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Review

Understanding the variegation of fat: Novel regulators of adipocyte differentiation and fat tissue biology ☆☆

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ABSTRACT

The differentiation of uncommitted cells into specialized adipocytes occurs through a cascade of transcriptional events culminating in the induction and activation of the nuclear receptor PPARγ, the central coordinator of fat cell function. Since the discovery of PPARγ, two decades ago, our views of how this molecule is activated have been significantly refined. Beyond the cell, we also now know that diverse signals and regulators control PPARγ function in a fat-depot specific manner. The goal of this article is to review the latest in our understanding of the early and late transcriptional events that regulate adipocyte development and their potential impact on energy storage and expenditure in different fat depots. This article is part of a Special Issue entitled: Modulation of Adipose Tissue in Health and Disease.

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1. Introduction

Obesity is a major risk factor for metabolic disorders such as insulin resistance, diabetes, cardiovascular and liver disease. Excess weight and obesity are caused by an imbalance between energy input and its output, that results in excessive adipose tissue expansion, secondary to hyperplasia of adipocyte precursor cells and hypertrophy [1]. In the past several years, a series of key molecular players that influence adipose tissue mass have been identified and renewed emphasis has been placed on understanding the mechanistic principles that govern the development of these tissues and orchestrate energy homeostasis. Humans and rodents have two major anatomically distinct types of adipose tissues, white and brown. These tissues derive from different cell lineages and exert opposite roles on lipid metabolism. While white fat stores energy, brown fat dissipates it by using lipids as fuel for thermogenesis. Furthermore, white fat is not uniform. Distinct white fat depots exhibit a range of features, including different developmental gene signatures, adipokine repertoire, lipid storage capacity and variable susceptibility to inflammation. Given that these distinctive features influence the onset of the metabolic syndrome, it seems that an in depth understanding of the mechanisms that control different fat tissue types and depots is critical for designing strategies to prevent and treat obesity and its complications.

2. Types of adipose tissues and depots

2.1. White adipose tissue

White adipose tissue is the primary site of energy storage. Adipocytes, specialized cells devoted to the accumulation of triglycerides, store excess of nutrients as fat so that fatty acids may be released during energy demand in times of scarcity. Morphologically, mature white fat cells are characterized by an unilocular lipid droplet surrounded by a thin cytoplasmic rim containing only a few mitochondria. Fat cells are extremely plastic, able to rapidly expand in size and number, and as a result fat tissue of individuals with high body mass index represents the second largest organ in the body after the skin. Developmentally, fat tissue has been believed to originate from the mesoderm, however recent studies have shown that fat tissue present in the face has a neuro-ectodermal origin [2] and that the vasculature represents the primary source of fat cell precursors [3,4]. Despite similar morphological appearance of white fat tissue in every part of the body, there are major regional differences spanning from distinct gene expression profiles to distinct adipokine production. Microarray molecular analyses have confirmed that both human and mouse white fat tissues from different anatomical locations differ in a large number of expressed genes, including developmental patterning genes [5–7]. It was recently shown that the homeobox gene Hoxc13 is exclusively expressed in gluteal

subcutaneous fat of both men and women and that its depot-selective expression is maintained in ex vivo cultures of preadipocytes and in differentiated adipocytes. These data suggest that different fat depots may be characterized by defined homeobox codes that identify them in specific locations of the body. Some of the molecular differences present in distinct depots manifest as distinct lipolytic capacity and differential responsiveness to sex hormones. It is now evident that gender-specific fat distribution exists: it has been demonstrated that men have increased propensity to expand their intra-abdominal fat depot, assuming an apple shaped phenotype, while women mostly enlarge the size of depots in the gluteal, hip and femoral area, giving rise to the characteristic pear shaped body conformation. These regional- and gender-specific differences in fat accumulation are particularly relevant given that increased fat in the subcutaneous region is associated with lower risk to develop the metabolic syndrome compared with increased fat deposition in the intra-abdominal area [8].

2.2. Brown fat

In response to beta-adrenergic stimuli, brown fat oxidizes lipids for thermogenic purposes. Smaller mammals, such as rodents, need brown fat to maintain their temperature during exposure to cold atmospheric conditions and so do human newborns who have little insulating white fat to protect them from low temperatures right after birth. The view that brown fat existed only in these conditions has undergone a dramatic change. In fact in adult humans, there are substantial amounts of cells containing molecular and functional footprints of brown fat especially in the neck and supraclavicular regions. These areas can be identified by PET scan as regions of high glucose uptake in a variety of patients, including in those exposed to thyroid replacement therapy or to cold temperatures [9–11]. These recent data implicate the existence of "brown fat-like" tissue in adult humans and have revamped the field of brown fat biology.

Brown fat cells are characterized by multilocular lipid content and by a high number of mitochondria. Brown fat burns lipids as fuel and uses UCP1, an inner mitochondrial membrane protein, to uncouple oxidative phosphorylation from ATP production causing the dissipation of energy as heat. The importance of UCP1 in vivo has been demonstrated through knock-out experiments showing that mice lacking UCP1 develop obesity at thermoneutrality [12]. The developmental origin of brown fat tissue was recently uncovered via elegant fate cell mapping analyses. Through these studies it was shown that brown fat has a distinct origin compared to white fat, and that it derives specifically from a population of Myf5 + precursor cells [13]. This muscle-like origin had been previously suspected since the analysis of gene signatures of brown and white fat tissues had shown a closer resemblance of brown fat to muscle than to white fat [14].

2.3. Brite/beige fat

In addition to white and brown fat cells, a new type of fat cell, called brite (short for brown in white) or beige, has been recently identified. It has been long established that subcutaneous white fat tissue is quite heterogeneous, with multilocular cells typically found in brown fat, interspersed between classic white unilocular fat cells [15]. These multilocular cells have been observed in white fat cells also in response to treatment with the antidiabetic drugs TZDs [16]. Immunostaining analyses have revealed that, although these "brown-like" cells express the characteristic brown fat marker UCP1, they are Myf5−, suggesting that they derive from a population of cell precursors distinct from that that gives rise to brown fat. To further define the molecular identity of the cells present in regions of browning within white fat tissue, the Spiegelman group embarked in a purification effort to clone and characterize these brite/beige cells. Through this approach, a population of cells, which express low UCP1 levels in the basal state, but can be induced to express it in response to cAMP stimulation, was

identified [17]. Further molecular characterization by microarray analysis revealed that these cells express unique gene signatures, suggesting that brite/beige cells are a new and distinct cell type. Among the brite/beige-selective genes, the cell surface markers CD137 and TMEM26 were identified and used to detect brite/beige cells in mouse white fat tissues [17] and were shown to be expressed also in UCP1 positive human fat tissues [17,18]. Interestingly, UCP1 positive depots obtained from adult donors with thyroid cancer showed the coexistence of both classical brown and some brite/beige cell signatures [8], while UCP1 positive biopsies of healthy subject exposed to cold or obtained post-mortem from children mainly expressed beige-selective genes with nearly undetectable levels of classic brown fat markers [17,18].

It is now established that the number of the UCP1 positive cells in white fat tissue seems to be regulated not only by cold temperature, beta-adrenergic stimulation and drugs such as TZDs, but also by hormones and secreted peptides, as demonstrated by the browning effects observed after administration of the myokine irisin [17,19], FGF21 [20] and after treatment with the cardiac natriuretic peptide ANP, activated by p38 [21]. There are currently a number of hypotheses on how brite/beige cells arise in white fat tissue. Both trans-differentiation of white adipose tissue cells [22] and proliferation of brite/beige precursors embedded in white fat in response to various stimuli have been proposed. Recent analyses of transgenic and knock-out mouse models have shown that browning of adipose tissue occurs more often than anticipated, pointing to novel molecular mechanisms regulating browning of white tissue [23–27]. The evidence that certain stimuli not only increase brown fat tissue but also induce browning of white fat and increase energy expenditure has become an area of intense investigation for therapeutic purposes.

3. Regulators of adipocyte differentiation and fat tissues function

3.1. Terminal differentiation regulators

3.1.1. PPARγ

Adipocyte differentiation in either white or brown fat is under the control of the transcription factor PPARγ. PPARγ's role in adipogenesis was discovered in the early nineties [28], when PPARγ was identified as the transcription factor binding to the fat specific enhancer present in the adipocyte fatty acid binding protein aP2 promoter [29]. In vitro gain of function studies subsequently demonstrated that ectopic expression of PPARγ is sufficient to confer the fat differentiation phenotype and to induce the expression of critical adipose tissue specific genes [28]. PPARγ has the typical nuclear receptor structure, characterized by a DNA binding motif, a ligand binding region and two activation domains, one ligand-independent located at the N-terminus, and the other at the C terminus functioning in response to agonists. PPARγ heterodimerizes with the nuclear receptor RXR and is able to activate transcription by binding to response elements characterized by direct repeats of the sequence 3′-PuGG/TTCA-5′ separated by 1 nucleotide, also called DR1. PPARγ's ligand binding pocket is large and can accommodate a wide array of ligands [30]. In the absence of agonists, PPARγ is bound to corepressors, such as NCor [31]. These repressive complexes are readily displaced after ligand binding, which results in PPARγ to undergo conformational changes to recruit coactivator complexes [32]. Recent advances in technology have permitted the global mapping of PPARγ targets via a systematic approach involving deep sequencing of adipocytes. These studies have confirmed that PPARγ controls lipid storage and allowed the identification of PPARγ targets at a genome-wide level [33–35]. Although PPARγ was discovered almost twenty years ago, its role as a central orchestrator of adipocyte function has remained uncompromised.

Regulation of PPARγ beyond ligand activation has been well documented. Modulation of CDK5-mediated phosphorylation levels of PPARγ at serine 273 by the partial agonists MRL24 has been shown to regulate selective PPARγ target genes, such as adiponectin and adipsin,

contributing to insulin sensitization, without altering PPARγ function on differentiation [36]. In addition, PPARγ's binding to coregulators that are necessary to induce the typical genes of white and brown fat can be modulated pharmacologically. For example, chronic treatment of mice with PPARγ synthetic ligands such as TZDs causes the induction of brown specific genes leading to browning of white adipose tissue [37]. It has been shown that TZD treatment affects the levels of PPARγ sumoylation at residues K107 and K395, via regulation of FGF21 [38], and PPARγ acetylation levels at lysines 268 and 293 through the control of SIRT1. PPARγ deacetylation was shown to regulate adipokine production, similarly to the effects observed by modulating CDK5-mediated phosphorylation at serine 273, and led to corepressor/coactivator exchange, causing the release of NCor binding and increasing PPARγ's ability to recruit the coactivator PRDM16. These SIRT1 mediated effects led to the induction of brown fat specific markers, such as UCP1, and browning of white fat tissue in vivo [25]. It is now clear that both the induction and modulation of PPARγ impacts energy metabolism (Fig. 1).

3.1.2. The CCAAT/enhancer-binding proteins

This family of transcription factors consists of several members, including C/EBPα, β, δ and CHOP. C/EBPα, β and δ proteins contain a DNA binding region and a leucine zipper dimerization domain. C/EBPβ and δ are rapidly induced after hormonal stimuli that induce differentiation and have been shown to participate in the early phases of adipogenesis. Although C/EBPβ expression has been shown to be sufficient to induce differentiation in vitro even in the absence of classic differentiation inducers, an analysis of C/EBPβ knock-out mice has demonstrated that C/EBPβ is not necessary for the formation of fat mass. Reduction in adipose tissue is only observed when both C/EBPβ and δ are genetically ablated (reviewed in Ref. [1]). C/EBPα is expressed at later stages of differentiation and participates in a positive feedback loop with PPARγ to sustain adipocyte maturation. Although C/EBPα appears to be partly required to maintain insulin sensitivity, its absence does not appear to severely affect adipocyte differentiation in vitro [39].

3.2. Depot-selective factors

3.2.1. PGC1α1

The peroxisome proliferator-activated receptor-gamma coactivator 1 alpha 1 (PGC1α1) is considered a critical metabolic coactivator (reviewed in Ref. [40]). It was originally identified in 1998 as a PPARγ interacting protein in brown fat and shown to be highly inducible after cold exposure [41]. It has been now demonstrated that PGC1α1 is one of four different isoforms generated by alternative promoter usage and splicing of the PGC1α gene [42]. Since its discovery, PGC1α1 has been implicated in regulation of metabolism in a variety of tissues including liver, skeletal muscle and heart, in cooperation with several transcriptional factors. PGC1α1 ectopic expression in fibroblast induces a plethora of brown fat specific genes including those involved in mitochondrial and peroxisomal function and in thermogenesis [43,44]. Both global and fat tissue specific deletion of PGC1α1 have confirmed its role in vivo in the regulation of adaptive thermogenesis [45,46]. Recent studies have demonstrated that PGC1α1 plays a critical role, not only in brown fat tissue, but also in the regulation of white adipose depots browning, as shown by the effect observed after administration of FGF21 on PGC1α1 protein levels and stability in white fat tissue and by the decreased levels of browning in PGC1α1 conditional fat depot knock-out mice [46]. The evidence accumulated so far indicates that PGC1α1 is a central regulator of energy metabolism and suggests that its induction in brown or white fat tissues could be therapeutically exploited.

3.2.2. PRDM16

In a search for specific brown fat regulators, Seale and colleagues identified PRDM16 via a genome wide screen of transcriptional regulators expressed in brown fat tissue and cell lines. Through

gain-of-function experiments it was demonstrated that PRDM16 induces the expression of brown fat specific genes via physical interaction and functional cooperation with PPARγ and PPARα and their coactivators PGC1α1 and β [47]. The role of PRDM16 in driving the thermogenic programs also in vivo has been demonstrated via the generation of transgenic mice expressing PRDM16 in fat tissues. These mice developed brown-like adipocytes selectively in their subcutaneous adipose depot. In addition they displayed an overall improved metabolic profile as demonstrated by increased energy expenditure and improved glucose tolerance in response to high fat feeding [23]. Conversely, mice haplo-insufficient for PRDM16 showed reduced white fat browning in response to beta-adrenergic stimuli. Interestingly, knock-down studies performed in primary brown fat cell cultures demonstrated that the reduction in PRDM16 levels caused cells to assume an elongated morphology, resembling that of myocytes, to have reduced thermogenic gene expression levels and to increase the expression of muscle specific genes, implicating PRDM16 as a critical regulator of brown fat cell identity via controlling the switch between brown fat and skeletal muscle cell fate [13,48].

3.2.3. Ebf2

Early B cell factors (Ebfs) have been previously shown to be involved in adipocyte differentiation via regulation of PPARγ [49–51]. Recent studies by the Seale group [52] have identified a more specific brown fat selective role for one family member, Ebf2, in the determination and maintenance of brown fat cell identity. Through genetic studies it was demonstrated that Ebf2 −/− embryos at E18.5 d.p.c. have reduced brown fat size, decreased mitochondrial density and loss of expression of brown fat genes, such as PRDM16, UCP1 and CIDEA, due to cell autonomous defects of primary brown fat preadipocytes. Functional in vitro studies demonstrated that norepinephrine-dependent respiration of cells obtained from Ebf2 −/+ mice is uncoupled, indicating a central role of Ebf2 for the respiratory function of brown adipocytes.

3.3. Commitment/early differentiation regulators

3.3.1. ZFP423

To identify novel molecular determinants of the fat cell lineage, Gupta and colleagues screened the expression levels of 1800 transcription factors in fibroblastic cell lines with different differentiation propensities and found that Zfp423, a zinc finger protein previously shown to regulate brain development, was expressed at higher levels in preadipocytes than in non-adipogenic fibroblasts. To test the role of this factor in inducing the commitment of cells to the adipocyte lineage, Zfp423 was ectopically expressed in NIH3T3 fibroblasts and shown to activate PPARγ expression thereby promoting adipocyte differentiation. Conversely, knock-down of Zfp423 in 3T3-L1 preadipocytes reduced PPARγ expression levels, affecting the differentiation capacity of these cells. Mechanistic studies revealed that Zfp423 regulates PPARγ expression through the amplification of the BMP signaling pathway. In vivo analysis of Zfp423 in embryos obtained from Zfp423-deficient mouse showed impaired differentiation of brown and white fat, supporting the notion that Zfp423 plays a critical role as a transcriptional regulator of preadipocyte determination of both brown and white fat depots [53]. Recently, analysis of murine adipose progenitor of the brown and white fat lineage labeled via expression of GFP from the Zfp423 genetic locus identified committed preadipocytes in endothelial and perivascular cells, suggesting that committed brown and white adipocytes may independently go through a pericyte lineage [4].

3.3.2. ZNF638

Using a candidate gene approach based on sequence homology with the transcriptional coactivator PGC1α1, our group identified the zinc finger protein ZNF638 as a novel regulator of adipocyte differentiation [54]. ZNF638 is a large multidomain protein containing an RS region and multiple RRM domains, which are motifs shared
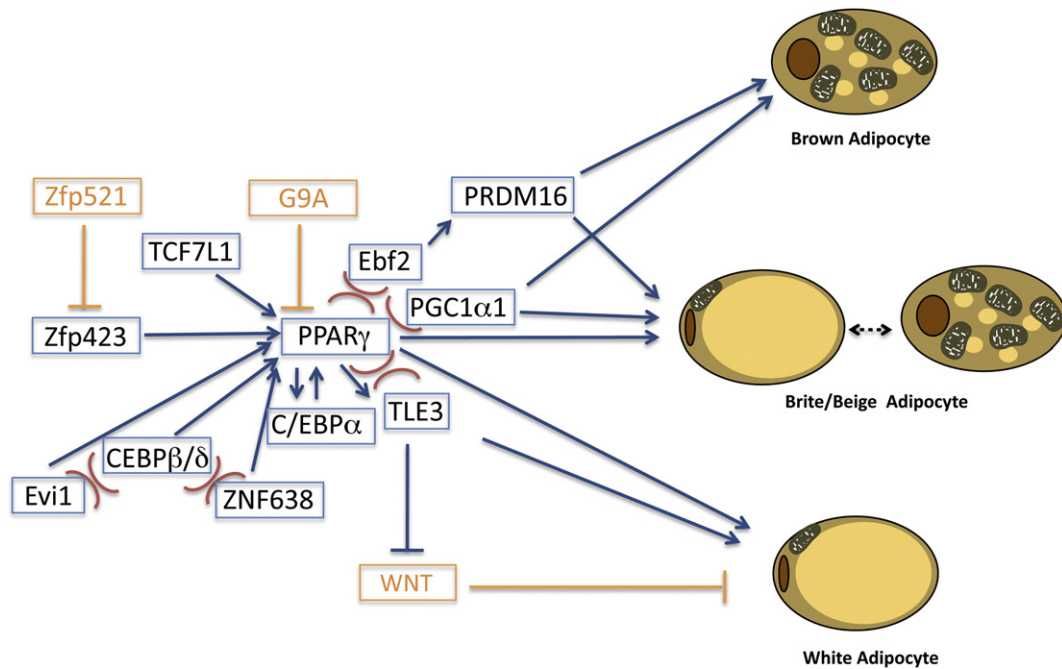
Fig. 1. Schematic depiction of the transcriptional regulators that affect the differentiation of white, beige and brown adipocytes. The red curved lines indicate protein–protein interactions.

with PGC1α1. In addition, ZNF638 contains a putative DNA binding domain, two zinc fingers and an acidic repeat. ZNF638 is induced transiently during early phases of adipocyte differentiation and via gain-of-function studies we demonstrated that ZNF638 is able to stimulate pluripotent mesenchymal cells to acquire the molecular and morphological features of adipocytes. Interaction studies revealed that ZNF638 physically binds to early regulators of PPARγ expression, C/EBPβ and δ. This finding was supported by chromatin IP studies indicating that ZNF638 can be found bound to the PPARγ promoter at the C/EBP responsive element in combination with C/EBPs, during the early phases of differentiation. Immunostaining studies of ZNF638 revealed that this protein is localized in the nucleus in a punctuated pattern, reminiscent of speckles, which are nuclear bodies considered to be storage areas of splicing factors [55]. Whether ZNF638 has the competency of regulating alternative splicing in addition to transcription, thereby contributing to differentiation by generation of fat depot-selective isoforms, remains to be determined.

3.3.3. Evi1

To identify novel upstream modulators of PPARγ in white fat tissue, Ishibashi and colleagues tested the role of Evi1, a member of the PR domain-containing family, closely related to the brown fat program regulator PRDM16, in adipocyte differentiation. They showed that Evi1 mRNA levels are increased in preadipocytes at the onset of differentiation and that forced expression of Evi1 in non-adipogenic cells led to their conversion into adipocytes. Loss of Evi1 function in preadipocytes suppressed adipocyte differentiation. Interestingly, overexpression of Evi1 in myoblast cells was able to force their conversion into adipocytes [56]. Mechanistically, Evi1 appears to stimulate adipogenesis through induction of PPARγ2 levels via physical interaction with C/EBPβ. In vitro functional assays combined with adipose tissue expression data suggested that Evi1 may play a role specifically in white fat tissue, where Evi1 expression is higher compared to brown fat, suggesting that PRDM16 and Evi1 may regulate similar processes in adipogenesis, respectively in brown and white fat lineages. It remains to be established whether Evi1 plays a role in browning of white adipose tissues, a process in which PRDM16 has been shown to be critically involved. Whether the balance between the levels of Evi1 and PRDM16 in white adipose tissue determines the expression of

white or brown fat programs downstream of these two factors remains to be determined.

3.3.4. Zfp521

To answer the question of what factors control cell fate choices, Kang and colleagues took a candidate gene approach. Given the known involvement of the zinc finger protein Zfp423 in adipose determination, they investigated the role of Zfp521, a close paralog of Zfp423. Overexpression of Zfp521 caused inhibition of adipogenesis, whereas its genetic ablation enhanced it. Zfp521 functions by binding to early B cell factor 1 (Ebf1) and by inhibiting the expression of Zfp423. Interestingly, Ebf1 was shown to bind to an intronic enhancer of Zfp521 and to repress Zfp521 expression. In vivo analysis of Zfp521 function reveled that embryos with genetic ablation of Zfp521 have increased interscapular brown adipose tissue and subcutaneous white adipocytes, suggesting a role for this factor in both depots [57].

3.3.5. TCF7L1

Cristancho and colleagues recently demonstrated that regulators of cell structure organization can control early events in adipocyte differentiation. Forced expression of the transcriptional repressor factor 7-like 1 (TCF7L1), which is normally induced by cell contact in adipogenic cell lines, is sufficient to commit non-adipogenic fibroblasts to the adipogenic fate. Conversely, depletion of TCF7L1 inhibited differentiation, demonstrating a role for TCF7L1 as an adipogenic competency factor. The mechanisms of TCF7L1's function involve the silencing of genes involved in cell structure which occurs after the addition of adipogenic stimuli [58]. These data indicate a role of TCF7L1 as a competency factor in adipogenesis.

3.3.6. TLE3

Through a high throughput screen for novel modulators of adipocyte differentiation, Villanueva and colleagues identified the Groucho family member TLE3 [59]. TLE3 expression was shown to be increased during adipocyte differentiation and by PPARγ agonists. Through gain- and loss-of-function studies it was demonstrated that TLE3 is able to regulate adipocyte differentiation. Mechanistically, TLE3 forms a transcriptional complex with PPARγ and synergistically cooperates with it at PPARγ target gene promoters. Interestingly, TLE3 also inhibits Wnt

target gene expression and reverses β-catenin-dependent repression of adipocyte genes, suggesting that TLE3 is a dual-function switch that facilitates the adipogenic program through the formation of both active and repressive transcriptional complexes. Transgenic mice expressing TLE3 in adipose tissue revealed that TLE3 expression in vivo ameliorates high-fat-diet-induced insulin resistance. Given that TLE3 is expressed at higher levels in WAT relative to BAT, it is possible that TLE3 may have a depot-selective function, specifically in white adipocytes.

3.3.7. Histone H3K9 methyltransferase G9a

Recent genome wide studies of histone methylation profiling in preadipocytes performed by Wang and colleagues identified the histone methyltransferase (HMT) G9a as a mediator of repressive epigenetic marks [60]. It was shown that H3K9me2 is selectively enriched at the PPARγ locus and its levels and those of G9a are decreased during adipogenesis, concomitantly to the increase in PPARγ mRNA levels. Loss of H3K9me2 through G9a deletion led to enhanced chromatin opening thereby facilitating the binding of the early adipogenic regulator C/EBPβ to the PPARγ promoter. Interestingly, while G9a repressed PPARγ expression, it positively affected Wnt10a, a known inhibitor of adipocyte differentiation. In vivo, deletion of G9a in mouse adipose tissues led to an increase in adipogenic gene expression programs and in the amount of epididymal, inguinal and brown fat depots.

4. Summary, open questions and future directions

In the last few years, adipose cell and tissue biology has flourished with remarkable discoveries. These discoveries have led to the elucidation of new pathways and molecules that control the early events guiding adipocyte differentiation and have provided evidence that fat tissues throughout the body, despite their appearance, are not equal and should be thought of as distinct depots. The evolution of thinking on this latter point is critical. If adipose tissue is inherently distinct based on its developmental origin and ability to respond to hormonal stimulation, then approaches to study it should take this into account. In this article we reviewed some of the novel factors influencing differentiation of adipocytes and contributing to fat depot formation. With the expansion of the adipose tissue biology field, there are many new unanswered questions. For example, given the pathological link between enlargement of specific adipose depots and development of the metabolic syndrome, a pressing question is related to the identity of depot-specific drivers of fat accumulation. This issue is particularly relevant if strategies to combat obesity aiming at finding ways of controlling the balance between fat accumulation and lipid oxidation for thermogenic purposes are desired. Anchoring experiments around upstream regulators and modulators of PPARγ function could be a fruitful strategy to better define the mechanisms that control selective depot expansion. In time, these may reveal potential targets to control or prevent fat deposition in a depot-selective manner.

Another open question is whether brite/beige cells arise from the proliferation of beige precursor embedded in white fat tissue or if there is intrinsic plasticity in white fat cells allowing their transdifferentiation into beige in response to select physiological cues or pharmacological stimuli. This is a relevant question not only from a developmental biology prospective but also because a clear answer defines how beige cells can be exploited to transform white fat depots from storage sites to energy burning furnaces. Current data indicate that beige cells share features with surrounding white fat cells but in addition express markers of brown fat. Lineage tracing will be necessary to definitively establish the origin of these brite/beige fat cells.

One potential antiobesity therapeutic intervention could consist of inducing/stimulating the "browning" of white adipose tissue. It remains to be assessed whether the induction of UCP1 protein in white fat is sufficient to increase the overall basal temperature to levels that are therapeutically exploitable and whether the heat production described in some of the animal models in which "browning" has been observed is

supplemented by muscle [61]. If we want to determine which tissue ultimately to target for future antiobesity intervention, understanding the distinction between these different thermogenic sources is critical.

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