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Genetic and Epigenetic Control of Adipose Development

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Abstract

White adipose tissue (WAT) is the primary energy storage organ and its excess contributes to obesity, while brown adipose tissue (BAT) and inducible thermogenic (beige/brite) adipocytes in WAT dissipate energy via Ucp1 to maintain body temperature. BAT and subcutaneous WAT develop perinatally while visceral WAT forms after birth from precursors expressing distinct markers, such as Myf5, Pref-1, Wt1, and Prx1, depending on the anatomical location. In addition to the embryonic adipose precursors, a pool of endothelial cells or mural cells expressing Pparγ, Pdgfrβ, Sma and Zfp423 may become adipocytes during WAT expansion in adults. Several markers, such as Cd29, Cd34, Sca1, Cd24, Pdgfrα and Pref-1 are detected in adult WAT SVF cells that can be differentiated into adipocytes. However, potential heterogeneity and differences in developmental stage of these cells are not clear. Beige cells form in a depot- and condition-specific manner by *de novo* differentiation of precursors or by transdifferentiation. Thermogenic gene activation in brown and beige adipocytes relies on common transcriptional machinery that includes Prdm16, Zfp516, Pgc1α and Ebf2. Moreover, through changing the chromatin landscape, histone methyltransferases, such as Mll3/4 and Ehmt1, as well as demethylases, such as Lsd1, play an important role in regulating the thermogenic gene program. With the presence of BAT and beige/brite cells in human adults, increasing thermogenic activity of BAT and BAT-like tissues may help promote energy expenditure to combat obesity.

Introduction

Adipose tissue plays a crucial role in mammalian metabolism. White adipose tissue (WAT) stores excess energy as triglycerides (TAGs) in a unilocular lipid droplet within adipocytes. WAT is also considered an endocrine organ that secretes adipokines to affect various processes including food intake and insulin sensitivity [1]. In contrast, brown adipose tissue (BAT) serves mostly as an oxidative tissue to regulate body temperature but also is beneficial to glucose and lipid homeostasis [2,3]. Brown adipocytes contain multilocular lipid droplets and abundant mitochondria with the unique protein Ucp1, which uncouples substrate

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oxidation from ATP synthesis to generate heat. In rodents, BAT is located primarily in the interscapular region, whereas WAT depots are found in various but specific regions in the body. More recently, “thermogenic” Ucp1 positive adipocytes, so called “beige” or “brite” cells, have been found in mainly subcutaneous WAT, following cold exposure or stimulation by β3-adrenergic agonists, drawing much attention due to their potential benefit in weight-loss [4–6]. This review will focus on the developmental origin of adipocytes, highlighting transcriptional and epigenetic control of brown and beige adipogenesis.

Developmental origin of WAT

Researchers have long puzzled over the origin of adipose tissue as well as its development. WAT is categorized into subcutaneous and visceral WAT. Subcutaneous WAT is found in inguinal (posterior) and intrascapular (anterior) regions, whereas visceral WAT is found in perigonadal (often referred as epididymal WAT in males), perirenal, epicardial, retroperitoneal, mesenteric and omental regions (Shown in Figure 1A). Subcutaneous and visceral WAT are believed to have distinct response mechanisms as well as health consequence upon high-fat diet-induced expansion [7–10]. In addition, subcutaneous as opposed to visceral WAT, is considered to be the major site of “browning” during cold exposure (will be discussed in detail in a later section). Moreover, although both subcutaneous and visceral WAT are believed to be of mesodermal origin, it has been unclear whether these WAT depots have the same origin or development.

Role of Pref-1 cells in adipose tissue development

To address the temporal and developmental origin of adipose tissue, Hudak et al. utilized lineage tracing with an inducible Preadipocyte factor 1 (Pref-1 or Dlk1) promoter coupled with two fluorescent reporters- H2BGFP for transient labeling and Rosa26-flox-stop-flox-tdTomato for permanent labeling in mice [11]. Pref-1 represents a useful tool for studying adipose tissue development since its expression is detected only in adipose precursors and is absent from differentiated adipocytes [12]. From transient labeling studies, it became apparent that Pref-1^+ cells first started appearing at E10.5 in mouse embryogenesis in the dorsal mesenteric region at the presumptive inguinal and dorsal subcutaneous depots. By E13.5 these cells formed a line at the dorsal edge of the embryo under the skin, were proliferative, but did not yet contain lipids. At E17.5, these precursor cells differentiated into lipid-containing adipocytes forming subcutaneous WAT. By E19.5, the number of lipid-filled cells more than doubled in this region, indicating hyperplasia as a mechanism for WAT expansion during embryogenesis. This temporal aspect of adipogenesis that occurs prenatally was confirmed using permanent labeling that showed that the lipid filled tdTomato positive cells in the subcutaneous region at E19.5 were descendants of Pref-1 cells. In contrast, no Pref-1 marked cells or lipid containing adipocytes were detected in the presumptive visceral WAT during embryogenesis in this study. It is not until P6 that Pref-1 cells were detected as lipid-laden adipocytes in the visceral WAT. This temporal difference in subcutaneous and visceral WAT development provided the first solid evidence that subcutaneous WAT starts its development perinatally, while visceral fat development takes place after birth. These findings are in agreement with studies utilizing the AdipoChaser model, an inducible system for permanent labeling with LacZ, relying on the adipocyte specific activity of the AdipoQ

promoter [13]. Regardless, in humans, since WAT is estimated to account for 16% of body weight of newborns, at least some of the adipose depots must develop prenatally [14]. Although genetic approaches cannot be used in humans, light microscopy examination showed the first traces of a fat organ between the 14th and 16th weeks of prenatal life [15].

Development of subcutaneous vs. visceral WAT

Given the temporal difference in the formation of subcutaneous versus visceral WAT, it is unclear whether they arise from the same or distinct precursors. However, a study reported that formation of six different visceral WAT depots, but not subcutaneous WAT or BAT, occurred from cells expressing Wilms Tumor 1 (Wt1) in late gestation [16]. This work also suggested that WAT depots associated with visceral organs have a mesothelial layer that serves as a source of adipocyte precursors, while, the subcutaneous depot was derived from cells marked by Prx1, a homeobox transcription factor expressed in embryonic limb and bud mesenchyme [17,18]. By lineage tracing using membrane fluorescent reporter, mT/mG, Myf5-Cre, which was thought to be active in muscle and interscapular BAT as described below, also labeled most or all cells of anterior subcutaneous and renal WAT, but not inguinal or perigonadal WAT [19]. Moreover, by utilizing Sox10-Cre and R26-YFP to trace neural crest cells, Billon et al. found in adult mice that cephalic WAT around salivary glands, but not subcutaneous, gonadal, perirenal or interscapular WAT, was labelled by Sox10-YFP$^+$ cells that co-expressed Perilipin, indicating that the craniofacial adipocytes arise from neural crest cells [20]. These studies, overall, highlight that a simplistic division of WAT into visceral and subcutaneous may need to be reconsidered. It is probable that different WAT depots may have different origins and even cells within the same adipose tissue may be heterogeneous in origin.

Adipose tissue expansion in adults

Numerous studies also investigated the origin of white adipocytes during adipose expansion in adults. Mural cells (pericytes and vascular smooth muscle cells) are mainly derived from mesodermal lineages and, within WAT, were shown first to be a likely pool of adipose progenitors [21–24].The evidence came from Tang et al. who utilized Pparγ locus driving GFP or LacZ expression that detected Pparγ$^+$ cells within blood vessels in adult WAT but not in other tissues [24]. These Pparγ$^+$ cells also closely resembled mural cells based on the expression of Pdgfrβ, neural/glial antigen 2 (Ng2) and smooth muscle actin (Sma). In a later study, the same group reported that Pparγ marked both developmental and adult WAT progenitors that derived from distinct lineages, but only adult adipose progenitors came from Sma$^+$ mural lineage [22]. However, Pparγ is known to be expressed in macrophages and dendritic cells [25,26] in addition to being master a regulator of adipogenesis [27]. Therefore, Pparγ lineage tracing gives some information about adipocyte origin, but potential contribution from macrophages and dendritic cells cannot be completely ruled out. Another study that followed cells expressing Zfp423, a zinc-finger transcription factor that regulates Pparγ expression, reported that adult adipose tissue was derived from not only mural cells but also from endothelial cells [28,29]. In a later study, Zfp423$^+$; Pdgfrβ$^+$ mural cells were shown to contribute to WAT hyperplasia in diet-induced obesity in a sex- and depot-dependent manner [30]. Additionally, two distinct pools of Perilipin$^+$ preadipocytes for both developmental and adult adipose expansion were identified but the majority of embryonic

preadipocytes were not derived from the Pdgfrβ$^+$ mural compartment [31], highlighting the importance of mural precursors for adult adipose tissue expansion. Recently, cells expressing Vstm2a, which is secreted from preadipocytes [32], were also associated closely with the vasculature, but interestingly, they did not express endothelial or mural markers. In addition, neural crest-derived adipose precursors were detected in subcutaneous WAT as well [33].

In addition to mural cells, other mesenchymal precursors have been shown to play a role in adult WAT expansion. Ablation of Pref-1$^+$ cells using diphtheria toxin in early embryogenesis resulted in a severe lack of adipose tissue postnatally, further indicating the importance of Pref-1 expressing mesenchymal precursors in embryonic and postnatal adipogenesis. Upon high-fat diet feeding, Pref-1$^+$ cells proliferated and contributed to WAT expansion through hyperplasia. However, Pref-1$^+$ adipose precursors did not express hematopoietic markers (Cd45) and were not of endothelial (Cd31$^-$, Ve-cadherin$^-$) or pericyte origin (Sma$^-$, Pdgfrβ$^-$ or Cd146$^-$) [11]. Permanent dual labeling with Tie2-GFP and Pref-1-tdTomato also showed that those cells labeled by Pref-1 were not co-stained with Tie2-GFP that labels vasculature, further excluding an endothelial origin of Pref-1$^+$ cells. This study showed that Pref-1 labels a population of early mesenchymal adipose precursors that do not coincide with precursor/preadipocyte populations associated with the vasculature. Similarly, Pdgfrα$^+$ adipose precursor cells that give rise to all adult WAT depots and proliferate in visceral WAT upon high-fat diet [34,35], albeit in close proximity to capillaries, were negative not only for Pparγ but also for Sma, Pdgfrβ, and the endothelial marker, isolectin Ib4 [34]. These data indicate that the Pdgfrα$^+$ progenitor cell population may reside outside of the mural compartment. These studies, overall, suggest that very early precursors expressing Pref-1 may migrate closer to the vasculature upon loss of Pref-1 and acquire mural or endothelial markers and then become Pdgfrα expressing cells. Alternatively, the mural cells or those cells in the vasculature may represent a different progenitor pool from Pref-1 or Pdgfrα positive pools. However, the exact relationship between adipose cell populations traced by various markers and their relative contribution to each WAT depot in development and in adults in various conditions still need further investigation.

Isolation of adipose precursors in SVF of adult WAT

In adipose tissue, in addition to adipocytes, there are multiple other types of cells, such as preadipocytes or adipose precursors, stem cells, fibroblasts, endothelial cells, macrophages, and leukocytes, often collectively called SVF based on the separation from lipid-containing adipocytes. Heterogeneity within SVF has long been an obstacle in isolating and characterizing pure precursor populations. Fluorescence activated cell sorting (FACS) using several stem cell markers allowed researchers to enrich SVF for the precursor/progenitor population and, thus, sorting for Lin$^-$ (Cd31$^-$, Cd45$^-$, Ter119$^-$) eliminated the majority of endothelial, hematopoietic cells and erythrocytes [36]. However, it is not clear whether these markers may label cells at different stages of differentiation, such as stem cells, committed preadipocytes, or those cells at early stage of differentiation.

Rodeheffer et al. isolated a Lin^{-}: $Cd29^{+}$: $Cd34^{+}$: $Sca1^{+}$: $Cd24^{+}$ population of proliferating adipose precursors that gave rise to $Cd24^{-}$ cells *in vivo* during adipogenesis [36]. In a later study, $Cd24^{-}$ cells of the precursor population were shown to represent preadipocytes that express Pparγ and C/ebpα, key adipogenic transcription factors [35]. Since $Pdgfr\alpha^{+}$ labeled both $Cd24^{+}$ and $Cd24^{-}$ precursor populations in WAT, isolation of Lin^{-}: $Pdgfr\alpha^{+}$ cells may represent a strategy to enrich for adipocyte precursors in adipose tissue [37,38]. In an attempt to characterize Pref-1^{+} cells in the context of the adipogenic lineage, Hudak et al. performed gene expression and immunostaining analysis of Pref-1^{+} cells and showed their mesenchymal origin ($Sox9^{+}$, $Cd29^{+}$, $Sca1^{+}$, $Cd105^{+}$ and $Cd34^{+}$) [11]. The transiently labeled Pref-1^{+} cells did not yet express the adipogenic transcription factor Pparγ or adipose commitment factor Zfp423, but were proliferative precursors based on expression of Cd24 and Ki-67 and incorporation of BrdU. Some of the permanently labelled Pref-1 cells had lipid laden morphology and expressed Zfp423, Pparγ and C/ebpα indicating that Pref-1 cells indeed are adipose precursors. Additionally, when SVF from Pref-1-tdTomato mice was injected into SCID mice, after 2 weeks these cells differentiated into adipocytes with a characteristic gene expressing signature. These findings suggest that Pref-1 precursors represent a very early stem cell-like population and highlight the usefulness of Pref-1 as a marker for FACS or conditional ablation from adipose precursors at early stages of adipogenesis. Further research will shed light on when and under which physiological and molecular cues Pref-1 expression is lost in order for adipogenesis *in vivo* to proceed. Overall, these studies suggest that adipose SVF contains a hierarchy of progenitor populations with different degree of progression from adipose commitment to differentiation. Additionally, obese individuals show excessive fibrosis in WAT [39,40]. It has recently been reported that myofibroblasts arising from diet-induced obesity came from highly proliferative $Cd9^{high}$, $Pdgfr\alpha^{+}$ cells with high Pref-1 expression. In contract, $Cd9^{low}$, $Pdgfr\alpha^{+}$ population was enriched for Pparγ and C/ebpα, with low Pref-1 expression, having low proliferative capacity but high adipogenic potential [41]. Overall, the relationship between cell populations identified based on the expression of various markers needs further investigation. Identification and characterization of stage specific markers may help to isolate and define various precursor populations and their relationship during WAT development.

Developmental origin of BAT

Given the morphological and functional differences between brown and white adipocytes, these two types of adipocytes may have different developmental origins. Indeed, interscapular BAT (iBAT) formation in mice starts earlier than WAT during embryogenesis and BAT is fully thermogenically-competent at birth, providing a defense mechanism against cold stress in newborns. As early as at E9.5, cells expressing engrailed 1 (En1), a homeobox domain containing gene that marks the central dermomyotome, were shown to give rise to iBAT [42]. Moreover, cells that express En1 at E10.5-11.5 gave rise to iBAT as well as dermis and epaxial muscle, indicating a very early specification of cells to BAT and that BAT and muscle cells may share the same progenitors in early development. The next evidence came from a study by Timmons et al. where, by microarray analysis of primary preadipocytes, they found that brown fat preadipocytes had a myogenic transcription

signature (expressing transcription factors for muscle differentiation, such as MyoD and Myf5), differing from immortalized adipogenic cell lines [43]. Seale and colleagues then utilized mice harboring Myf5-Cre coupled with R26R3-YFP and found that Myf5 expressing cells gave rise to not only muscle but also iBAT and perirenal BAT [4]. Later, a study with Myf5-Cre coupled with mT/mG, showed that Myf5$^+$ cells also gave rise to some WAT depots in the anterior subcutaneous, interscapular subcutaneous, and retroperitoneal regions. However, not all BAT depots may come from Myf5$^+$ cells, with only interscapular and subscapular BAT being fully labeled by Myf5-Cre, and only a population of cells in cervical BAT and none from periaortal or perirenal BAT [19]. Additionally, a study using a CreER^{T2} knock-in allele at the paired-homeodomain transcription factor- Pax7 locus combined with a LacZ reporter, showed that somatic Pax7 expressing cells marked at E9.5 gave rise to dorsal dermis, BAT, trunk muscle, and diaphragm muscle. After E12.5 these cells become restricted to the muscle lineage [44]. Moreover, although Pref-1 is required for WAT development and expansion, only a few cells labeled by Pref-1 were detected in BAT, further indicating an early divergence of BAT precursors in development prior to Pref-1 expression [11]. Overall, it can be viewed that interscapular and subscapular BAT and muscle originate from common Myf5$^+$, Pax7$^+$ progenitors, while brown adipocytes from periaortic and perirenal BAT as well as the most well studied WAT depots, such as inguinal and epididymal do not share the same developmental origin.

“Browning” of WAT

Typical adipocytes in WAT have a unilocular lipid droplet morphology and few mitochondria (Summarized in Figure 1B). Upon cold exposure or β-adrenergic stimulation, some cells in WAT acquire Ucp1 expression and have multilocular lipid droplets and abundant mitochondria. Thus, WAT may undergo “browning” with the appearance of thermogenic “beige” or “brite” adipocytes that share some similar features with brown adipocytes [4–6]. Historically, the first evidence of existence of these multilocular adipocytes in WAT came from a study in 1984, in which cold acclimation caused the appearance of brown fat like cells in the parametrial WAT depot of female BALB/c mice judged by morphology in EM and presence of Ucp1 [45]. Later in 1992, a report showed Ucp1 expression in various WAT depots of rats, that was induced by cold exposure [46]. Furthermore, mouse subcutaneous inguinal WAT depot is much more susceptible to “browning” even with mild stimulation compared to visceral epididymal WAT. Additionally, capacity to “brown” depends on the mouse strain: the A/J strain showed a marked induction in Ucp1 in WAT depots following β3-adrenergic stimulation compared to C57BL/6J [47].

Transdifferentiation as a potential mechanism for “browning”

A study by Himms-Hagen et al. showed that multilocular brown-like adipocytes in retroperitoneal WAT following a 7-day treatment with β3-adrenergic agonist- CL-316,243, did not incorporate BrdU [48]. The authors concluded that these brown-like adipocytes did not derive from actively proliferating cells, and rather came from conversion of white adipocytes. Consistently, 95% of brown-like multilocular cells with numerous mitochondria also did not incorporate BrdU in retroperitoneal WAT in rats following β3-adrenergic stimulation [49]. More recently, Rosenwald et al. showed the appearance of Ucp1$^+$ cells in

ingWAT after one week cold exposure [50] by using two mouse models, Ucp1-GFP for transient labeling of Ucp1 expressing cells and Ucp1-CreER-ROSA-tdRFP for permanent labeling. They also reported that beige/brite adipocytes were not eliminated by apoptosis and reverted to a white adipocyte unilocular morphology with characteristic gene expression after 5 weeks of warm adaptation. Furthermore, if subjected to a one-week cold exposure again, these cells could convert back to beige/brite cells. However, after restimulation, only half of the beige/brite adipocytes detected were from warm–adapted white, but previously beige adipocytes. These data suggested that in addition to transdifferentiation some of the beige adipocytes may have formed from *de novo* differentiation of precursor cells. In line with this evidence for transdifferentiation, another study showed that most if not all beige/brite adipocytes in ingWAT, but not abdominal WAT, following cold exposure did not arise from recruitment or proliferation precursors and rather came from unilocular adipocytes [51]. All together, these studies showed transdifferentiation to be a mechanism for "browning" of WAT.

De novo differentiation of beige/brite adipocytes

In contrast to the concept of transdifferentiation, various researchers have reported that, upon cold exposure or β3-adrenergic stimulation, a subset of SVF cells isolated from WAT by using different markers, such as Pdgfrα and Ebf2, proliferate and become Ucp1$^+$ beige or brown-like cells [34,52]. The use of the AdipoChaser model also revealed that most of the "browning" in subcutaneous WAT following cold exposure or β3-adrenergic stimulation occurred from *de novo* adipogenesis [13]. Overall, these studies indicate the presence of precursors cells in WAT that upon cold or β3-adrenergic stimulation may undergo browning and acquire Ucp1 expression. Are beige precursors distinct from typical white or brown adipocyte precursors? Unlike brown adipocytes, the Ucp1$^+$ cells in WAT were originally believed to come from a Myf5$^-$ lineage [4]. However, a more recent study using Myf5-Cre coupled with fluorescent reporters (R26R3-YFP, R26R-LacZ or mT/mG) showed that beige/brite adipocytes can come from Myf5$^+$ or Myf5$^-$ lineages depending on the WAT depot and the type of stimulation [4,19]. To understand the molecular identity of beige precursor cells, Wu et al. isolated clonal lines from ingWAT SVF to compare gene expression signature of adipogenic clones. This analysis revealed the existence of a subset of cells in ingWAT that showed gene expression patterns more similar to *bona fide* brown fat cell lines than other ingWAT cell lines, suggesting the presence of a distinct pool of progenitors that generate "beige" cells in ingWAT that are more similar to classical BAT progenitors. These "beige" progenitors in basal conditions had low expression of thermogenic genes. However, upon stimulation with cAMP, they responded by an increase in Ucp1 expression to levels similar to brown fat cells, having enhanced respiration [53]. Additionally, Pdgfrα$^+$ cells from abdominal WAT, which represent white adipose precursors, can also become Ucp1$^+$ cells in response to β3-adrenergic stimulation but only in abdominal WAT [34]. Early B-cell factor 2 (Ebf2), a transcription factor critical for BAT development, was shown also to mark SVF cells in ingWAT capable of acquiring Ucp1 upon differentiation in culture [52]. The number of these Ebf2$^+$ cells in ingWAT increased upon cold-exposure contributing to *de novo* beige adipogenesis. In another study, beige/brite adipocytes were shown to share a molecular signature with smooth-muscle cells not observed in classical brown adipocytes [54]. A fate-mapping approach based on the myosin heavy chain 11 (Myh11) promoter active in smooth

muscle or smooth muscle-like cells marked some of the beige adipocytes in ingWAT following 2-weeks of cold exposure [54]. Gene expression analysis from Myh11$^+$ cells of WAT revealed that these cells were not enriched in pericyte, endothelial or hematopoietic markers, but they expressed some precursor/preadipocyte genes (Sca1, Pdgfrα, Zfp423). The authors concluded that the smooth muscle lineage may overlap with previously described preadipocyte populations. Another study showed that cells expressing the mural marker Pdgfrβ which has been demonstrated to play a role in WAT adipogenesis were also recruited to become beige adipocytes after prolonged cold exposure [30]. Interestingly, after a short-term cold exposure (1 week), however, very few beige cells came from Pdgfrβ$^+$ cells, indicating that distinct "browning" mechanisms may exist in short versus long term cold exposure. However, it remains unclear whether they represent a distinct pool or share precursors with classical WAT or BAT cells. Interestingly, it has also been reported that, following cold exposure, eWAT exhibited an increase in adipogenesis; however, the newly formed adipocytes in this depot appeared to be Ucp1$^-$ further proving evidence that "browning" of WAT is depot- dependent [13]. Together these studies suggest the presence of a subpopulation within WAT precursors that is capable of differentiating into Ucp1$^+$ adipocytes. Further investigation is needed in order to establish markers that distinguish this cell population predisposed to "browning" to affect energy metabolism *in vivo*.

How can such a discrepancy in the origin of beige/brite adipocytes be explained? Potentially, different degrees of innervation exist between subcutaneous and visceral WAT and may play a role in different mechanisms governing "browning" between the two depots. Indeed, Granneman and coworkers reported that Pdgfrα$^+$ precursors from abdominal WAT proliferated to become Ucp1$^+$ cells, while brown-like adipocytes emerged in subcutaneous WAT (ingWAT) upon stimulation were from transdifferentiation of unilocular white adipocytes [34,51]. Regardless, results explaining either transdifferentiation or *de novo* adipogenesis for "browning" may partly arise due to technical limitations such as caveats associated with long lasting effect of tamoxifen when using Cre-ER or difficulty in substrate penetrance for β–galactosidase when using LacZ reporter mice [5,51]. Overall, all these studies point to the emerging complexity in the origin of brite or beige adipocytes. The mechanism of their formation may be highly dependent on the mouse strain and sex, the specific WAT depot analyzed, as well as the specific stimulation applied. Better studies using lineage-tracing and co-labeling with multiple precursor markers may help determine the relative contribution of each mechanism.

Human BAT: beige or brown?

Is human BAT considered beige or classical brown? While BAT as an organ was originally described in 1551 [55], it was not described to be present in all mammals until the 20th century. The presence in humans was discovered almost 40 years ago but was limited to outdoor workers, skid row alcoholics, and those with pro-brown adipogenic tumors [56,57]. Indeed, a breakthrough in 2009 showed that BAT in humans is either widespread or universal under cold stimulus and could be metabolically relevant[58–63]. In infancy, human BAT is localized to interscapular and perirenal depots, the molecular signature of which closely resembles classical rodent iBAT [60]. Recent studies suggest that in adult humans UCP1$^+$ adipocytes can be found in various depots around the body that are typically

heterogeneous, also containing UCP1^{-} adipocytes. Brown-like adipocytes have been detected in the supraclavicular region, being the most enriched for UCP1^{+} adipocytes, and also around the aorta, carotid artery and subscapular region, as well as others [2,6]. Whether these UCP1^{+} adipocytes in human adults represent beige or true brown adipocytes remains controversial. On the gene expression level, supraclavicular UCP1^{+} adipocytes more closely resembled mouse beige adipocytes. Lee et al. showed that these adipocytes expressed some common BAT markers such as UCP1, PGC1α, PRDM16 and DIO2, but not MPZL2 which is thought to only be expressed in classical iBAT of rodents [64]. On the other hand, these cells expressed beige fat-specific genes such as Tmem26 and Hoxc9. Another study confirmed the "beige-like" molecular signature of human UCP1^{+} adipocytes and discovered novel markers K3 (KCNK3) and mitochondrial tumor suppressor 1 (MTUS1) to be enriched in human beige cells versus white adipocytes [65]. However UCP1^{+} adipocytes from other human depots such as cervical and perirenal regions, showed expression of classical BAT markers, ZIC1 and LHX8 [66,67]. All these studies point out heterogeneous composition of human BAT as well as an emerging need for novel markers that will allow researchers to clearly distinguish classical BAT vs beige cells.

Transcriptional regulation of BAT development and "browning" of WAT

Since BAT mass is inversely correlated with BMI in humans, increasing BAT activity could be a promising strategy for weight-loss and management of obesity-associated diseases [2,68]. With greatly higher mass of WAT in comparison to BAT, increasing WAT "browning" may improve insulin sensitivity and reduce weight gain under high fat diet as shown in mice [3,69–73]. This section summarizes the transcriptional regulation involved in BAT development and "browning" of WAT. The well-established transcriptional program for white adipocyte differentiation has been extensively reviewed elsewhere (Rosen and Spiegelman, 2014; Farmer, 2006).

General adipogenic transcription factors, Prdm16, and Zfp516

Early work describing the transcriptional regulation of BAT centered on the norepinephrine-β3-adrenergic receptor-cAMP- cyclic AMP response element binding protein (CREB)/ p38 MAP kinase axis central to the response to cold. Thus, several target genes of this signaling pathway have been described such as Pparγ-coactivator 1α (Pgc1α), CCAAT-enhancer binding protein β (C/ebpβ), diodinase 2 (Dio2) as well as Ucp1 itself [74–76]. Further work showed that BAT gene regulation requires general adipogenic machinery including peroxisome proliferator-activated receptor gamma (Pparγ), RXR, and the aforementioned C/ebpβ [77]. Importantly, by RT-qPCR analysis of WAT and BAT to identify transcription-related genes enriched in BAT, Seale et al. first identified Prdm16 as a BAT-enriched coregulator of the BAT gene program [78]. Prdm16 interacts with a wide variety of transcription factors and cofactors including C/ebpβ, C-terminal binding protein 1 and 2 (Ctbp1, Ctbp2), histone deacetylase 1 and 2 (Hdac1/2), mediator complex subunit 1 (Med1), Pgc1α, Pparγ, and Zfp516 as well as epigenetic regulators-euchromatic histone lysine methyltransferase 1 (Ehmt1) and lysine specific demethylase 1 (Lsd1) [71,78–84]. Interestingly, Harms et al. showed that while Prdm16 is required for maintenance of brown adipocyte identity, Prdm16 is not required for BAT development. While there may be some

compensatory activity of Prdm3, prenatal development was not affected by Prdm3/Prdm16 double knockout [85]. Med1 that interacts with Prdm16 may mediate the active chromatin structure at BAT specific genes bringing distal enhancers to proximity to the transcription start site facilitating transcriptional activation [86]. However, markers of active transcription such as H3K27ac were only mildly affected by Prdm16 knockout. Interestingly, Prdm16 is not regulated by cold and must rely on interacting factors to facilitate Prdm16-mediated BAT gene induction. One such factor is Zfp516, which is induced upon cold exposure. Zfp516 binds and activates the Ucp1 promoter in response to cold to upregulate Ucp1[71]. In this regard, Zfp516 KO embryos show dramatically reduced BAT mass, while mice overexpressing Zfp516 in adipose tissue demonstrate enhanced “browning” of WAT even at room temperature. Although Zfp516 directly binds to Prdm16 and may be responsible for cold-inducible Prdm16-mediated activity, Dempersmier et al. showed that Zfp516 can induce Ucp1 expression in the absence of Prdm16. Perhaps in the absence of Prdm16, Zfp516 binding to a Prdm16 related protein, such as Prdm3, or a yet to be identified factor, may drive BAT gene expression.

Other factors involved in the cold-inducible regulation of Ucp1 include Pgc1α, a cofactor known to regulate mitochondrial biogenesis [87]. However, Pgc1α-deficient adipose tissue shows only a mild thermogenic defect [88]. Another cold inducible factor, Irf4 interacts with Pgc1α to drive expression of Ucp1, and Irf4-deficient mice have a greater thermogenic deficiency [89]. However, Irf4 has been shown to be important for general adipogenesis and thus, the BAT specific role is unclear [90]. Recent work identified a critical role for histone deacetylase 3 (Hdac3) in basal and cold-inducible activation of thermogenic gene promoters including Ucp1. While normally working as a transcriptional repressor, Hdac3 was shown to deacetylate Pgc1α resulting in activation of thermogenic gene transcription even in the absence of thermogenic stimuli [91]. Another factor recently identified to play a role in regulation of thermogenic gene expression through inducing Pgc1α in response to acute cold exposure is the sirtuin, Sirt6. Yao et al. found that Sirt6 was induced by cold where it interacts with phosphorylated activating transcription factor 2 (Atf2) to drive Pgc1α promoter activity. Adipocyte specific Sirt6 knockout mice had significantly reduced Pgc1α levels as well as Pgc1α binding to the Ucp1 promoter. Interestingly, Sirt6 ablation did not result in decreased Pgc1α acetylation, which has been shown to be important for Pgc1α transcriptional coactivity, and the authors did not examine the role of Sirt6 deacetylase activity in regulating Pgc1α promoter activity[92].

Ebf2 as a pioneer factor for the BAT gene program

While analyzing differential binding of Pparγ in WAT and BAT, Rajakumari et al. found that BAT-specific Pparγ response elements (PPRE) coincide commonly with early B-cell factor *cis*-elements and thus identified a critical role of Ebf2 recruiting Pparγ to the Prdm16 promoter for Prdm16 transcription [93]. Further work identified Dpf3 as an Ebf2-interacting histone reader, which identifies genes of the BAT program, leading to Ebf2-mediated recruitment of the chromatin remodeler, Brg1, resulting in opening of chromatin for transcription [94]. Thus, Ebf2 appears to be working as a pioneer factor during adipogenesis leading to activation of the BAT gene program.

Transcription factors specifically regulating “browning”

While most of the transcriptional regulators mentioned above are common for brown and beige adipogenesis in order to induce thermogenic genes such as Ucp1, several genes were reported to be specifically important for induction or repression of “browning”. For example, Myocardin-related transcription factor A (Mrtfa) was shown to inhibit beige adipocyte differentiation, and Mrtfa KO mice show an increased number of beige adipocytes in WAT. These mice were protected from diet induced obesity and insulin resistance [95]. Additionally, Smad3, a key mediator of Tgfβ signaling was shown to inhibit beige adipocyte differentiation [96,97]. Klf11 was reported to activate selective beige gene expression by cooperation with Pparγ at superenhancers [98]. Interestingly, transcription factor Hes1, which is activated by Notch signaling, repressed Prdm16 and Pgc1α transcription during beige but not classical brown adipogenesis [99]. These studies describe common as well as distinct transcriptional mechanisms involved in BAT and beige adipogenesis. Given that not all brown and beige cells have the same origin, it is tempting to speculate that tissue-specific early developmental transcription factors may exist to establish brown versus beige cell differentiation and development.

Epigenetic control of the BAT gene program

As in most biological processes, interactions between genes and the environment, such as temperature or diet [100,101], may influence BAT gene expression and thermogenesis, by involving epigenetic events, i.e., heritable changes in traits without changes in DNA sequence. The broad umbrella of epigenetics research includes both DNA and histone modifications as well as microRNA and long noncoding RNA (lncRNA) either inhibiting or enhancing transcription. In this regard, DNA is wrapped around histone proteins- the building block of the nucleosome (the histone octamer core contains 2 copies of H2A, H2B, H3 and H4) the structure of which is critical for regulation of transcription [102,103]. Histone modifications, such as methylation and acetylation, affect transcription by altering nucleosome compaction, changing the chromatin landscape, and thus DNA accessibility of transcription factors and co-regulators [104,105]. For example, H3K4me3 is a well-recognized hallmark of transcription activation, whereas H3K9me3 represents a repressive mark, all at the tail region of H3 [106,107]. The field of BAT epigenetics has been extensively reviewed of late [108–112] and this section focuses on writers and erasers of histone methylation marks, specifically, methyltransferases and demethylases, which are critical components of epigenetic regulation.

Mll3/4

Lysine Methyltransferase 2C (KMT2C, Mll3/4), a H3K4 methyl transferase, has been shown to be involved in adipogenesis, and mice with catalytic dead mutations show reduced adiposity. However, recent studies into the role of Mll3/4 in immortalized brown adipocytes found that Mll3/4 identifies critical super enhancers for BAT and general adipogenic genes and recruits Cbp/p300 to poised enhancers to drive transcription [113,114]. Interestingly, this study showed a broad spectrum of histone modifications in both brown preadipocytes and brown adipocytes, with differential patterning of H3K4me1/2, H3K9me2, H3K27me3,

H3K27ac, and H3K36me3 implicating many yet to be identified histone methyltransferases in this process.

Ehmt1

Ehmt1, identified as a binding partner for Prdm16, catalyzes the methylation of H3K9 di- or tri-methylation (H3K9me2/3). Loss of Ehmt1 resulted in loss of BAT gene expression and decreased BAT tissue mass [81]. However, the proposed mechanism of action was not due to the methylation activity, but due to stabilization of Prdm16 protein, a mechanism proposed for the Pparγ agonist and "browning" agent, rosiglitazone [115]. Thus, the relative importance of Prdm16 stabilization versus the potential Ehmt1 demethylase activity in the BAT gene program remains to be studied.

Lsd1

Another H3K9 demethylase, that has been shown to regulate the BAT gene program by histone modification, is lysine-specific demethylase 1 (Lsd1) which catalyzes the demethylation of mono- and dimethylated H3K9. Through direct interaction with Zfp516, Lsd1 is recruited to BAT gene promoters to promote transcription *in vivo* [82]. Indeed, BAT of Lsd1 KO mice using Ucp1-Cre showed reduced Ucp1 expression, which accompanied increased H3K9 mono and demethylation at the proximal Ucp1 promoter, a site where both Lsd1 and Zfp516 were bound. Therefore, BAT-specific Lsd1 ablation compromised BAT gene expression as well as development of BAT, with the tissue resembling WAT with reduced thermogenic activity. Thus, Ucp1-driven Lsd1 ablation resulted in obesity with impaired glucose tolerance upon high fat feeding. Interestingly, later work by Zeng et al. identified an LSD1 complex containing Zfp516 and Prdm16 as well as Ctbp1/2, Hdac1/2 and others [84]. This complex was found to suppress WAT-specific genes by colocalizing in regions of H3K4me1 demethylation. Another group identified an Lsd1 interaction with Nrf1 to drive mitochondriogenesis and thermogenic transcription in brown adipocytes maintaining their BAT identity [116].

Jhdm2a

Jumanji-C domain containing histone demethylase 2A (Jhdm2a, also known as Jmjd1a), a mono-and di-methyl H3K9 demethylase, was originally identified as a regulator of thermogenic gene expression when a global knockout, generated to identify the role of Jhdm2a in spermatogenesis, developed an obese phenotype. A closer examination of these mice showed that Jhdm2a was recruited to the Ucp1 promoter during cold and isoproterenol treatment to remove H3K9 methylation resulting in promoter activation [117,118]. Interestingly, later work showed that the H3K9 demethylation activity of Jhdm2a may actually be a secondary activity and that Jhdm2a primarily functions as a PKA-mediated, phosphorylation-dependent scaffold protein that interacts with SWI/SNF complex members Arid1a, Brg1, and Baf60b as well as Pparγ in multiple locations of target promoters. Interestingly, non-phosphorylatable Jhdm2a mutants were unable to be recruited to target promoters resulting in decreased UCP1 expression and activity *in vitro* and *in vivo* [119]. The authors hypothesized that this may be facilitating long-range chromatin interaction, but further work using chromosome conformation capture (3C) needs to be done to validate these claims.

Demethylation of trimethylated H3K27: potential players

Several recent papers have identified the importance of the demethylation of trimethylated H3K27. Interestingly, two different enzymes have been attributed to be responsible for this activity, Jmjd3 and ubiquitously transcribed tetratricopeptide repeat on chromosome X (Utx). In cells, knockout of Jmjd3 as well as chemical inhibition of Jmjd3 enzymatic activity results in a decrease in Ucp1 mRNA and protein. However, *in vivo*, transgenic expression or chemical inhibition of Jmjd3 only affected Ucp1 expression in aged mice [120]. Similarly, Utx, which increases during brown adipogenesis and cold exposure, was found to decrease H3K27me3 at the Ucp1 enhancer and transcription start site (TSS). It is proposed that Utx then recruits Cbp to acetylate H3K27 thereby promoting BAT gene transcription [121]. This pro-thermogenic program of Utx is antagonized by the activity of Hdac1, which deacetylates H3K27 and recruits Ezh2 and Suz12 to facilitate H3K27 methylation while preventing Utx binding [122]. However, both studies into the function of Utx were performed *in vitro* so the relative contributions of Jmjd3 and Utx *in vivo* have yet to be discerned. Given the overlap in functionality, the lack of a strong phenotype in the Jmjd3 knockout mice could be due to compensatory activity of Utx. Further studies are needed to verify this hypothesis. These studies, overall, indicate a critical role for both transcriptional and epigenetic regulation of thermogenic gene expression. Further studies will provide molecular details of the chromatin modifications required for the thermogenic gene program (See figure 2).

Future directions

Understanding WAT and BAT development and the underlying mechanism to promote “browning” of WAT may provide targets for combating and preventing obesity and associated diseases. For some of the markers of adipose precursors recently identified, further investigation is needed to establish their contribution in embryogenic versus postnatal adipogenesis. Better FACS using multiple markers coupled with immunostaining and lineage tracing approaches will be needed. Moreover, single cell level genomic studies would allow the study of the possible mosaic developmental origin of WAT as well as potential mechanisms for “browning” of WAT. Additionally, many of the studies have focused on a specific depot or condition, e.g. adipose expansion during high fat diet or “browning” after cold exposure. Given that different WAT depots may have different developmental origins it would be beneficial to examine those markers in various depots and different conditions. In addition, as various “browning” mechanisms may exist depending on the type of stimulus applied, the relative contribution of transdifferentiation versus *de novo* differentiation need to be assessed under different conditions. Moreover, the relationship among transcription factors involved in transitioning from completely “closed” silent chromatin to open/poised chromatin in brown/beige adipocytes needs further investigation. With striking differences in histone modifications during brown/beige adipose development, the enzymes catalyzing these modifications and their contribution to thermogenic gene regulation remains to be elucidated.

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List of abbreviations

WAT	white adipose tissue
BAT	brown adipose tissue
Ucp1	uncoupling protein 1
Pref-1	preadipocyte factor 1
TAG	triglycerides
H2BGFP	histone2B fused green fluorescent protein
EM	electron microscopy
BMI	body mass index

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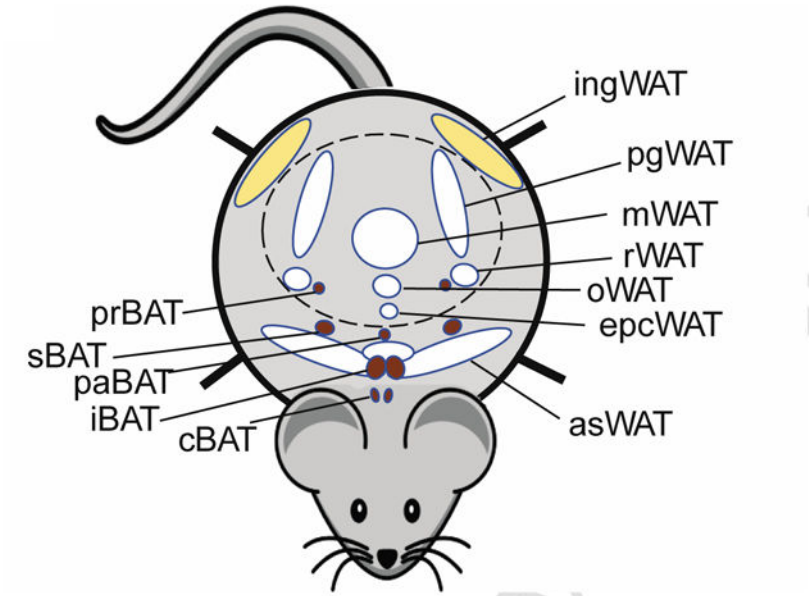

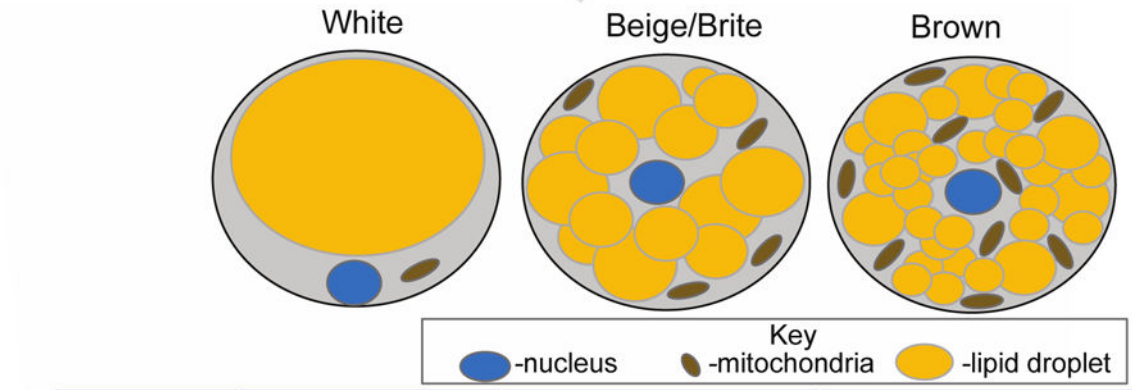

	White	Beige/Brite	Brown
UCP1 expression	-	+	+
Mitochondria Density	Low	High	High
Lipid droplet	One large	Multilocular	Multilocular
Function	Energy storage Endocrine (Leptin, AdipoQ, Resistin)	Thermogenic Endocrine?	Thermogenic Endocrine (Fgf21)

Figure 1.

Anatomical location of murine adipose tissue and three types of adipocytes. A. Anatomical location of mouse white adipose tissue (W AT) and brown adipose tissue (BAT). ingWAT-inguinal WAT also known as posterior subcutaneous, pgW AT-perigonadal WAT, rWAT-retroperitoneal WAT, mWAT-mesenteric WAT, oWAT-omental WAT, epcWAT- epicardial WAT, asWAT- anterior subcutaneous WAT. pgWAT, rWAT, mWAT, oWAT, epcWAT are collectively called visceral WAT, while ingWAT and asWAT are combined into subcutaneous

WAT. prBAT- perirenal BAT, sBAT-subscapular BAT, paBAT-periaortal BAT, iBAT-interscapular BAT, cBAT-cervical BAT.
The peritoneum is depicted as a dotted line. B. Functional and morphological differences between 3 types of adipocyte: white, beige/brite and brown.

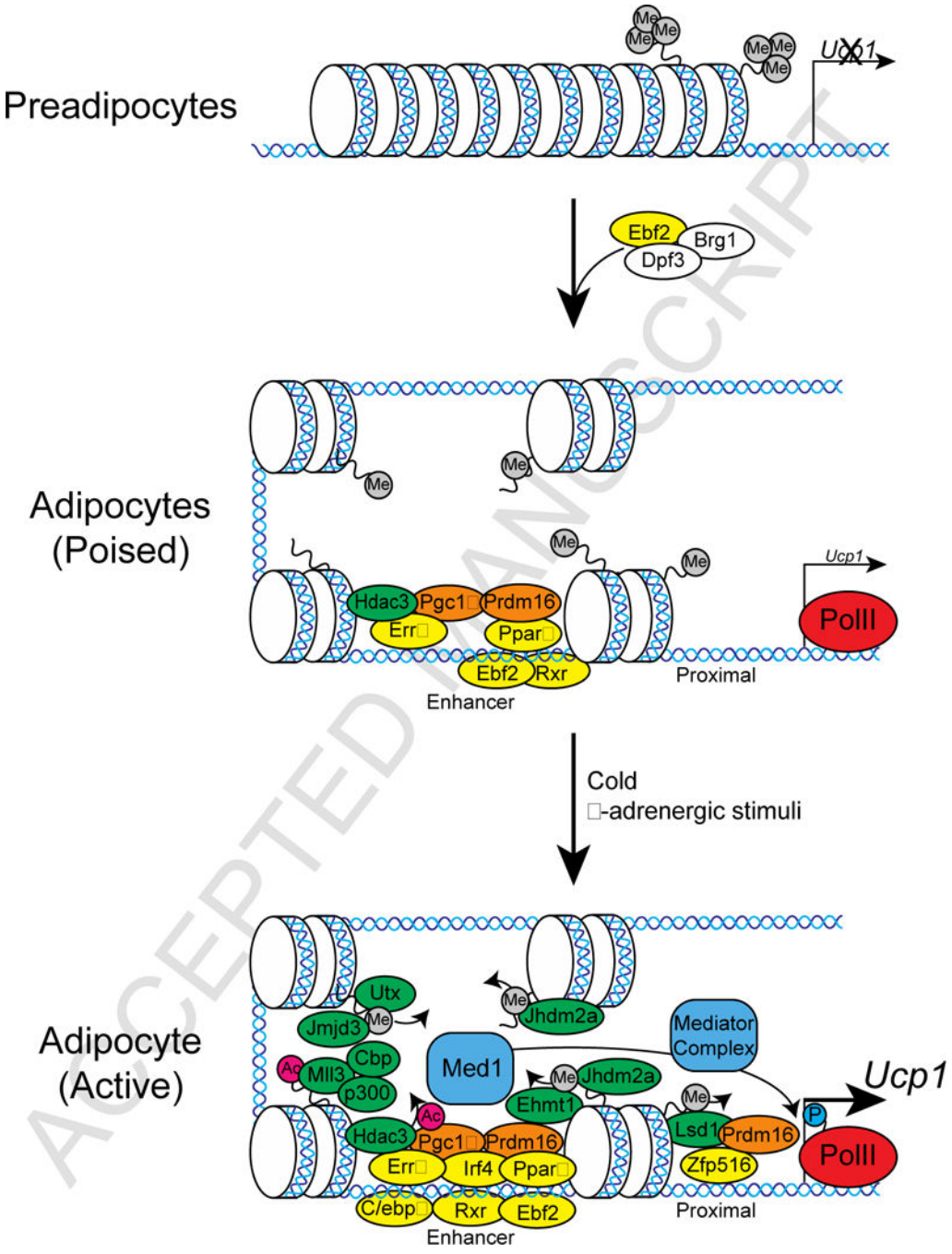

Figure 2.

Graphical Representation of Transcriptional and Epigenetic Regulators binding to the UCP1 promoter in beige/brown adipocytes. The UCP1 promoter is silent in preadipocytes. During differentiation, chromatin remodeling occurs and basal transcriptional machinery is recruited to the UCP1 promoter (poised). During a cold challenge, further transcriptional activators are recruited for the activation of the UCP1 promoter (active).

Table 1

Genes involved in adipose tissue expansion and development

Marker	Embryonic origin	Tissue marked	Expression in adult			Ref
			Precursor	Preadipocyte	Adipocyte	
Cd24	Mesoderm	WAT	+	–	–	35,36
En1, Pax7	Dermomyotome	iBAT and muscle	+	?	–	42,44
Myf5	Dermomyotome	iBAT, muscle, asWAT	+	?	–	4,19,43,123
Ng2	Dermomyotome	Subcutaneous WAT	?	+	–	24
Pdgfrα	Mesoderm	Subcutaneous and visceral WAT, BAT?	+?	+	–	34,35,51
Pdgfrβ	Mesoderm	ingWAT, visceral WAT, retroperitoneal WAT	?	+	–	24,30,31
Perilipin	Mesoderm	WAT?	–	+	+	31
Pref-1	Mesoderm	Subcutaneous and visceral WAT	+	+	–	11
Pparγ	Mesoderm	All WAT and BAT	–	+	++	24
Prx1	Embryonic limb and bud mesenchyme	Subcutaneous WAT	+	?	–	17
Sma	Dermomyotome	Subcutaneous and visceral WAT	?	+	–	22,24
Vstm2a	Mesoderm	?	–	+	–	32
Wt1	Mesothelium	Visceral WAT	+	++	–	16
Zfp423	Mesoderm	?	–	+	+	28–30

Table 2

Genes involved in the genetic and epigenetic regulation of thermogenic transcription

Gene	Activity	Promoter State	Reference(s)
Brg1	Chromatin remodeler	Poised/Active	94
C/EBPβ	DNA binding	Active	74–78
Creb	DNA binding	Active	74–76
Ctbp1	DNA binding	Poised/Active	79,84
Ctbp2	DNA binding	Poised/Active	79,84
Ebf2	DNA binding	Poised/Active	93,94
Ehmt1	H3K9 methyl transferase	Active	81,115
Errα	DNA binding	Poised/Active	91
Hdac1	H3K27 deacetylase	Poised/Active (?)	84,122
Hdac3	Pgc1a Deacetylase	Poised/Active	91
Hes1	DNA binding	Poised/Active	99
Irf4	DNA binding	Active	89,90
Jhdm2a	H3K9 demethylase	Active	117–119
Jmjd3	H3K27 demethylase	Active	120
Klf11	DNA binding	Poised/Active (?)	98
Lsd1	H3K9 demethylase	Active	82,84,116
Med1	Cofactor	Active	86
Mll3/4	H3K4 methyl transferase	Active	113,114
Mrtfa	DNA binding	Poised/Active	95
Pgc1α	Cofactor	Poised/Active	64,71,74–76
Pparγ	DNA binding	Poised/Active	71,77–84
Prdm16	Cofactor	Poised/Active	71,78–86
Rxr	DNA binding	Poised/Active	77
Sirt6	Cofactor	Active	92
Smad3	DNA binding	Poised/Active	96,97
Utx	H3K27 demethylase	Active	121,122
Zfp516	DNA binding	Active	70,71