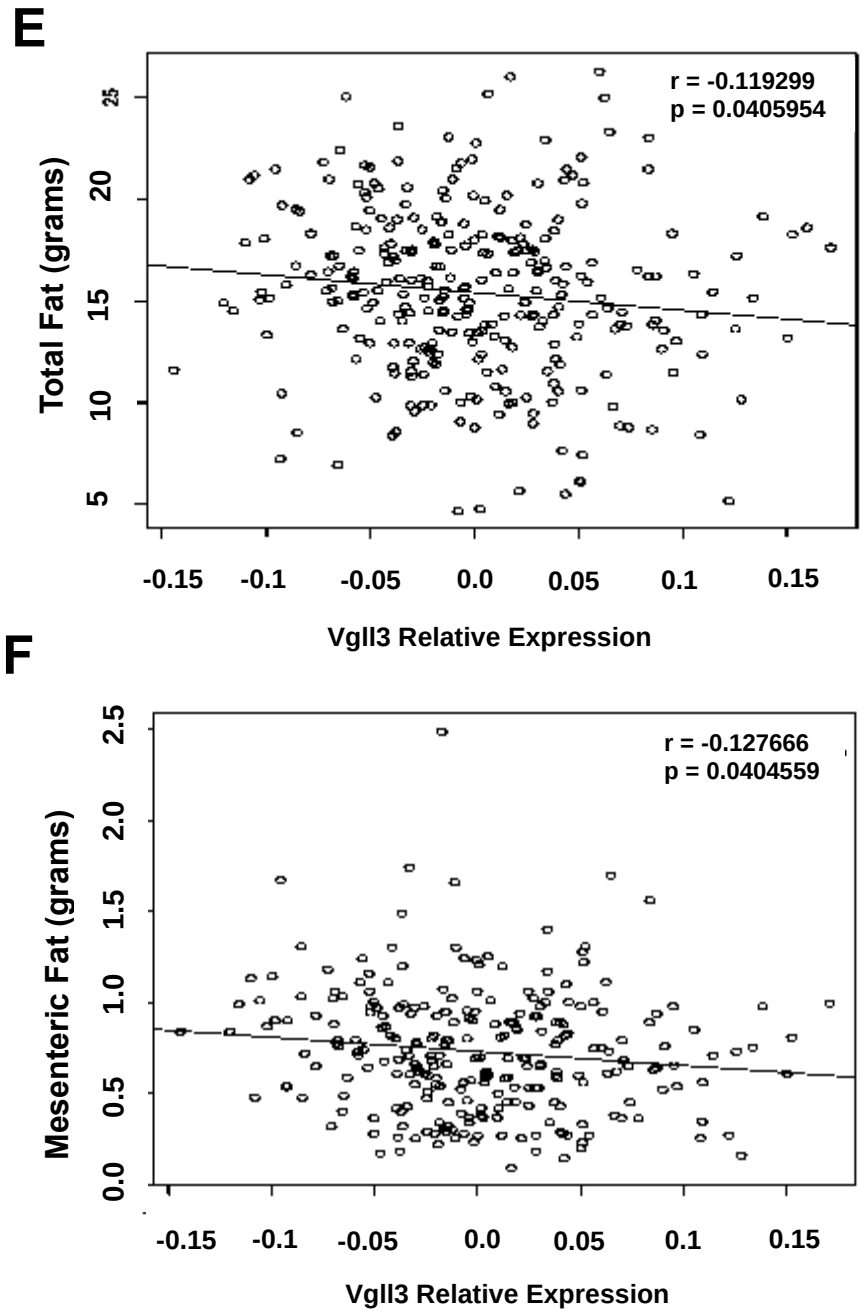


Figure 5 continued

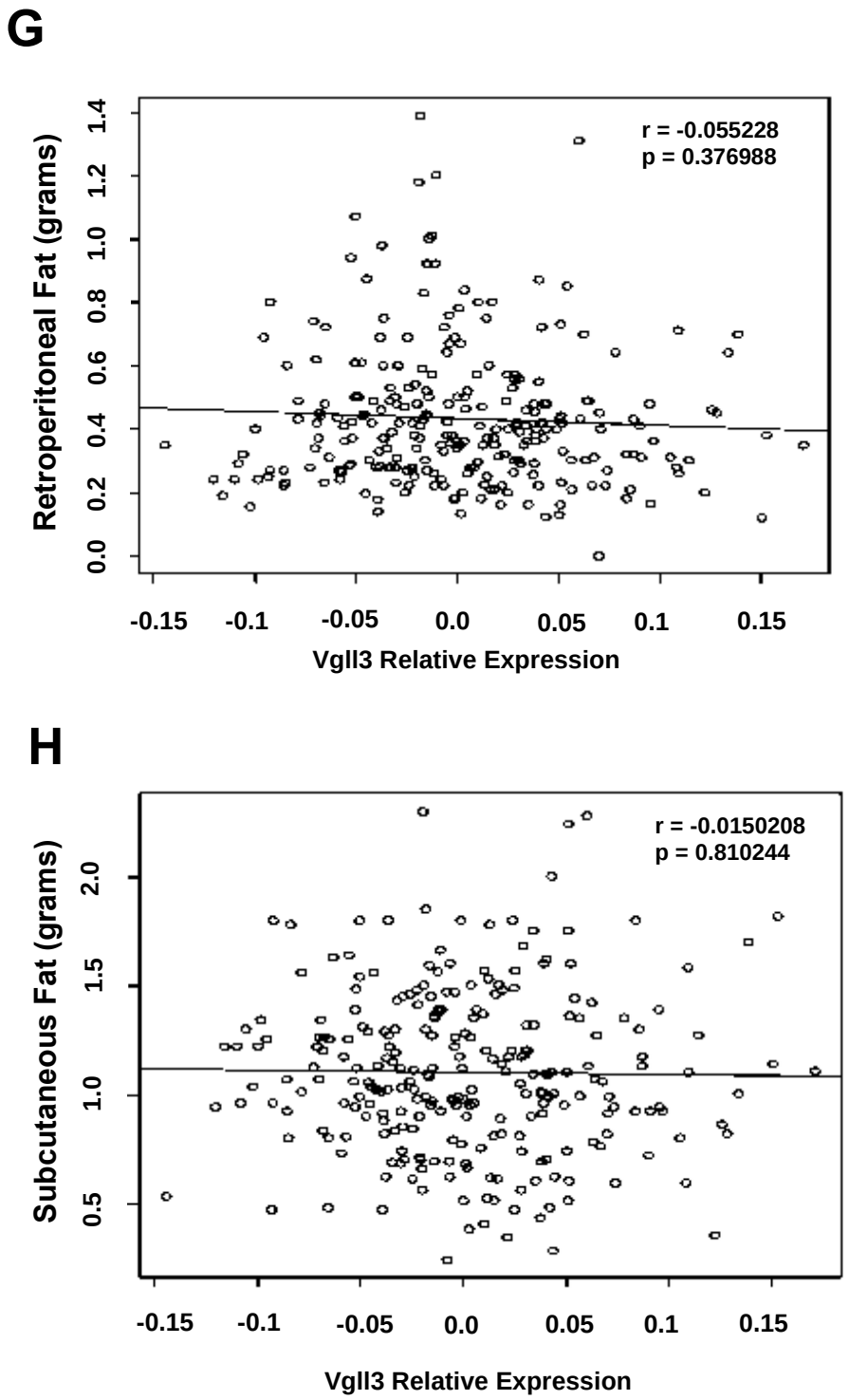


Figure 6

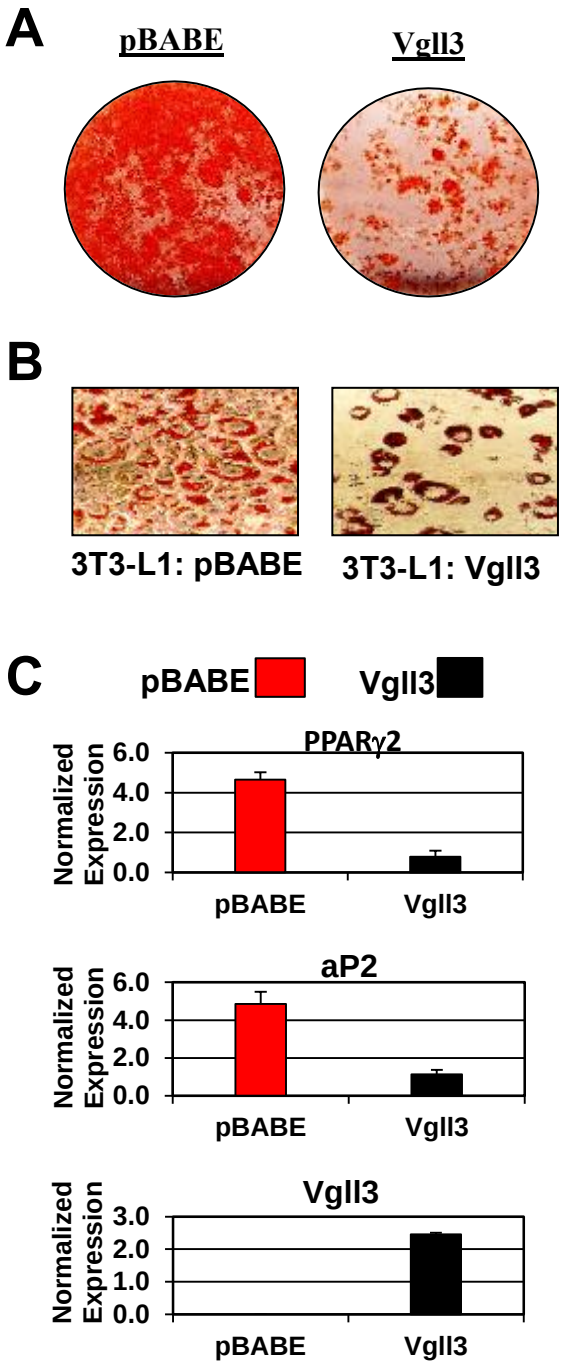

A
pBABE
Vgll3
B
3T3-L1: pBABE
3T3-L1: Vgll3
C
pBABE
Vgll3
PPARγ2
Normalized Expression
6.0
4.0
2.0
0.0
pBABE
Vgll3
aP2
Normalized Expression
6.0
4.0
2.0
0.0
pBABE
Vgll3
Vgll3
Normalized Expression
3.0
2.0
1.0
0.0
pBABE
Vgll3

Figure 6 continued

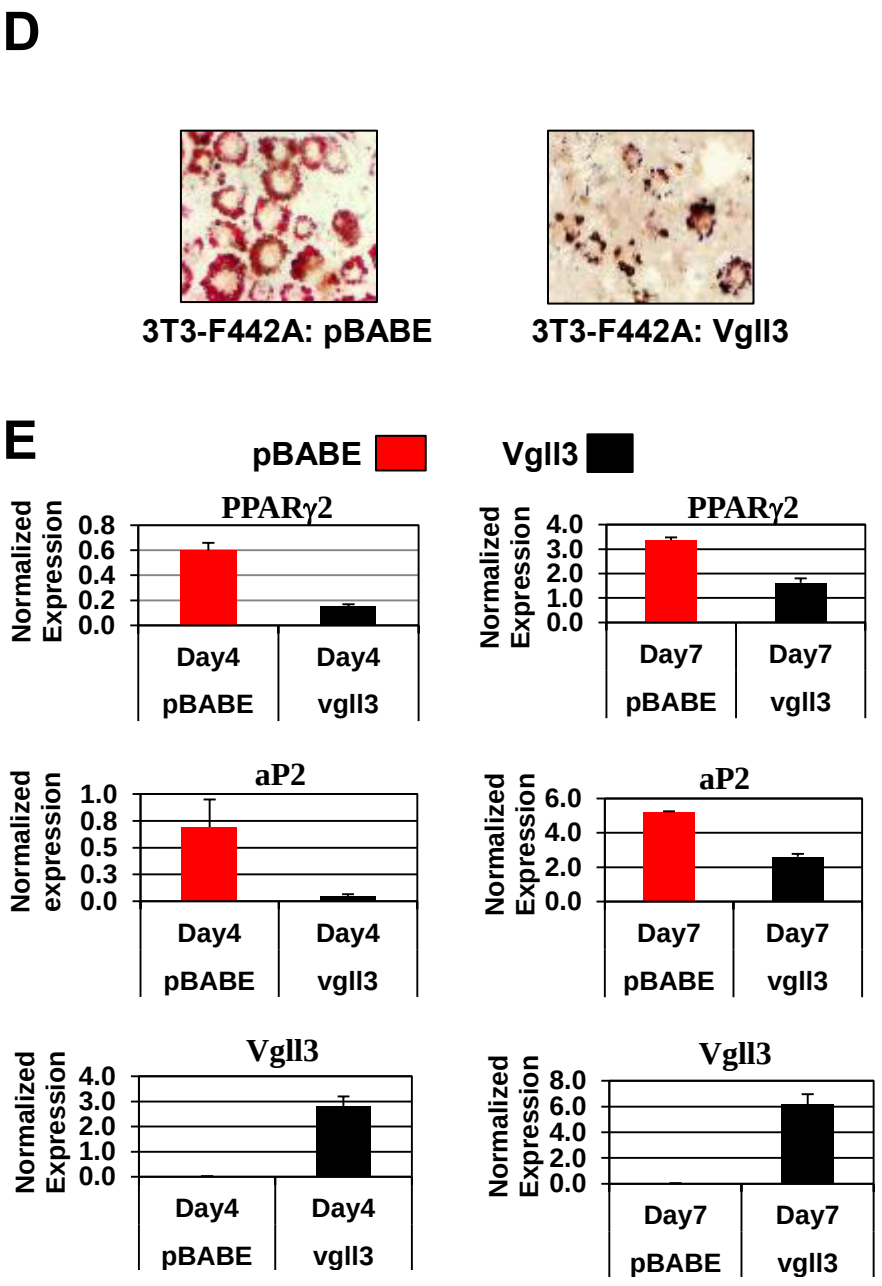


Figure 7

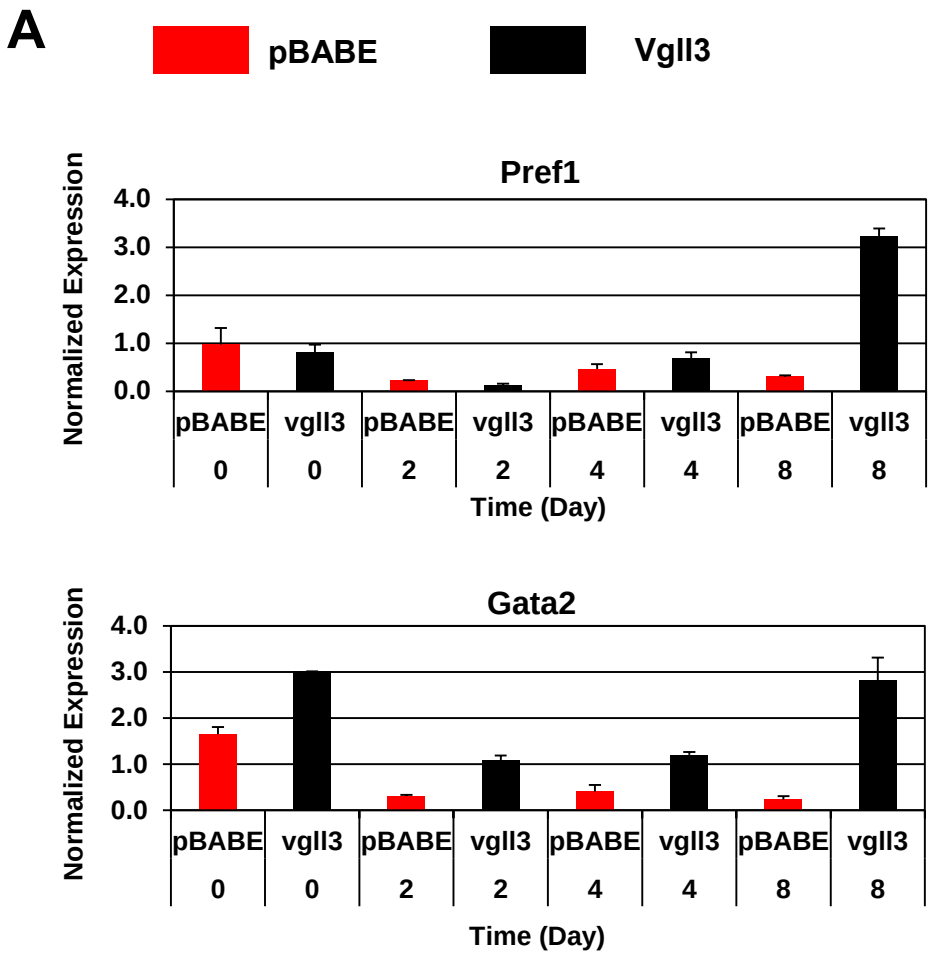

A
pBABE
Vgll3
Pref1
Normalized Expression
4.0
3.0
2.0
1.0
0.0
pBABE 0
vgll3 0
pBABE 2
vgll3 2
pBABE 4
vgll3 4
pBABE 8
vgll3 8
Time (Day)
Gata2
Normalized Expression
4.0
3.0
2.0
1.0
0.0
pBABE 0
vgll3 0
pBABE 2
vgll3 2
pBABE 4
vgll3 4
pBABE 8
vgll3 8
Time (Day)

Figure 7 continued

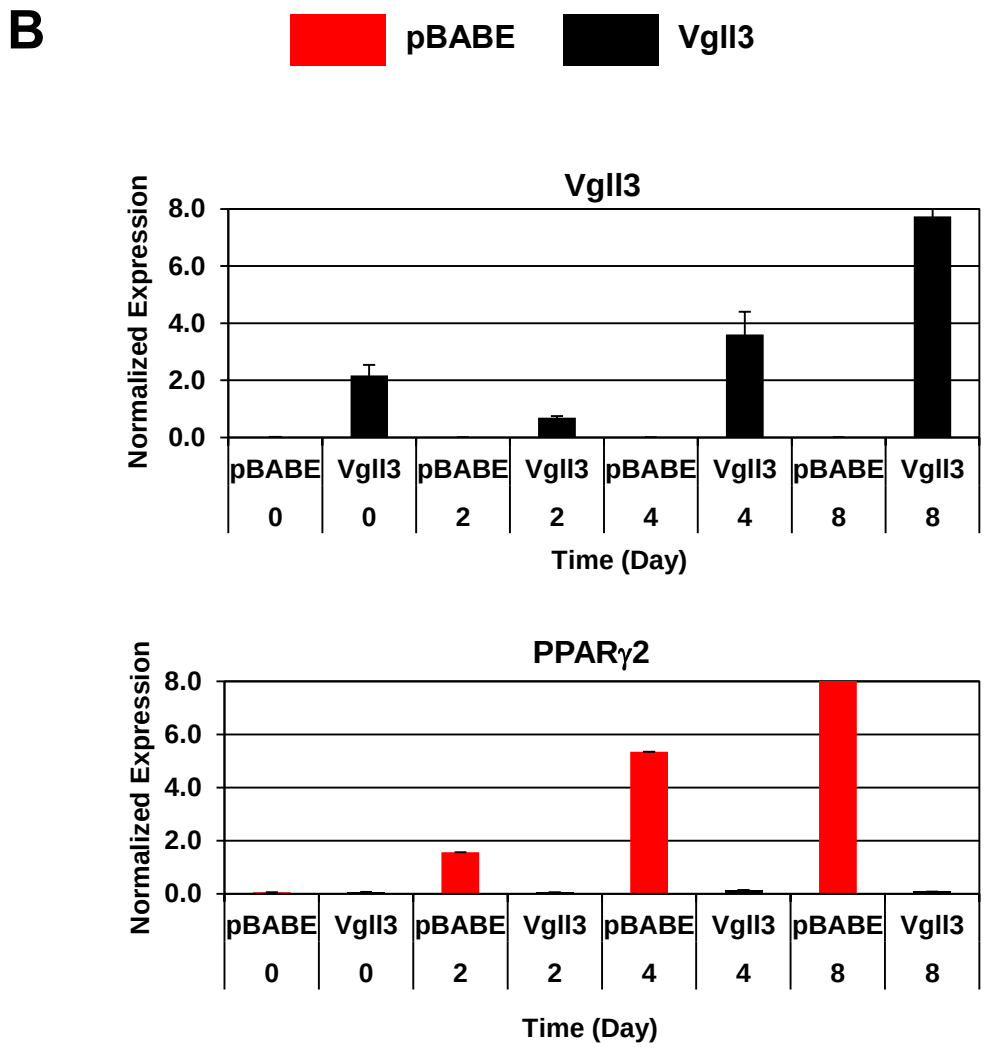


Figure 8

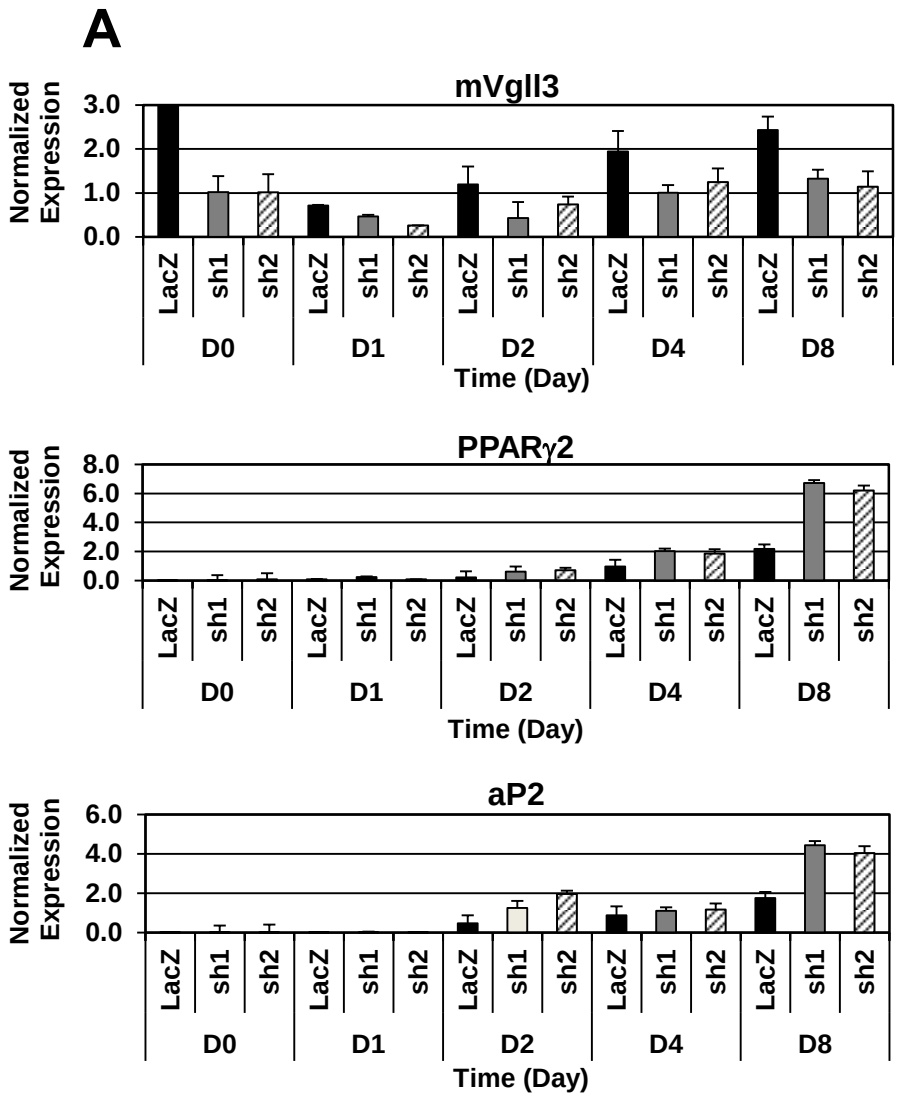

A
mVgll3
Normalized Expression
3.0
2.0
1.0
0.0
LacZ
sh1
sh2
D0
D1
D2
D4
D8
Time (Day)
PPARγ2
8.0
6.0
4.0
2.0
aP2

Figure 8 continued

B

Figure 9

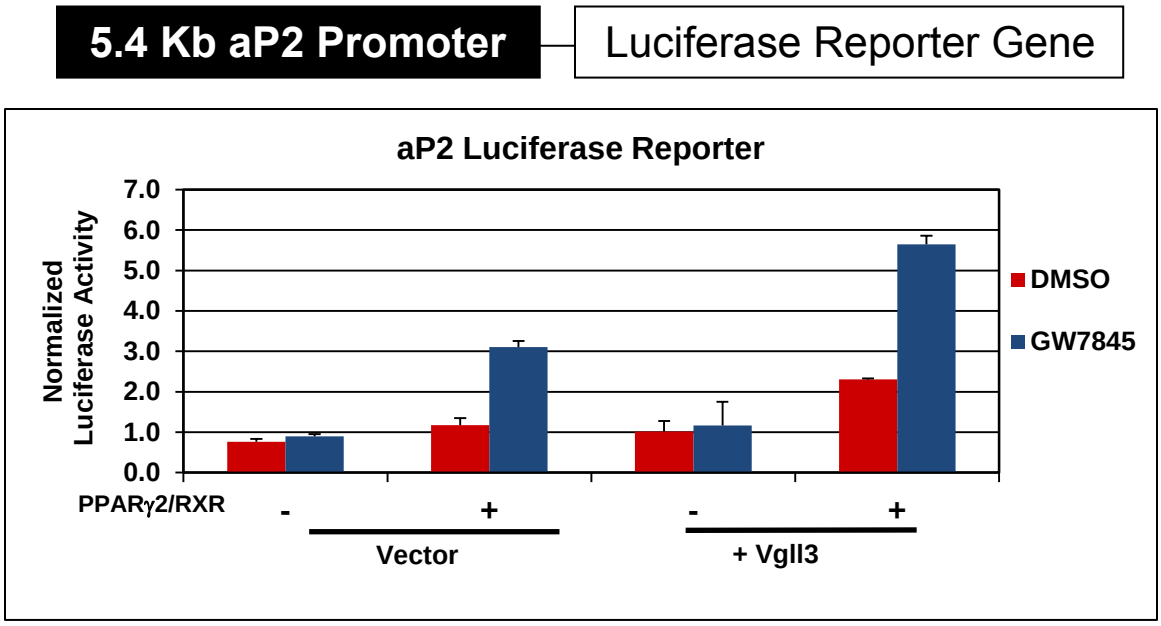

5.4 Kb aP2 Promoter
Luciferase Reporter Gene
aP2 Luciferase Reporter
Normalized Luciferase Activity
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
DMSO
GW7845
PPARγ2/RXR
-
+
-
+
Vector
+ Vgll3

Figure 10

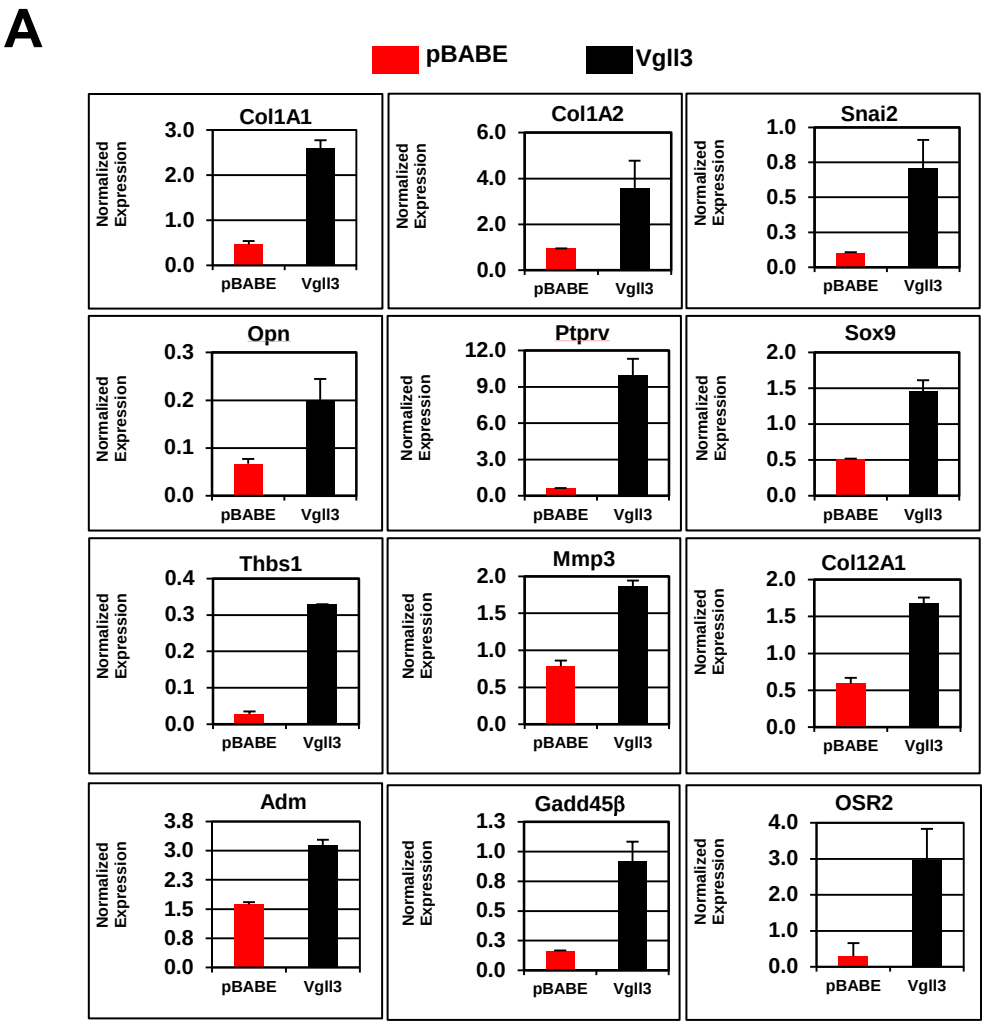
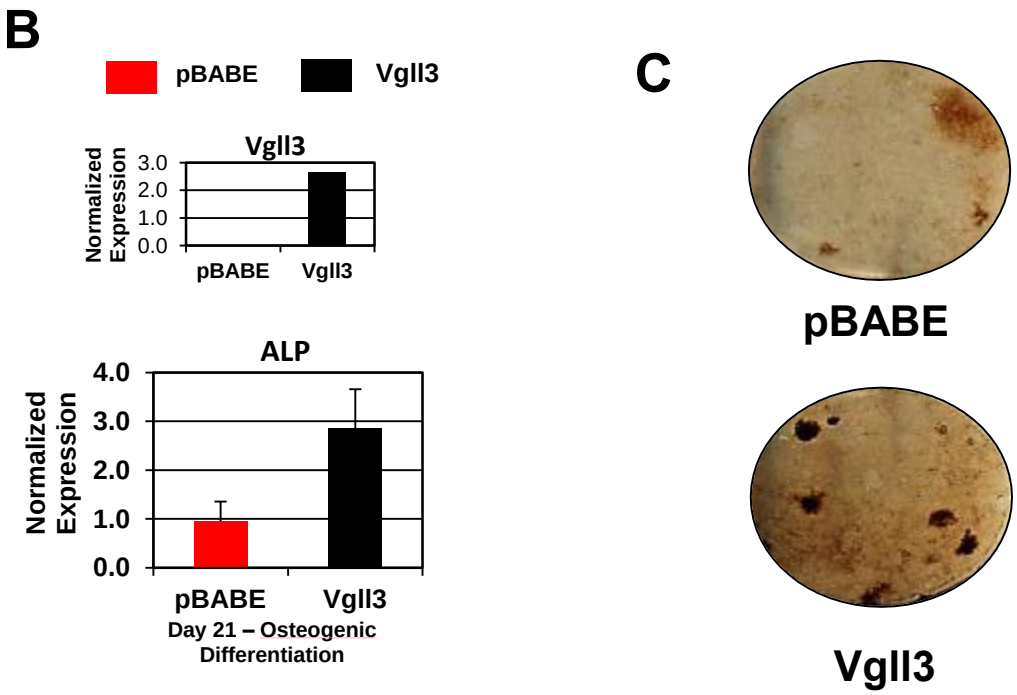

A
pBABE
Vgll3
Col1A1
Col1A2
Snai2
Opn
Ptprv
Sox9
Thbs1
Mmp3
Col12A1
Adm
Gadd45β
OSR2
Normalized Expression
pBABE
Vgll3

Figure 10 continued



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Chapter 4

Discussion

The enormity of the crisis in metabolic disease has come to represent a clarion call to vigorously uncover knowledge of the mechanisms that underlie adipocyte development and function. Looking forward into the future, groundbreaking new methods for treating metabolic disease will require innovative use and advancement of classic and modern *in vitro* and *in vivo* experimental techniques and approaches to answer key questions regarding basic adipocyte biology. For example, more specific understanding of the adipocyte developmental lineage will absolutely be important. More specifically, discovery of exactly when and where adipocyte precursors occur during embryonic development will represent a key milestone in the study of adipogenesis. Such knowledge will likely shed light on whether white and brown adipocyte precursors might share a common mesenchymal origin. In addition, this could also help to fully validate if and when the total number of adipocytes is definitively "set" and why different adipose depots seem to differentially express particular sets of genes.

The discovery of so-called "beige" adipocytes raises the provocative question as to whether it may be possible to modulate the efficiency of systemic energy metabolism. If so, the ability to therapeutically direct the loss of energy in the form of heat could in theory represent another clinical modality for use in combating the sharp societal incidence in obesity. Clearly, the study of beige adipocytes is only in its nascent stage and there are still many questions that need to be answered before therapeutically targeting this newly discovered cell population can become a real possibility. In particular, how similar are they to brown adipocytes? Can the production of beige adipocytes be activated specifically? How do beige adipocytes actually develop? Are there small molecules that can convert white fat cells into beige fat cells? In attempting to begin to address these fundamental questions, cyclooxygenase (COX)-2, a rate-limiting enzyme in prostaglandin synthesis, has been found to be necessary for the induction of

brown adipose tissue (BAT) in white adipose tissue (WAT) depots[1]. In addition, ectopic expression of COX-2 in WAT is able to induce the production of BAT, elevate systemic energy expenditure, and protect mice against high-fat diet-induced obesity. Moreover, it has also been recently reported that deletion of the prolactin receptor can cause reduced fat mass associated with the appearance of massive brown-like adipocytes that exhibit increased PRDM16 and PGC1α gene expression[2]. Therefore, such preliminary investigations and future studies like these may someday provide the basis for the precise manipulation of specific pathways that foster the formation of beige adipocytes.

The discovery of the endogenous ligand of PPARγ would also be a huge step in finding new ways to modulate adipogenesis. Indirect evidence of an endogenous ligand that is dependent on the increase in cAMP early in adipogenesis has been reported[3]. In addition, accumulated published data suggests that ADD1/SREBP1c, a pro-adipogenic transcription factor that regulates lipogenic genes, is possibly involved in endogenous ligand production. In one study, PPARγ transcriptional activity was observed to be increased when ADD1/SREBP1c was co-expressed with PPARγ using a reporter assay system[4]. In a subsequent study, conditioned media obtained from cultures of fibroblasts transfected with ADD1/SREBP1c was found to possess a factor(s) that binds to and activates a GAL4–PPARγ ligand binding domain fusion protein[5]. Thus, given the variety of known fatty acid-based PPARγ ligands, the endogenous ligand for PPARγ is likely to be a lipophilic, hydrophic molecule that is synthesized *de novo* in response to a rise in cAMP levels and mediated in part by ADD1/SREBP1c activity in differentiating pre-adipocytes.

Finally, uncovering novel factors and mechanisms that regulate adipogenesis is an essential part of furthering the knowledge of this critical biological process. In this thesis, two strategies were presented that sought to address this problem. In Chapter 2 of this thesis, generation of a transgenic mouse line expressing a LacZ reporter vector containing conserved non-coding genomic sequences was described. This was carried out in order to determine if these sequences might contain an enhancer capable of directing adipose-specific gene expression. However, no reporter gene expression or activity was detected in adipose tissues from mice determined to carry these sequences. Challenges were encountered during this investigation that likely contributed to the inability to detect reporter gene expression and/or activity in adipose tissue. First, out of three founder mice obtained, only one was able to mate and produce progeny. Second, it has been well-established that active promoters and enhancers located at specific sites in the genome are much more accessible compared to adjacent sequences that are restricted from transcriptional machinery due to the local remodeling of chromatin structure. Therefore, it is not possible to completely rule out whether the reporter vector may have been inserted into a transcriptionally inactive region of the mouse genome. Finally, using sequence comparisons to surmise experimental hypotheses regarding the location of functional enhancers has been recognized to have limitations[6]. For example, one study has found that genomic locations of binding sites for some key regulatory proteins (Oct4 and Nanog) are poorly conserved across the mouse and human genomes, despite their generally supposed fundamental importance as vital regulators of gene expression[7]. Thus, it is likely that not all functional regulatory elements correspondingly match with highly conserved sequences present in genomes of different species. Future studies will perhaps need to collectively integrate information from genome-wide studies utilizing approaches such as Chromatin Immunoprecipitation-Sequencing

(ChIP-Seq) and/or Dnase I HyperSensitive-Sequencing (DHS-Seq) in conjunction with cross-species sequence comparisons to more accurately predict where enhancers that regulate tissue-selective expression may reside.

In Chapter 3 of this thesis, transcriptional profiling of isolated sub-clones of the 3T3-F442A cell line was carried out. This uncovered a previously unidentified inhibitor of adipogenesis, Vestigial-like 3 (Vgll3). Ectopic expression of Vgll3 in 3T3-L1 cells was found to up-regulate genes associated with other mesenchymal-based cell fates. Interestingly, many reports have documented the ability of cells that derive from the mesenchymal lineage to be directed to alternative cell fates or to "transdifferentiate" under particular experimental conditions[8]. It has also been observed clinically that age-related osteoporosis is simultaneously associated with an elevated number of adipocytes in bone marrow[9]. A more complete understanding of the mechanisms that regulate the conversion between adipogenesis and osteoblastogenesis is now warranted given the expected higher incidence of osteoporosis in an increasingly aging population. In fact, one study has already shown that homozygous PPARγ-deficient embryonic stem (ES) cells fail to differentiate into adipocytes but do unexpectedly differentiate into osteoblasts[10]. Additionally, PPARγ haploinsufficiency was demonstrated to promote osteoblastogenesis *in vitro* and to enhance bone mass *in vivo*. It would be fascinating to ask whether Vgll3 could have any involvement in the promotion of osteoblastgenesis in PPARγ-deficient and/or haploinsufficient embryonic stem cells. Furthermore, it would be interesting to experimentally analyze ES cells and/or other multipotent stem cells constitutively expressing Vgll3. Other questions to be addressed in future studies include: what transcription factor(s) does Vgll3 co-activate? How is Vgll3 expression modulated in other cell differentiation programs? Do Vgll3 transgenic mice have reduced adiposity? Do Vgll3 knockout mice exhibit lower bone

density? This thesis has presented the discovery of Vgll3 as a gene that requires careful regulation in developing adipocytes. The data presented in this work now strongly suggests that Vgll3 should be considered as a potential factor that may facilitate critical cell fate decisions in the developing mesenchyme.

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