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# Transcriptional targets in adipocyte biology

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## Abstract

The global burden of metabolic disease demands that we develop new therapeutic strategies. Many of these approaches may center on manipulating the behavior of adipocytes, which contribute directly and indirectly to a host of disease processes including obesity and type 2 diabetes. One way to achieve this goal will be to alter key transcriptional pathways in fat cells, such as those regulating glucose uptake, lipid handling, or adipokine secretion. In this review we look at what is known about how adipocytes govern their physiology at the gene expression level, and we discuss novel ways that we can accelerate our understanding of this area.

## 1. Introduction

We have experienced an enormous increase in the prevalence of obesity and its attendant metabolic complications, including Type 2 diabetes (T2DM), dyslipidemia, and cardiovascular disease. Once a problem solely of wealthy nations, obesity has spread from the developed to the developing world, and for the first time in human history overnutrition has surpassed undernutrition as a global cause of morbidity and mortality[1]. Even our children are not immune, as >40% of U.S. children are now considered overweight or obese[2]. This situation has provoked intense study of all aspects of metabolism, including adipocyte biology.

Adipose tissue has long been recognized as a site for storage of excess energy derived from food intake. During fasting, adipocytes release energy in the form of fatty acids for other organs to consume. However, the discovery of leptin in 1994[3] suggested that adipose tissue could also function as an endocrine organ, and there are now thousands of publications that demonstrate important physiological roles for a wide variety of adipocyte-derived products[4-7]. These substances, often collectively referred to as adipocytokines, include leptin, adiponectin, retinol binding protein 4 (RBP4), in addition to several other factors. Not all of these factors are proteins; a recent study identifies the lipid palmitoleate as a 'lipokine' that shares some of the functional properties of the classic adipose-derived peptide hormones, and may be the prototype for more lipid-derived mediators yet to be discovered[8]. These adipokines exert a variety of effects on many aspects of nutrient homeostasis, including appetite, satiety, fatty acid oxidation, and glucose uptake. In addition, adipocytes secrete hormones that regulate non-metabolic processes such as immune function, blood pressure, bone density, reproduction, and hemostasis.

Given the important role played by adipocytes in the regulation of systemic energy balance and nutrient homeostasis, it is reasonable to imagine that these cells might be therapeutic targets for metabolic diseases. In fact, the antidiabetic thiazolidinedione (TZD) drugs provide proof-of-concept for such an approach. TZDs exert their beneficial effects on hyperglycemia and insulin resistance by activating a specific transcription factor, the nuclear receptor peroxisome

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proliferator-activated receptor γ (PPARγ)[9]. There is debate about which cell acts as primary mediator of the antidiabetic effects of TZDs, but it seems clear that PPARγ in adipocytes account for at least part of their actions. PPARγ is a master regulator of adipogenesis and adipocyte biology, and virtually all aspects of adipose metabolism are affected directly or indirectly by TZDs. These agents improve adiponectin secretion, promote lipogenesis, block lipolysis, and enhance insulin-stimulated glucose uptake. In fact, genome-wide location analysis of PPARγ in mature 3T3-L1 adipocytes identifies binding sites near an extremely large number of metabolically relevant genes[10, 11].

While TZDs have been an important addition to our armamentarium for metabolic disease, they are not always highly effective. Furthermore, they can induce adverse effects such as edema and coronary events in some cases[12]. This indicates that additional options are needed for therapy; at least part of the reason that we don't have such options is that we don't fully understand the transcriptional pathways that fat cells use to control their metabolic actions.

An important point that needs to be mentioned: the goal is not to prevent adipogenesis, to enhance fat cell apoptosis, or to otherwise reduce fat cell number. Adipocytes serve as a safe place to store excess calories. Animals and humans who have a reduced ability to make fat cells are lipodystrophic, and they suffer from a range of undesirable effects including ectopic lipid deposition in muscle and liver, severe insulin resistance, and cirrhosis[13]. We need our fat cells. The goal is to manipulate adipocytes in selective ways that promote health.

In this review, we will look at some of the major metabolic functions of fat cells, with an emphasis on the transcriptional pathways that regulate these processes. We will then discuss newer strategies that are being brought to bear on this issue, which we believe will result in a broader understanding of how adipocytes regulate their own behavior, and may ultimately provide novel targets for drug therapy.

## 2. Key Functional Pathways in Adipocytes

Fat cells perform a wide array of functions that affect systemic metabolism. In this section, we review the major pathways that regulate these functions with emphasis on what is known about the transcriptional regulation of key genes within those pathways.

### 2.1. Adipogenesis

Adipogenesis, or the formation of new fat cells, is not really a physiological function in the strict sense of the term, but it is worth reviewing briefly because most of the key adipogenic transcription factors also play important roles in the functions of mature adipocytes. PPARγ, C/EBPα, KLFs, and other pro-and anti-adipogenic transcription factors (discussed below) are not simply required for initiating and regulating the transcriptional cascade that regulates the development of new fat cells. These same factors regulate the expression of key metabolic enzymes, signaling components, and adipocytokines in the mature fat cell, and thus maintain the differentiated state by enabling the full range of functions performed by fat cells.

It is worth making the point, however, that adipogenesis *per se* does not contribute to obesity, except in the most literal sense that one cannot be obese without differentiated fat cells. However, obesity cannot be caused by excessive adipogenesis. This is because obesity represents an excess of stored calories, which results from an imbalance between energy taken in and energy burned. This simple restatement of the First Law of Thermodynamics makes it clear that simply making more fat cells does not cause excess adiposity; a theoretical doubling of fat cell number without concomitant changes in food intake or energy expenditure would result in a fat pad containing twice as many cells of half the size.

Adipocyte differentiation is regulated by a few key signaling pathways that exert their actions on a cascade of transcription factors. Pro-adipogenic extracellular signals include insulin and FGF, while the Wnt and hedgehog pathways are anti-adipogenic. Detailed reviews of the adipogenic transcriptional cascade have been recently published[14-16], but we will review a few critical players here.

**2.1.1. Peroxisome proliferator-activated receptor γ (PPARγ)—**The nuclear hormone receptor PPARγ (NR1C3) is as close to a master regulator of adipogenesis as we are likely to discover. Virtually all known activators and inhibitors of adipogenesis act at least in part by regulating the expression or activity of PPARγ, and cells that lack PPARγ cannot be converted into adipocytes even when other powerfully pro-adipogenic factors are ectopically expressed. For example, overexpression of C/EBPα or EBF1 cannot compensate for the absence of PPARγ in PPARγ$^{-/-}$ fibroblasts[17, 18]. PPARγ is the only transcription factor that is absolutely necessary and sufficient for adipogenesis[19-22].

In cultured cell models of adipogenesis, PPARγ mRNA is directly induced by factors that act early in the cascade, such as C/EBPβ, C/EBPδ, EBF1, and KLF5. Conversely, early repressors of adipogenesis such as GATA2, KLF2, and CHOP act in part by reducing PPARγ expression. Other factors, such as SREBP1c and the enzyme XOR may act to increase PPARγ activity (as opposed to actual mRNA or protein levels), perhaps by regulating production of a still elusive endogenous ligand[23-25]. PPARγ itself induces the expression of transcription factors that induce genes of terminal differentiation, including lipid handling enzymes and other mediators of adipocyte physiology. Additionally, PPARγ itself directly binds to regulatory regions flanking the majority of these same genes, in a classic example of a 'feed-forward' loop.

The study of PPARγ *in vivo* has been hampered somewhat by the confounding fact that this protein is required for normal placentation; PPARγ$^{-/-}$ embryos thus die relatively early in embryogenesis[19, 20]. Nonetheless, a requirement for PPARγ in adipogenesis *in vivo* has been ascertained from several studies involving chimeric animals, or mice in which placentation was rescued using tetraploid aggregation[19, 21]. Furthermore, humans with dominant negative mutations in PPARγ suffer from lipodystrophy[26], confirming a role for this factor in our own species as well.

There are two major isoforms of PPARγ, designated PPARγ1 and PPARγ2, which are formed by alternative promoter and first exon usage. Both proteins are induced during adipocyte differentiation, though PPARγ2 appears to be more adipose-specific. There are conflicting data on the relative importance of these two isoforms. Selective knockdown of PPARγ1 and PPARγ2 promoters using engineered zinc finger proteins suggested that only PPARγ2 can induce adipogenesis[27], yet others showed that both isoforms are more or less equal in their ability to induce adipogenesis in PPARγ$^{-/-}$ MEFs[28]. The story is no clearer *in vivo*, as one study of PPARγ2 selective knockout mice showed decreased fat mass with impaired adipogenesis while another study found normal adiposity[29, 30].

**2.1.2. CCAAT-enhancer-binding proteins (C/EBPs)—**Multiple members of the bZIP family of CCAAT-enhancer-binding proteins (C/EBPs) are involved in adipogenesis. C/EBPβ, C/EBPδ, and C/EBPα are all pro-adipogenic, while C/EBPγ and CHOP repress differentiation[31]. C/EBPβ and C/EBPδ are expressed at very early stages of differentiation, and together they induce expression of both C/EBPα and PPARγ. C/EBPα and PPARγ have an important synergistic relationship. First, they induce each other's expression in a mutually reinforcing positive feedback loop that maintains the differentiated state. In addition, both factors bind to a highly overlapping complement of target genes at locations often quite close to one another[10, 11]. C/EBPα is therefore of particular importance in adipogenesis and adipocyte physiology. Nonetheless, it remains true that PPARγ can promote differentiation in

the absence of C/EBPα (although the resulting adipocytes are insulin resistant; see below)[32], but the converse is not true[18].

**2.1.3. Krüppel-like factors (KLFs)—**The Krüppel-like factors (KLFs) are a large family of Cys2/His2 zinc finger DNA-binding proteins related to the *Drosophila melanogaster* segmentation gene product, Krüppel. KLFs are important regulators of erythropoiesis, T cell activation, vascular development, lung development, and skin development. Many KLF proteins are expressed in adipose tissues, and they show a wide variety of expression profiles during adipocyte differentiation. In keeping with this, several KLFs have been shown to play an active role in adipogenesis. These include KLF2 and KLF7, which negatively regulate adipogenesis[33, 34]. On the other hand, KLF3, KLF4, KLF5, KLF6, and KLF15 all promote adipogenesis to one degree or another[35-39]. One intriguing feature of the relationship between KLFs and adipocyte differentiation is the transitional nature of the expression of each factor. Some factors, like KLF4, act early in the transcriptional cascade, while KLF15 is induced quite late. KLF5 occupies an intermediate position in the cascade, after C/EBPβ and C/EBPδ induction but prior to PPARγ. Target genes for the KLFs in adipocytes are not clearly defined, although KLF15 is known to activate Glut4 directly[40], and KLF5 induces PPARγ [36]. Much remains to be discovered about the role of KLF action in adipocyte biology.

**2.1.4. Early B cell factors (EBFs)—**Early B cell factors (EBFs) are atypical helix-loop-helix factors important for the control of B lymphocyte specific genes and for the transcriptional regulation of genes in olfactory receptor neurons. Mammals have four EBF proteins encoded by distinct genes, designated EBF1-4. EBF1, 2 and 3 are expressed in adipose tissue, with increasing levels seen as differentiation progresses. EBF1 stimulates adipogenesis in NIH-3T3 fibroblasts, and expression of a dominant-negative EBF-fusion protein blocks 3T3-L1 differentiation[41]. Knockdown experiments showed that EBF1 and EBF2 are required for adipogenesis[17]. C/EBPα and PPARγ have been showed to be direct EBF targets[17], but similar to KLFs, we still lack a comprehensive understanding of their target genes.

**2.1.5. GATA factors—**GATA transcriptional factors play important roles in a variety of developmental processes. GATA2 and GATA3 are specially expressed in mouse preadipocytes, and the expression and GATA2 and GATA3 decrease during adipogenesis[42]. Overexpression of GATA2 and GATA3 in preadipocytes inhibits terminal differentiation into mature adipocytes. GATA3-deficient embryonic stem cells have enhanced adipogenic capacity. These effects are mediated through the direct suppression of PPARγ expression as well as via inhibition of C/EBPα through protein-protein interactions[42, 43].

**2.1.6. Forkhead proteins—**Several members of the forkhead family of winged helix proteins are expressed in adipose tissue, and many have been shown to have significant effects on adipogenesis and adipose physiology. The FoxO proteins FoxO1, FoxO3a, and FoxO4 are all induced during adipogenesis, although forced expression of FoxO1 inhibits the process; conversely, a dominant negative FoxO1 promotes differentiation[44]. Overexpression of the same dominant negative protein in adipose tissue in vivo causes the formation of smaller adipocytes associated with enhanced glucose tolerance and insulin sensitivity[45]. FoxA2 is also expressed in adipose tissue, and this is dramatically enhanced by high-fat feeding. FoxA2 has divergent effects on differentiation, which it inhibits, and on metabolism in mature adipocytes[46]. Expression of FoxA2 in mature cells induces Glut4, HSL, and other adipocyte-specific genes. FoxC2 is another interesting family member. When expressed in preadipocytes, FoxC2 blocks differentiation[47, 48], but in mature cells FoxC2 overexpression sensitizes adipocytes to β-adrenergic signaling, thus promoting lipolysis and leanness[49].

## 2.2. Lipogenesis

Lipogenesis refers to the process by which fatty acids are synthesized de novo and the subsequent esterification of fatty acids into triglycerides. Fatty acids can also be imported into fat cells from the circulation to be used as substrate for triglyceride accumulation, and for the sake of simplicity we consider the process of fatty acid transport to be part of the overall lipogenic pathway. Lipogenesis is often confused with adipogenesis because lipid accumulation is often used as a marker of fat cell differentiation. Nevertheless, it is important to make the distinction as one can see lipid deposition in cells without concomitant expression of other genes that mark adipocytes. Conversely, it is possible to promote adipogenesis without allowing lipid accumulation, for example, by limiting the supply of biotin (which acts as an essential cofactor of the rate-limiting enzyme of lipogenesis, acetyl CoA carboxylase). Species differences can also be important. In humans, de novo lipogenesis occurs primarily in the liver, with a relatively small contribution from adipose tissue. Rodents, however, generate a higher percentage of lipid directly in adipose tissue[50].

Lipogenesis in adipocytes is activated by a high carbohydrate supply and by the actions of insulin[51]. The biosynthesis of fatty acids involves acetyl-CoA transport across the inner mitochondrial membrane into cytoplasm followed by conversion to malonyl-CoA by the multifunctional polypeptide acetyl-CoA carboxylase (ACC). Then the fatty acid synthase (FAS) complex performs a series of enzymatic reactions that generates C16 palmitate. Longer chains are synthesized and double bonds are introduced by a variety of microsomal enzymes. Fatty acids thus produced are incorporated into triglycerides by a series of reactions catalyzed by enzymes such as glycerol 3-phosphate acyltransferase (GPAT) and several isoforms of acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase (AGPAT), diacylglycerol:acyl-CoA acyltransferase (DGAT) and others. Fatty acids can also be imported into adipocytes from the plasma using both passive mechanisms as well as via protein carriers called fatty acid transport proteins (FATPs).

Adipocyte lipogenesis is regulated by the nutritional environment. In general, feeding promotes lipogenesis, and fasting reduces it. Glucose and insulin both promote lipid formation, while polyunsaturated fatty acids (PUFAs) repress lipogenesis.

**2.2.1. Sterol regulatory element binding proteins (SREBPs)—**A number of studies have shown that lipogenesis is mediated in part by a transcription factor called sterol regulatory element binding protein 1c (SREBP1c; also known as adipocyte determination and differentiation factor 1 (ADD1)). SREBPs belong to the basic helix-loop-helix-leucine zipper class of transcription factors, and are represented by three isoforms: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1c is the form most highly expressed in adipocytes, and plays an important role in the control of genes involved in the biosynthesis of fatty acids, such as fatty acid synthase. Specifically, studies *in vitro* have identified SREBP1c as an important mediator of the effect of insulin on *de novo* lipid synthesis[52]. Surprisingly, the study of SREBP-1c knockout mice showed normal fat mass, and no alteration of lipogenic gene expression[53]. These results suggest that SREBP1c may be more important for nutritional changes in lipid synthesis, as compared to basal lipid synthesis. Furthermore, massive overexpression of SREBP-1c under the control of aP2-promoter induces severe lipodystrophy[54]; these discrepancies remain unresolved.

**2.2.2. PPARγ—**PPARγ was discussed earlier as a key regulator of adipocyte differentiation, and as mentioned, it also plays an important role in the maintenance of the differentiated state. This includes the expression of lipogenic genes, and PPARγ binding sites have been discovered in the flanking sequence of a large number of lipogenic enzymes and regulators[10, 11]. This

includes proteins involved in lipid transport and intracellular shuttling, such as FABPs and FATPs. Consistent with this, TZDs promote lipid accumulation in mature adipocytes.

**2.2.3. Liver X receptor (LXR)—**LXRs are nuclear hormone receptors that affect cholesterol metabolism and lipid biosynthesis. There are two LXR genes that encode the factors LXRα and LXRβ. LXRα is expressed in liver, spleen, kidney, small intestine and adipose tissue, while LXRβ is more widely expressed. LXR agonists increase lipid accumulation in fat[55], and LXRα/β null mice have exhibit reduced lipid accumulation[56].

**2.2.4. Carbohydrate response element binding protein (ChREBP)—**ChREBP is another member of the basic helix-loop-helix family of transcription factors. Originally discovered as a sucrose-responsive factor that promotes lipid accumulation in liver, ChREBP is also expressed in adipocytes. There are limited data available on ChREBP in fat, but it appears to be induced by glucose, insulin, and TZDs, and repressed by fatty acids[57].

### 2.3. Lipolysis

Lipids deposited in adipose tissue during periods of nutrient availability must be broken down and released as fatty acids to supply other tissues during periods of fasting. This process involves the sequential deesterification of fatty acids from the glycerol backbone by a succession of lipases followed by release of glycerol and free fatty acids (FFAs) into the circulation. FFAs and glycerol are substrates for gluconeogenesis and ketogenesis, respectively, in the liver, and FFA is used by skeletal muscle and heart as an energy source[58].

The conversion of triacylglycerol (TAG) to diacylglycerol (DAG) is performed primarily by the recently discovered adipocyte triglyceride lipase (ATGL)[59]. The best-known lipase involved in lipolysis is hormone sensitive lipase (HSL), which converts DAG to monoacylglerol (MAG), which is in turn hydrolysed by monoglyceride hydrolase (MGH). Other enzymes may also participate in lipolysis in adipocytes, such as carboxyesterase 3, also known as triglyceride hydrolase (TGH).

Historically, the metabolic control of lipolysis has been believed to be strictly under the control of signal transduction cascades that respond quickly to a change in nutritional status, rather than regulation at the level of gene expression. The classic example is HSL, which is phosphorylated in fasting by protein kinase A (PKA), leading to translocation of HSL from the cytosol to the lipid droplet. More recent studies, however, suggest that other lipolytic enzymes, such as ATGL, may be subject to nutritional regulation of gene expression. The expression of ATGL, for example, is sharply repressed by insulin[58], which likely accounts for some of the anti-lipolytic actions of that hormone.

Another way that lipolysis is regulated transcriptionally is by the induction of glycerol kinase (GyK) by TZDs in isolated rodent adipocytes. GyK catalyzes the phosphorylation of glycerol to generate glycerol-3-phosphate, which is the first step in the resynthesis of acylglycerols. GyK was long believed to be absent in adipocytes, consistent with the idea that such activity would lead to futile cycling. Lazar and colleagues showed that GyK is abundantly expressed when PPARγ is activated, resulting in reduced FFA release[60]. Contrary data exist in human adipocytes, however, suggesting that GyK activation by TZDs does not contribute to the beneficial effects of these agents in our species[61].

Lipolysis can also be stimulated by activation of PPARδ in adipocytes, either by adding a PPARδ-specific ligand or by expressing a pre-activated PPARδ allele[62]. Mice expressing PPARδ fused to the viral activating protein VP16 specifically in adipocytes are protected from obesity, and ectopic expression of the same fusion protein in cultured adipocytes increases both

lipolysis and fatty acid oxidation[63]. Interestingly, global PPARδ knockout mice are more prone to obesity than wild-type controls, but adipocyte-specific knockout mice do not show this effect[64]. One way to reconcile these data is to suggest that PPARδ is not required for basal lipolysis in adipocytes, but activation of this receptor can cause enhanced lipid hydrolysis and oxidation. In this scenario, increased adiposity in the global knockouts would reflect altered PPARδ-mediated fatty acid oxidation in liver, muscle, and possibly other non-adipose tissues. It has been proposed that the cannabinoid receptor CB1R may be a relevant target of PPARδ in white adipose tissue[62]. PPARδ represses CB1R expression, and CB1R is antilipolytic. This is a plausible pathway but there is little direct experimental evidence to support it at this time.

### 2.4. Insulin-stimulated glucose uptake

The ability to take up glucose in an insulin-dependent manner is a cardinal feature of adipocytes. This process involves a signal transduction cascade beginning with the insulin receptor and culminating with the translocation of Glut4-containing vesicles to the cell surface. The number of proteins known to be involved in insulin signaling is large and ever-growing, but relatively little is known about the transcription factors that regulate the expression of these proteins in adipocytes.

Adipocytes (along with muscle cells) are unique in utilizing the specialized glucose transporter Glut4, which is normally located in vesicles below the cell surface in the basal state. In the presence of insulin, these vesicles translocate to the cell surface. Insulin resistant humans show a significant reduction in adipocyte Glut4 expression, the cause of which is uncertain. This can be reversed by treatment with TZD. Transcription of Glut4 mRNA is regulated by a number of transcription factors implicated in cell metabolism, including C/EBPα, KLF15, SREBP-1c, MEF2C, NF-1, EBF1 and LXR[40, 65, 66]. Other factors directly inhibit Glut4 expression, including IRF4[67].

One interesting insight was obtained by Wu, et. al., who discovered that cells lacking C/EBPα could be induced to differentiate by expressing PPARγ [32]. Despite their morphological appearance as adipocytes, however, these cells lacked normal insulin-stimulated uptake. Part of the defect was traced to the fact that Glut4-containing vesicles were inappropriately located at the cell surface in the absence of insulin, suggesting that C/EBPα may be required for the expression of a key (and as yet undetermined) intracellular vesicle tethering protein[68]. C/EBPα also regulates the expression of several other genes required for insulin-stimulated glucose uptake, including the insulin receptor itself, as well the signaling intermediate IRS-1[69].

### 2.5. Adipokine secretion

As mentioned earlier, the ability of adipocytes to secrete hormones that coordinate a wide variety of metabolic functions throughout the body has only been recently appreciated. The expression of several of these adipokines is regulated at the transcriptional level by fasting and feeding, by obesity, and by drugs like TZDs.

**2.5.1. Leptin—**The anorexic adipokine leptin is expressed almost entirely by adipose tissue. Leptin performs a wide variety of functions, but the best understood is its role in the regulation of appetite and satiety. Leptin binds to its receptor and activates JAK/STAT signaling in target neurons in several parts of the brain, including the arcuate nucleus of the hypothalamus[70]. Leptin activates pro-opiomelanocortin (POMC) neurons, which produce the anorexic peptide α-melanocyte-stimulating hormone (αMSH), and conversely suppresses the expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP) in orexigenic NPY/AgRP neurons[70]. Serum leptin levels positively correlate with the mass and lipid content of adipose tissues in rodents and humans. Importantly, obese individuals have elevated serum leptin levels

but simultaneously exhibit an impaired leptin response, termed leptin resistance. The expression of leptin is also acutely suppressed by fasting and is activated by refeeding, outside of changes in adiposity.

Amazingly, despite thousands of papers written about leptin, we know very little about the specific transcription factors and pathways that regulate its expression. There is a C/EBP site in the proximal leptin promoter, although this is insufficient to account for the high degree of adipose specificity of leptin expression[71]. Similarly, nutritional regulation of leptin expression is at least in part mediated by insulin signaling, which acts through the activation of PI3K and MAP kinase in brown adipocytes[72]. It has been suggested that the transcription factors SREBP1c and Sp1 mediate the insulin effect on leptin transcription[52, 73]. Leptin is also induced by chronic exposure to glucocorticoids and inflammatory cytokines[74]. Acute elevation of glucocorticoid, however, does not have an effect on leptin expression[75].

**2.5.2. Adiponectin—**This adipokine is highly tissue-restricted to adipocytes, and circulates in the plasma in various higher-order multimeric complexes[4, 7]. Adiponectin promotes glucose uptake and utilization as well as fatty acid oxidation in muscle. It also inhibits gluconeogenesis in liver. These actions are mediated through the binding of two seven-transmembrane receptors and subsequent activation of 5'-AMP-activated protein kinase (AMPK). In contrast to leptin, serum adiponectin levels are positively correlated with insulin sensitivity and negatively correlated with obesity; the latter effect is paradoxical given the large increase in adipose mass in the obese state. Also unlike leptin, serum adiponectin concentration is induced by fasting and reduced by feeding[76]. Adiponectin expression is also modulated by a variety of cytokines and hormones[77-79]. For example, TNFα and IL-6 suppress adiponectin transcription whereas IGF-1 induces adiponectin expression. Glucocorticoids and β-adrenergic agonists also down-regulate adiponectin transcription, suggesting one mechanism by which these drugs may cause insulin resistance.

Adiponectin gene expression is regulated by a variety of transcription factors, many of which have been implicated in adipogenesis. For example, the pro-adipogenic transcription factors PPARγ [80], C/EBPα [81, 82], and SREBP1c[83] activate adiponectin expression whereas the anti-adipogenic factors KLF7[33] and Id3[84] inhibit adiponectin promoter activity. Other factors that act on the adiponectin promoter have also been discovered, including the nuclear factor of activated T-cells c4 (NFATc4)[82], activating transcription factor 3 (ATF3)[82], nuclear factor Y (NF-Y)[81] and liver receptor homolog-1 (LRH-1)[85]. While LRH-1 alone does not induce adiponectin expression, it enhances the effect of PPARγ on adiponectin transcription. Treatment with a TZD causes results in increased serum adiponectin levels, although this effect is only partly mediated by enhanced transcription; PPARγ also enhances the secretion of preformed adiponectin.

**2.5.3. RBP4—**Retinol binding protein-4 (RBP4) is a protein secreted by liver and adipose tissue. Its role in metabolism was first suggested by a search for adipokines whose expression was altered in response to the changes in adipose Glut4 expression. RBP4 levels are upregulated in adipose Glut4$^{-/-}$ mice and down-regulated in adipose-Glut4 overexpressers[86]. The serum level of RBP4 is elevated in multiple insulin-resistant mouse models due to elevated expression of RBP4 specifically in adipose tissue. RBP4 transgenic mice or mice injected with recombinant RBP4 display insulin resistance, whereas RBP4 heterozygous or knockout mice are more insulin-sensitive, indicating that circulating RBP4 may cause insulin resistance[86]. Studies in humans also support the notion that elevated RBP4 level is correlated with insulin resistance[87]. Preferentially expressed in visceral fat, RBP4 expression is dramatically elevated in obese subjects. There are now a large number of papers looking at the connection between RBP4, obesity, and insulin resistance in various human populations using a variety of technical approaches; not all of these studies have confirmed the proposed relationship (summarized

in[88]). Some of the discrepancies may be due to shortcomings in available commercial assay techniques[89].

There is little known about the transcriptional pathways regulating RBP4. Retinoids reduce expression of RBP4 in fat[90], as does the PPARα ligand fenofibrate[91], although the specific mechanisms involved are still murky.

## 3. Brown fat: a cell of a different color

All of the functions mentioned above were discussed in the context of white fat, which is the form of adipose tissue that accumulates in obesity. There is another type of fat cell, however, called the brown adipocyte, which is distinguished from white cells by their enhanced metabolic rate associated with increased numbers of mitochondria and the presence of uncoupling protein-1 (UCP-1). UCP-1 dissipates the proton gradient that accumulates across the mitochondrial membrane during electron transport; this results in energy expenditure in the form of heat loss. Animals without UCP-1 are prone to obesity, and ectopic expression of UCP-1 in white adipocytes has a strong anti-obesity effect. This has led to interest in the idea that 'trans-differentiation' of white fat to brown could have therapeutic implications.

Brown adipogenesis is controlled by a factor called PRDM16, which acts as a coactivator of PPARγ[92]. Overexpression of PRDM16 in white fat precursors leads to the acquisition of a brown cell phenotype, complete with UCP-1 expression[93]. Conversely, RNAi-mediated reduction of PRDM16 in brown adipocytes causes the loss of the brown phenotype. Interestingly, PRDM16 regulates an important development switch involving fat in vivo, but it is not a brown-white switch. Rather, PRDM16 appears to control whether a cell becomes a muscle cell or a brown adipocyte. Mice lacking PRDM16 have abnormal brown fat with enhanced muscle gene expression, and the presence or absence of PRDM16 in cultured myoblasts determines which pathway they follow[94].

Other transcriptional regulators are also involved in the full expression of the brown fat phenotype, including pRB, which is reduced in brown fat[95], and PGC-1α, which promotes expression of a variety of brown fat genes, particularly those involved in mitochondrial biogenesis and oxidative phosphorylation[96].

## 4. Identifying novel transcriptional pathways in adipocytes

It is worth considering how we have learned what we know about transcriptional pathways in adipocyte biology. Generally speaking, key transcription factors have been identified in one of a few ways. For example, some factors were identified because they were highly or specifically expressed in fat. Initially, these studies involved Northern or Western blotting, but newer microarray-based techniques have enhanced our ability to focus on factors based on their expression pattern. For example, the pro-adipogenic factor KLF4 was pulled out of a microarray study looking at genes expressed during various timepoints of adipogenesis[37]. Other factors were identified because they are orthologous to factors discovered to control metabolism in lower organisms. The *Drosophila* protein *Serpent (Srp)*, for example, was shown to enhance larval fat body formation in that species[97]. This led to the hypothesis that GATA factors, which are the mammalian orthologs of *Srp*, might also be involved in adipogenesis. This, in fact, proved to be true, although interestingly, GATA2 and GATA3 are anti-adipogenic and not pro-adipogenic as was predicted from the known activity of *Srp*[42, 43]. Transcriptional pathways have also been inferred from the results of knockout studies in mice, which sometimes yield an unexpected metabolic phenotype. Mice lacking the transcriptional co-repressor RIP140, for example, were lean and resistant to high-fat diet induced obesity[98]. This proved to be due at least in part to an effect of RIP140 on lipogenic gene expression in adipose tissue.

Newer technologies are also bearing fruit in this area. This includes siRNA screening, which independently identified RIP140 as a player in adipocyte transcriptional pathways involved in fatty acid oxidation and mitochondrial biogenesis[99]. Tontonoz and colleagues performed a small molecule screen looking for compounds that might enhance adipogenesis, and identified an agent called harmine[100]. Interestingly, harmine activates PPARγ-mediated pathways by affecting PPARγ expression, rather than as a PPARγ ligand. Although the specific molecular target of harmine is still uncertain, this study illustrates that small molecule screening can provide insights in this area. Another new technology that has been applied to adipocyte biology is genome-wide assessment of transcription factor binding using chromatin immunoprecipitation followed by microarray hybridization ('ChIP-chip') or massively parallel sequencing ('ChIP-Seq'). This has been accomplished for PPARγ and C/EBPα during 3T3-L1 adipogenesis, demonstrating a role for these factors in regulating the expression of a huge number of metabolically relevant genes in fat[10, 11]. Importantly, this approach can enable deeper understanding of how a particular transcription factor regulates adipocyte physiology, but it requires that one have *a priori* knowledge of which factor to look at.

Another way to approach this problem is not to look directly for novel transcription factors, but to focus on regions of the genome that appear to be important for adipocyte gene expression. By identifying specific motifs of interest in the DNA sequence, one can then infer what the cognate trans-acting factors might be, thus working 'backward' to fill in important pathways. This, in fact, was the approach used by Spiegelman and colleagues to identify PPARγ as the key adipogenic transcription factor[101]. Those experiments involved focusing on a critical region of the FABP4 (also called aP2) promoter known to drive adipose-specific expression of a reporter gene. We have used a conceptually similar approach on a broader scale, using changes in chromatin structure.

Chromatin exists in one of two major forms within a cell. Tightly packed heterochromatin is highly condensed and is generally inaccessible to transcription factors, and is thus considered to be 'silenced'. Euchromatin, on the other hand, is in a more relaxed conformation and contains regions that are being actively transcribed. Even within euchromatin, however, one can make further distinctions based on a variety of epigenetic modifications. These include direct methylation of DNA on CpG residues, where hypermethylation is associated with reduced gene expression[102]. There are also a wide range of modifications that occur on histone proteins, including methylation, acetylation, and phosphorylation, and there has been extensive work done to understand the basics of the resulting histone code and how it regulates gene expression[102, 103]. Importantly, these modifications are perpetrated by a large group of ubiquitous nuclear proteins that do not bind directly; the specificity of chromatin marks within a particular cell type is thus dependent upon the complement of sequence-specific transcription factors that are active at that place and time. Changes in chromatin marks therefore 'tag' regions where such transcription factors bind, and motifs discovered within those regions can help identify the relevant factors.

We performed a 'proof-of-concept' study of this approach using a medium-throughput assay for DNAse hypersensitive sites flanking a select group of genes chosen for their relative adipose selectivity[67]. Regions of DNA that have unwound from nucleosomes and are "open" can be preferentially digested with a small amount of the enzyme DNAse I, and are thus considered hypersensitive to DNAseI. These sites correlate well with histone acetylation and other marks denoting important features such as promoters and enhancers. Traditional DNase-hypersensitivity analysis involves iterative Southern blotting, a cumbersome technique which limited the utility of this approach for decades. The use of quantitative PCR (qPCR) to quantify the degree of DNAse sensitivity, however, greatly simplifies the process. In our study, we examined DNAse sensitivity of over 250 highly conserved DNA regions (~ 70 bp in length on average) in the proximal 50 kb upstream of the transcriptional start site and the first intron of

27 adipose-selective genes in mature adipocytes as well as pre-adipocytes. We identified areas that were DNAse sensitive in adipocytes but not precursor cells, and subjected these regions to computational motif finding. Motifs that were overrepresented in DNAse hypersensitive regions included those for the high-mobility group protein HMGI-Y and PPAR, both of which were already known to promote adipogenesis[22, 104]. Among the unexpected motifs discovered were those corresponding to binding sites for interferon regulatory factors (IRFs) and the orphan nuclear receptors called chicken ovalbumin upstream promoter-transcription factors (COUP-TFs). We went on to show that IRF3, IRF4, and COUP-TFII are all expressed in adipose tissues and all exhibit anti-adipogenic activity in differentiation assays[67, 105]. Despite the fact that these experiments were performed using only a limited amount of flanking sequence from a small number of adipocyte genes, they demonstrate the power of using epigenetic marks as guides to identify novel transcriptional pathways.

Going forward, new technologies will allow us to perform these sorts of studies in a more comprehensive fashion. For example, we are now performing ChIP-Seq to determine the modified histone landscape at several time-points during both human and murine adipogenesis. These studies will allow us to look at events that mark enhanced and repressed regions of chromatin throughout the differentiation process, and will enable us to draw conclusions that are not specific to a single model of adipogenesis. Motifs that are over-represented in regions of interest will be used to suggest interesting transcription factors. One limitation of these studies is the limited representation of transcription factors in the motif databases. There are close to 2,000 transcription factors in the mammalian genome, only several hundred of which bind to defined motifs[106]. For this reason, advances in motif identification are a prerequisite for further progress. An alternative approach might entail direct proteomic identification of factors binding to regions of interest. This has been a difficult task in the past, but new strategies may make this more tractable in the near future.

Other approaches discussed above will also need to be employed to gain a more complete understanding of transcriptional pathways in adipocytes, including siRNA and small molecule screens. These techniques are becoming more broadly available, and as their cost comes down, more adipocyte biology groups will be empowered to attempt this sort of unbiased screening approach.

## 5. Expert Opinion

Our understanding of adipose tissue has come a long way from the simplistic notion of a passive energy storage depot. Given its central role in energy homeostasis and nutrient regulation, adipose tissue will remain a critical target in drug development for metabolic disease. Furthermore, we believe strongly that one of the most exciting areas to pursue in this regard is the control of adipocyte gene expression. It bears repeating that the goal of this approach is not to simply make fewer adipocytes; adipocytes serve a critical purpose as a safe place to store excess calories, and life without them would be characterized by significant metabolic dysfunction.

What functions of adipocytes would be most favorable to manipulate? Certainly it would be advantageous to provoke white fat cells to behave more like brown adipocytes. By enhancing lipid breakdown and fatty acid oxidation, while simultaneously releasing energy in the form of heat, one could promote leanness and enhanced insulin sensitivity. Alternatively, some conditions might actually be helped by increasing lipid storage in adipose tissue, as long as this was accompanied by simultaneous reductions in ectopic lipid deposition in liver and muscle. Other strategies could involve changing the synthesis and secretion of selected adipokines. Increasing leptin and/or adiponectin would be beneficial, as would decreasing some of the factors that inhibit global insulin action, like RBP4.

In order to bring some of these ideas closer to the medicine chest, we need to overcome several obstacles. The first is that we still have incomplete knowledge of the role played by adipose tissue in global physiology. While we have learned an extraordinary amount in the last fifteen years, we are still on the steep part of the curve here. Second, we know even less about the transcriptional pathways used by adipocytes to govern these functions. This has been the primary focus of this review, and it should be clear that the community is making strides in this area but that all the pieces of puzzle are not yet on the table. Third, there are obstacles that bedevil drug development in every area. How can transcription factors (other than nuclear receptors) be targeted pharmacologically? How can we achieve tissue specificity so that we affect fat cells but not other cell types? How can we predict unintended adverse consequences, such as the fluid overload sometimes seen with TZD use?

It is important not to oversell the promise of adipocyte-based therapies, but continued research will, we believe, help to solve some of these vexing issues. The result is certain to be exciting, and will lead to new insights that translate to better therapies for metabolic disease.

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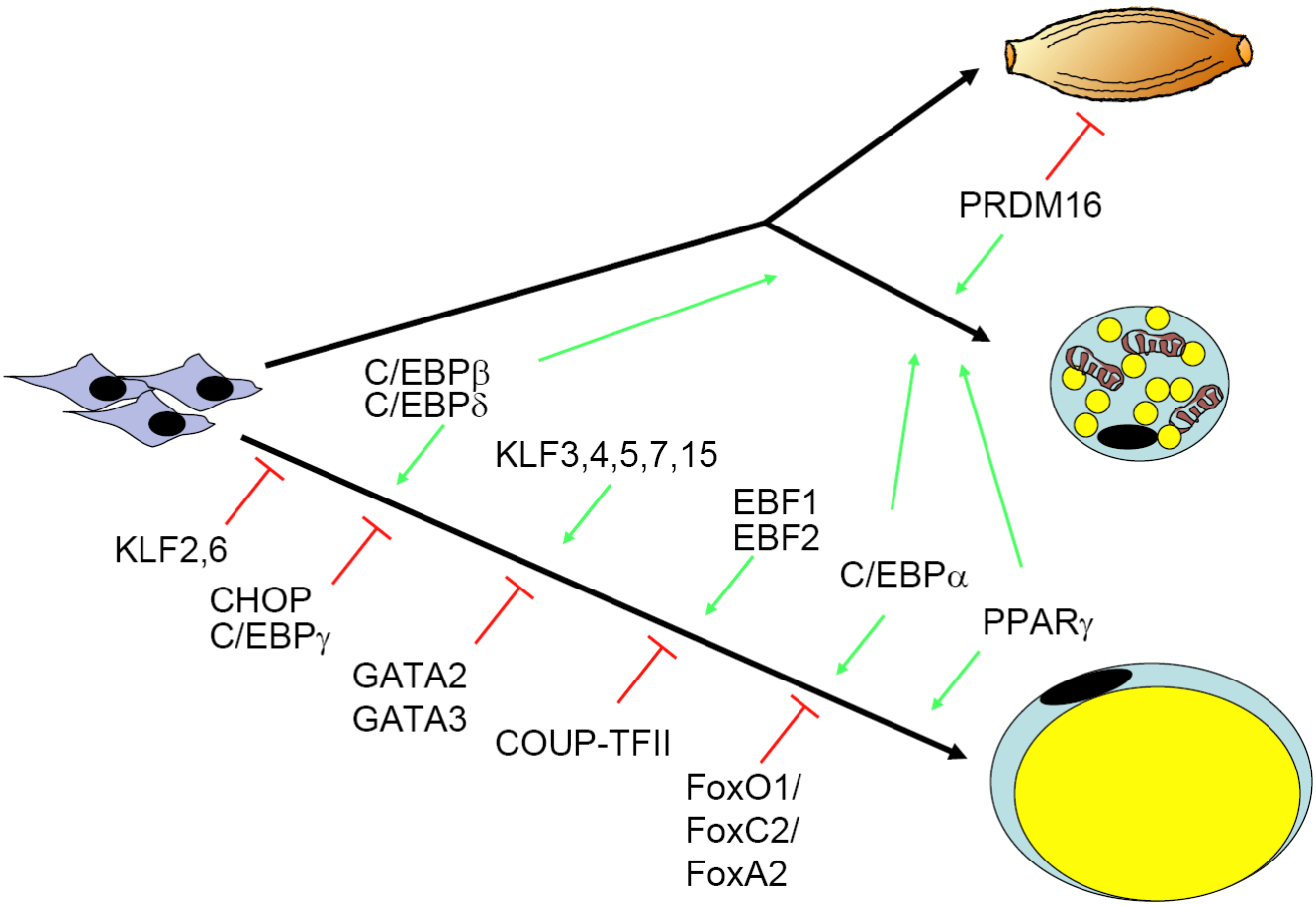


**Figure 1.**

Mesenchymal precursor cells can give rise to multiple cell types, include brown and white adipocytes. Recent data indicate that brown fat is ontologically closer to skeletal muscle than white fat, despite many phenotypic similarities between the two types of adipocytes. Prdm16 is a major determinant of the switch between brown fat and muscle. Most of what we know about adipogenesis derives from studies in cell culture models of white fat, although many of the same factors operate in brown fat as well. Key transcription factor families involved in this process include C/EBPs, forkhead proteins, KLFs, and several nuclear receptors, including the master regulator of adipogenesis PPARγ. See text for more details.

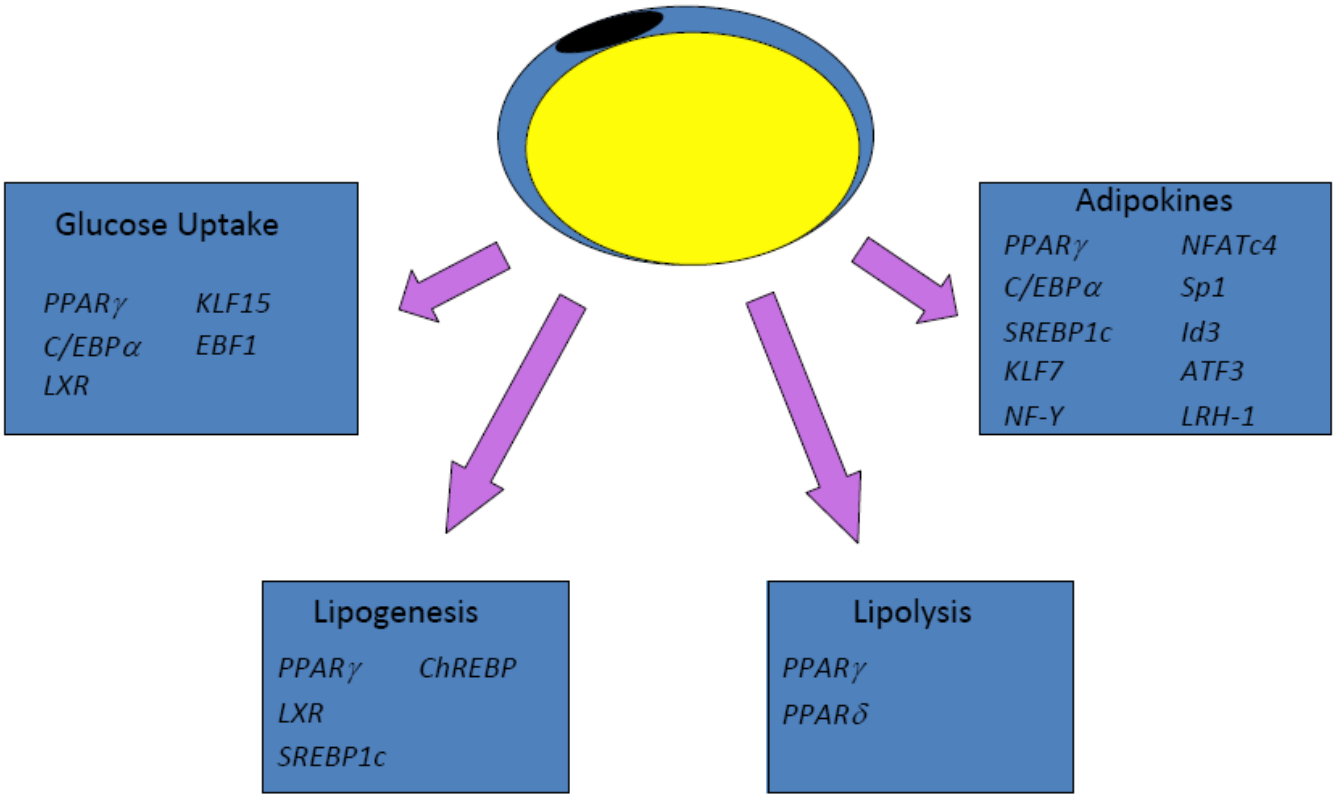


**Figure 2.**

Adipocytes perform a variety of functions that are under the transcriptional control of distinct yet overlapping sets of factors. Glucose uptake (particularly in response to insulin) and lipogenesis are most prominent in the fed state, while lipolysis occurs in the fasted state. Adipokine secretion by fat cells helps to integrate global metabolism, and is under the control of a large and growing number of transcriptional pathways. Please see text for details.