Review

A systems perspective on brown adipogenesis and metabolic activation

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Summary
Brown adipocytes regulate energy expenditure via mitochondrial uncoupling. This makes these fat cells attractive therapeutic targets to tackle the burgeoning issue of obesity, which itself is coupled to insulin resistance, type 2 diabetes, cardiovascular and fatty liver disease. Recent research has revealed a complex network underlying brown fat cell differentiation and thermogenic activation, involving secreted factors, signal transduction, metabolic pathways and gene regulatory components. Given that brown fat is now reported to be present in adult humans, it is desirable to harness the knowledge from each network module to design effective therapeutic strategies. In this review, we will present a systems perspective on brown adipogenesis and the subsequent metabolic activation of brown adipocytes by integrating signaling, metabolic and gene regulatory modules with a specific focus on known ‘druggable’ targets within each module.

Keywords: Brown adipogenesis, gene regulatory network, metabolism, signal transduction.

Introduction

With the increase in the global incidence of obesity and its associated pathologies, collectively termed the metabolic syndrome, there is renewed interest in exploring pathways that modulate energy balance in mammalian systems (1). Disproportional energy intake over expenditure leads to energy imbalance, excessive fat storage in the white adipose tissue (WAT), and disturbance of whole body homeostasis, igniting intense investigation into pathways that would enhance energy homeostasis (2,3). The latter is in part mediated by fat depots via secretion of cytokines and interaction with various organs of the body. WAT, known as an endocrine-type storage organ, is capable of secreting adipokines such as leptin and adiponectin (4), and communicating with different organs such as the brain, muscle and liver to regulate energy homeostasis as well as the immune response. Excessive energy intake leads to WAT hyperplasia (increase in the number of mature adipocytes) and/or hypertrophy (adipose tissue expansion). Given its status as an energy reservoir, WAT has therefore been the primary target to combat obesity. However, results have been discouraging due to lipid divergence, leading to the accumulation of lipids in other organs such as skeletal muscle, kidney and liver, ultimately causing the development of metabolic disorders. For instance, in response to the adipocyte-specific deletion of peroxisome proliferator-activated receptor gamma (Pparg), severe lipoatrophy is observed in mice, but the mass of Pparg null mice and their wild type littermates is similar, with the Pparg null mice demonstrating lipid divergence and afflictions such as fatty liver, diabetes and severe insulin resistance (5). This underscores the importance of designing effective therapies that lack adverse systemic side effects. While dietary restriction and exercise have a positive impact on adipose mass leading to diminished fat storage (6), novel strategies to improve whole body homeostasis and restore metabolic balance are being explored to aid metabolically challenged patients. With the recent positron emission tomography – computed tomography-based (PET-CT) discovery of metabolically active brown fat and recruitable brown-in-white fat depots in adult humans (7), current work in the field has turned to
explore ways to maximize energy expenditure via the brown adipose tissue (BAT) to combat excessive fat accumulation and restore energy homeostasis.

The presence of a second type of adipose tissue, BAT, has been originally described both in rodent models and in human infants, as the thermogenic activity of BAT is essential for the endurance of small mammals in cold environments. A brown adipocyte is characterized by densely packed, iron-rich mitochondria, rendering it brown in color, and is capable of converting free fatty acids into heat via the action of the uncoupling protein 1 (UCP1) in response to various stimuli, most notably cold and diet. Apart from classical brown fat, WAT also harbors pockets of UCP1-expressing cells with thermogenic capacities that arise in response to various stimuli. These adipocytes are known as beige or brite (brown-mogenic capacities that arise in response to various stimuli also harbors pockets of UCP1-expressing cells with ther- cold and diet). Apart from classical brown fat, WAT also harbors beige or brite (brown-in-white) or recruitable BAT and display similar characteristics of beige (or recruitable) BAT rather than classical BAT. This is unlike the interscapular depots of human infants that have been shown to resemble classical BAT found in rodents using high resolution imaging, as well as histological and biochemical analyses. To design effective therapies promoting BAT activity in metabolically challenged human subjects, it is important to have a thorough understanding of the molecular mechanisms underlying brown fat cell development and activity. This review is an attempt to integrate the signaling, metabolic and transcriptional modules governing brown fat differentiation and metabolic activation with an additional focus on the white to brown axis. We envision that this network-based perspective will contribute to the identification of unexplored pathways or incomplete mechanistic details regarding key regulators and signaling molecules, aiding in the development of targeted therapeutic approaches.

Part 1: Metabolic activity of brown adipose tissue

Uncoupling protein 1, the core player in brown adipose tissue activation

The process of ‘uncoupling’ substrate oxidation from adenosine triphosphate (ATP) synthesis, i.e. thermogenesis, occurs in brown adipocytes and is catalyzed by the mitochondrial protein UCP1. UCP1 is a multi-pass mitochondrial inner membrane protein and member of the mitochondrial carrier protein family. Ucp1 null mice show little resistance to cold and are not capable of sustaining their body temperature during prolonged cold exposure, suggesting a crucial role for UCP1 in cold-induced thermogenesis. Thermogenesis at the molecular level begins when free long chain fatty acids (LCFAs) liberated from the abundant multilocular fat droplets within the brown adipocyte are activated by acyl-CoA synthase to acyl CoAs and transferred into the mitochondria. This results in beta-oxidation of LCFAs (now acyl-CoAs), which, in combination with the Krebs cycle, produces the reduced electron carriers, Flavin adenine dinucleotide (FADH2) and Nicotinamide adenine dinucleotide (NADH). The latter are subsequently oxidized within the respiratory chain, which causes the protons to be pumped out of the mitochondria, ultimately resulting in the formation of a proton-motive force. UCP1 then mediates the influx of protons back into the mitochondrial matrix without ATP being synthesized. Rather, the energy stored in the proton-motive force is liberated as heat. More specifically, the mechanism by which UCP1 transports H+ ions has recently been elucidated using the patch clamp technique. The latter allowed direct measurement of UCP1 currents in the native inner membrane of BAT mitochondria, demonstrating that UCP1 is a symporter of LCFAs/H+. Thus, LCFAs serve as primary substrates that associate with UCP1 via hydrophobic interactions and enable UCP1 to transport H+ ions, thereby mediating thermogenesis.

Part 2: Known therapeutic interventions in humans

Non-drug-based approach

Methods that are employed to induce BAT development and activation, aiming to improve whole body energy homeostasis, include the following:

Cold exposure

Cold exposure, to our knowledge, is the most efficient approach to induce BAT development and activation, and it does so via the sympathetic nervous system (SNS) as well as the noradrenaline (NE)-dependent signal transduction pathway. Numerous studies showed that the effects of chronic cold exposure are compelling and significantly improve systemic metabolic homeostasis, including insulin sensitivity as well as glucose and lipid profiles.
Nevertheless, results of adult human-based studies revealed important discrepancies, perhaps due to varying study conditions and experimental designs (10). This illuminates the need for standardized experimental protocols to better assess and compare the actual impact of cold exposure on human BAT activity across individuals and studies.

Figure 1  Schematic representation of factors that promote development and activity of brown and beige adipocytes. Shown are factors with therapeutic potential as BAT potentiators. Physiological stimuli originating at the systemic level can be divided into 1) cold exposure-derived factors namely norepinephrine (NE) that is secreted by the SNS; 2) exercise-induced myokines including irisin, Meteorin-like (METRNL), interleukin 6 (IL-6), lactate and β-aminoisobutyric acid (BAIBA); and 3) insulin as a post-prandially secreted factor. In addition, endocrine stimuli are also visualized including thyroid hormone T3, natriuretic peptides (NP), fibroblast growth factor 21 (FGF21), bone morphogenetic protein 7 (BMP7), bone morphogenetic protein 8b (BMP8b), orexin (OX), vascular endothelial growth factor (VEGF) and prostaglandins (PG). Finally, several synthetic molecules are also shown that can act as BAT inducers but that are no longer used given their adverse secondary effects: mitochondrial uncoupler: 2,4-dinitrophenol, β3-adrenoreceptor agonist: CL316,243 and PPARγ activators: thiazolidinediones (TZDs). The presented list of factors activating BAT is not exhaustive. A few organ schemes originate from Servier Medical Art. Figure legend: Organs from top (clockwise): Brain, Skeletal muscle, WAT, Heart, Liver, Thyroid, BAT and Pancreas.
Exercise
The role of exercise in enhancing and maintaining overall fitness, health and well-being is incontestable. It is an established physiological method for improving muscle strength and cardiovascular system condition, decreasing body weight and allowing its maintenance, protecting against type 2 diabetes, as well as awarding enjoyment through endorphin production (28,29). In light of recent interest in BAT physiology, the cocktail of metabolic factors that are secreted during exercise was analysed in various studies in the context of BAT development and activation (12). This led to the identification of a number of molecules such as Irisin, Meteorin-like, IL-6, lactate, beta-aminoisobutyric acid amongst others (30–35) (Fig. 1). However, as with chronic cold exposure, it remains to be established how much of the beneficial effect of exercise occurs via BAT activation.

Bioactive food ingredients
Another non-drug-based factor discussed in the context of BAT activity enhancement is diet, which along with exercise has a powerful beneficial impact on overall health and fitness. Alternative dietary options, including substances such as green tea, ginger, capsaicin (36) amongst others, have been reported to induce BAT activity. Green tea, for instance, contains caffeine and remarkable quantities of catechin-polyphenols (notably epigallocatechin gallate). These molecules interact to induce 24 h energy expenditure and fat oxidation, thereby implicating them as effective activators of sympathetically mediated thermogenesis (37). Similarly, ginger, with its bioactive constituents gingerols and shogaols (38), was shown to enhance the thermic effect of food (39). Although it was reported that ginger-supplemented high-fat diet fed rats presented lower insulin, glucose and lipids versus the control rats (38), no such effect was observed in an equivalent study conducted in humans (39).

Drug-based approach
The abundance of brown fat and its activation by cold vary according to the extent of obesity of human subjects (7,24,26,40). Studies that directly measure oxidative metabolism in BAT by \(^{15}\text{O}\) PET blood flow demonstrated that the level of cold-inducible thermogenesis in most individuals is less than 10 kcal/day (41–42). In contrast to cold adapted rats where the blood flow to BAT can reach up to 1,000 ml/min/100 g (43,44), in acute cold-stressed, but chronically warm-adapted humans, the rate is 15–20 ml/min/100 g (41,42,45). To achieve a similar effect, for instance, via exercise, the thermogenic activation of BAT in humans needs to be increased 40–50-fold (46). Hence, drug-based therapies have been proposed to enhance the activity of BAT in metabolically challenged individuals where diet and exercise regimen alone is not sufficient to promote the metabolic activity of BAT. These include chemical uncouplers such as dinitrophenol, which was used extensively but is associated with complications such as hyperthermia and has since been banned. Alternatively, several groups demonstrated the effect of UCP1-specific activators and other futile cycles that may provide metabolic benefit within a tolerable safety profile. This is evidenced by the directed release of mitochondrial protonophores that can improve insulin resistance, diabetes, hypertriglyceridaemia and hepatic steatosis by safely uncoupling in the liver of rats (47). Other drugs include beta-3 selective adrenergic agonist CL-316,243 that can potently activate brown and beige fat in rodents and is known to improve insulin action in human clinical trials (48). Currently, acute dosage of Mirabegron, a drug that has been used to treat an overactive urinary bladder, has been shown to activate brown fat. Mirabegron is therefore a candidate in consideration for a putative drug under the condition that its effect is constant upon chronic dosing and without adverse cardiovascular side effects (49). Thiazolidinediones (TZDs), well-known PPARg agonists, are known to cause browning of white fat by stabilizing PR domain containing 16 (PRDM16), a transcriptional co-regulator of the early brown fat differentiation program. However, they also have a rather negative impact such as weight gain, fluid retention and cardiovascular events, reducing interest in their use. Additional molecules of interest include bone morphogenetic proteins such as BMP7 and BMP8b, cyclooxygenase-2 (COX2) and natriuretic peptides, and fibroblast growth factor 21 (FGF-21). However, these molecules have pleiotropic effects, underscoring the importance of delineating signaling and transcriptional pathways both upstream and downstream of the targeted factor. The following sections will therefore focus on key signaling and gene regulatory modules and mechanisms as well as highlight putative ‘druggable’ targets.

Part 3: Secreted factors for brown adipose tissue recruitment
Numerous factors were demonstrated to positively recruit BAT (or beige adipose tissue) activity via endocrine, paracrine or autocrine mechanisms (10,50). These include (i) natriuretic peptides that bind to natriuretic receptors on brown or beige adipocytes, and that trigger lipolysis and thermogenesis by activating cyclic GMP-dependent protein kinase (PKG) (10,51); (ii) irisin, a newly identified hormone that is induced in mouse and human muscle by exercise and that stimulates UCP1 expression (10,50); and (iii) orexin, a neuropeptide that triggers the brown fat cell differentiation program by impacting the sympathetic outflow via p38 MAPK as well as the bone morphogenetic protein receptor 1-A-dependent Smad1/5 signaling (10,52) and FGF family members including...
Part 4: Signal transduction pathways

Although lipid-storing cells evolved late during the course of evolution, it has been observed that evolutionarily ancient factors and pathways are highly active during adipogenesis and have either an activatory or inhibitory effect on adipocyte development. Some pathways are also known to exhibit dichotomic effects and are poorly characterized in the context of adipogenesis, particularly regarding how they converge on key transcriptional programs (54). As these signaling pathways control tightly regulated transcriptional cascades, further investigation into the transmission of signals is valuable. The core adipogenic circuit comprises the master regulator PPARα along with CCAAT/enhancer binding protein alpha (CEBPα), and many signaling events are focused on the activation of this regulatory circuit. On the other hand, initiation of brown fat cell differentiation and activation of the thermogenic circuit require additional transcriptional regulators and co-regulators such as PRDM16, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) that are also stimulated by specific signaling further promotes the activation of different signaling pathways in brown adipocytes. Of the three subtypes of beta-adrenergic receptors, beta 3 adrenergic receptor (ADRB3) is predominantly found in mature brown adipocytes, while beta 1 adrenergic receptor (ADRB1) is found in brown pre-adipocytes. Beta-adrenergic receptors are known to couple to G proteins of the Gs subtype and execute the thermogenic signaling cascade via adenylyl cyclase activation. It has been demonstrated that ADRB1 and -3 mediated thermogenic activation and upregulation of oxidative metabolism are highly effective in elevating whole-body thermogenesis in both rodents (44,55) and humans (56).

Thyroid signaling

Thyroid hormones bind to thyroid hormone receptors (TR) α1–2 and β1–2 to execute pleiotropic effects such as growth and metabolism in several target tissues throughout the body, such as liver, bone, fat and heart. Particularly in brown fat, Thyroxine or T4 is converted to its metabolically active form, Triiodothyronine or T3, by type 2 deiodinase (DIO2) following sympathetic stimulation (57). T3 further acts in concert with norepinephrine to stimulate Ucp1 gene expression in the BAT in rats (58,59). To determine the TR isoforms regulated by T3, pharmacological and genetic screening approaches have been used, leading to the identification of TRβ-1 agonist Gc-1, capable of stimulating Ucp1 expression but not affecting the regulation of whole body temperature. Further, genetic mouse models showed that TRα activation is important for thermogenesis while TRβ is necessary for cholesterol metabolism (60). Thus, drug designs targeting thyroid metabolism must take this into account.

Gq signaling

G protein-coupled receptors (GPCRs) are a family of seven transmembrane proteins that regulate biological processes in different tissues such as the adipose tissue, via the action of heterotrimeric G proteins composed of three subunits: alpha (α), beta (β) and gamma (γ). Upon activation of GPCRs, Ga dissociates from the Gbg dimer causing the activation of downstream signaling events determined by G protein coupling. Of the four classes of Ga proteins, Gq protein is known to activate multiple intracellular signaling pathways such as ERK, IP3/DAG and RHO/ROCK (61,62). A GPCR profiling-based approach revealed Gq-coupled GPCRs to be one of the highly expressed groups of signaling molecules in murine brown adipocytes that inhibit adipocyte differentiation through the RHO/ROCK signaling cascade. This phenotype effect was manifested via the Endothelin-1/Endothelin receptor type A (ET-1/EDNRa) signaling axis. Further, overexpression of Gq in vivo induced a phenotype similar to the effect of ‘whitening’ in BAT, preventing ‘browning’ of WAT in cold-exposed mice (63–65). Given that approximately 25% of the drugs available in the market target GPCRs, it seems feasible to target receptors coupled to Gq to enhance browning in white fat depots (66).

Cell fate and development processes are influenced by the para- and autocrine activity of the Wnt family of secreted glycoproteins. The binding of Wnt proteins to frizzled receptors triggers beta-catenin-dependent, canonical signaling pathways. Canonical Wnt signaling ligands such as Wnt10b promote osteogenesis in mesenchymal stem cells (MSCs) and suppress both white and brown adipogenesis. It has previously been demonstrated that Wnt10b blocks brown adipose differentiation and affects the expression of Ucp1 via repression of Pgc1α in mature brown adipocytes leading to a switch to white adipocytes. Wnt signaling...
Figure 2 (Continues)
Additional transcriptional regulators mediating brown adipogenesis are represented in the network. Nodes in bold italics represent genes, while the ones in bold represent proteins that interact with regulators to control downstream brown-specific and general adipocyte gene expression. The gene regulatory network comprises the following circuits (in chronological order of execution): 1. Early differentiation; 2. Core differentiation and 3. General adipogenic network are as follows:

- Insulin receptor substrate, Irs:
Pi3K
Hedgehog ligands, Smo:
Smoothened,
Tgfβ:
Bone morphogenetic proteins 2/4/7/8b,
Bmpr1a:
Homolog 3,
Schnurri 2,
Camp:
Transient receptor potential (TRP) ion channel,
Trpv:
B3Ar:
Beta adrenergic receptor 3,
Norepinephrine,
Dll:
Delta like,
Nicd:
Notch intracellular domain,
Triiodothyronine, T3:
P42/44 Mapk:
Mitogen-activated protein kinase 1,
P38 Mapk:
Protein kinase A,
PR domain containing 16,
Prdm16:
C/Ebpb:
CCAAT/enhancer binding protein beta,
C/Ebpd:
Early B-cell factor 1,
Ebf1:
Early B-cell factor 2,
Zfp521:
Zinc finger protein 521,
Zfp423:
Zinc finger protein 423,
Zeb1:
Krox24:
Preadipocyte factor 1 (aka Dlk1),
Sox9:
Placenta-specific 8,
Plac8:
Ews/Ybx1:
EWS RNA-binding protein 1/Y box binding protein 1,
Klf11:
Kruppel like factor 7,
Klf15:
Klf6:
Retinoid X receptor, Rxr:
Globin transcription factor 2/3,
Gata2/3:
Carbohydrate response element binding protein,
: Sterol regulatory element binding transcription factor 1,
Foxo:
Forkhead box O1,
Foxa2:
Forkhead box A2,
: Interferon regulatory factor 4,
Rbp-Jk:
C/ebpg:
CCAAT/enhancer binding protein gamma,
Chop:
C/EBP-homologous protein 10,
Ctbp1/2:
C-terminal binding protein ½,
Med1:
Diator complex subunit 1,
Setdb1:
Bifurcated 1,
Chd7:
Nemo like kinase,
Nlk:
Asxl1:
Additional sex comb like 2,
Ppara:
Carbohydrate response element, Chrebp:
Carbohydrate response element,
Fasn:
Fatty acid synthase,
Acc1:
Hormone-sensitive lipase,
Lipoprotein lipase,
Fabp4:
Fibroblast growth factor 2/3,
Vdr:
Vitamin D receptor,
Rbp-Jk:
Hes family BHLH transcription factor 1,
Nuclear hormone receptor NUR/77,
Rar:
C/ebpg:
CCAAT/enhancer binding protein gamma,
tofacitinib is sanctioned in the United States for rheumatoid arthritis (73). Additionally, because the JAK–STAT pathway is involved in immune system regulation, local administration of the drug or ex vivo transplantation of cells post-treatment is recommended to limit deregulated immune activation.

**Transforming growth factor-beta signaling**

Transforming growth factor beta (TGF-b), myostatin and bone morphogenetic proteins regulate the differentiation of many cell types including adipocytes. While subsequent signaling events have common properties, specific TGF-b family members influence distinct cell fate decisions by binding to serine/threonine kinase receptors and functioning through SMAD-dependent and -independent mechanisms. Transcriptional regulation of target genes is executed by receptor-regulated SMAD1 and SMAD3 TFs, which, upon phosphorylation, engage in dimer formation with SMAD4, and subsequently translocate to the nucleus. In adipogenesis, the canonical TGF-b signaling pathway has an unclear molecular function. While TGF-b expression correlates positively with obesity in humans, it inhibits in vitro adipogenesis of 3T3-F442A cells by signaling through SMAD3. It has been observed that supplementing wild-type mice with exogenous TGF-b1 reduced thermogenic gene expression in fat (74), implicating TGF-b signaling in the negative regulation of browning. This has been exploited for therapeutic purposes as evidenced in a study where a dominant negative form of activin receptor type II-b fusion protein was used to promote thermogenesis in mice by binding to TGF-b, thereby preventing downstream signaling (75).

**Bone morphogenetic protein signaling**

Bone morphogenetic proteins (BMPs) are categorized as a family within the TGF-b superfamily with the ability to stimulate white and brown fat cell differentiation programs. While BMP2 and BMP4 are known to induce commitment to the white adipocyte fate when supplemented by a hormonal induction cocktail, BMP7 is known to singularly support brown fat cell differentiation in both MSC progenitors and committed pre-adipocytes even in the absence of a differentiation cocktail via the p38 MAPK pathway (76). p38 MAPK further executes the thermogenesis program by phosphorylating and activating distinct nuclear receptors (NRs), ATF2 and PGC1a, which in turn promote *Ucp1* expression by binding to a cAMP and PPAR-response element that resides in a key enhancer of the *Ucp1* gene (77). This phenotypic effect was also observed in *vivo*, because *Bmp7* null mice show a reduction in brown fat mass that was rescued upon adenovirus-mediated overexpression of Bmp7, thereby protecting the mice from diet-induced obesity while triggering energy expenditure (76). BMP7 is also known to function synergistically with rosiglitazone, a TZD, to give rise to inducible brown adipocytes from SCA1+ adipocyte progenitor cells that arise from different fat depots as well as skeletal muscle in mice (78). Additionally, *in vivo* studies showed that BMP7 enhances *Ucp1* expression in WAT in combination with the beta3-adrenergic agonist CL-316,243 to levels higher than those achieved by CL-316,243 treatment alone (78). This study underscores the importance of the BMP7 signaling pathway in inducing browning and as a viable therapeutic option.

BMP8b is yet another BMP signaling molecule capable of regulating whole body energy metabolism both centrally, through AMPK activation in hypothalamic nuclei, and peripherally, by activating the p38 MAPK signaling pathway in differentiating and mature brown adipocytes. Using *Bmp8b* knockout mice, it was demonstrated that BMP8b increases the peripheral response of BAT to adrenergic stimulation and acts centrally to increase sympathetic output to BAT. BMP8b signaling specifically acts on the thermogenic pathway, as *Bmp8b* knockout mice do not show gross abnormality in BAT morphology. Additionally, it has been proposed that AMPK has a counter-regulatory role, acting in opposition to BMP8b to regulate energy expenditure, supported by the observation of greater thermogenic activation upon localized expression of the dominant negative AMPK alpha isoform. As AMPK is a known druggable target in peripheral tissues to treat insulin resistance (79), its relationship with BMP8b offers new opportunities in targeted drug design. It is worth noting that the ability of BMP8b to execute central actions to reduce whole body weight without compensatory food intake offers an exciting premise for new therapeutic options, demonstrating the importance of understanding thermogenesis at a central regulatory level.

**NOTCH signaling**

The Delta-like (DLL) and Serrate/Jagged (JAG) family of membrane bound ligands bind to transmembrane NOTCH receptors causing gamma-secretase-mediated proteolytic cleavage of NOTCH, leading to the nuclear translocation of the NOTCH intracellular domain (NICD). Once in the nucleus, the NICD activates the RBP-jk transcriptional complex and subsequently its downstream targets such as the *Hes* and *Hey* family genes (80). Constitutive activation of NOTCH signaling inhibits *Pgc1a* and *Prdm16* transcription in white adipocytes via HES1-mediated transcriptional repression and causes ‘whitening’ of BAT. In contrast, inhibition of NOTCH signaling is associated with ‘browning’ of WAT, improvement of insulin sensitivity and whole-body energy homeostasis (81). This differential response is intriguing and highlights the involvement of different gene regulatory programs that are cell-context dependent. As NOTCH signaling is an evolutionarily conserved pathway, these findings can be extended to human studies; however, this merits further investigation into identifying molecular
mechanisms operating in human white to brown conversion.

Part 5: Gene regulatory networks

Once adipogenic precursors commit to the adipogenesis program, a transcriptional circuit is switched on, leading to the expression of genes associated with fat cells. While the white and brown differentiation programs share a set of regulators and adipogenic stimuli, they both have unique requirements that determine their phenotypic differences. It is therefore important to delineate both, i.e. the core adipogenic differentiation and the thermogenic programs, to get a full picture of the gene regulatory events that give rise to fully functional brown and beige fat. In this section, we present an overview of the individual circuits such as the early and core differentiation, thermogenic and adipogenic modules within the overall gene regulatory network. A schematized overview is presented in Fig. 2.

Early differentiation circuit

**Early B-cell factors**

The early B-cell factors (EBF), especially EBF1 and EBF2, play an important regulatory function in the early differentiation circuit along with Zinc Finger Protein (ZFP) 423 and its close paralog ZFP521, both implicated as early regulators of adipose commitment and differentiation (82,83). ZFP521 functions in a repressive feedback loop with EBF1, while also repressing ZFP423, thereby influencing early regulatory events in the lineage switch between bone and fat (83). ZFP423, on the other hand, functions by promoting Pparg expression through amplification of the BMP signaling pathway via SMAD binding (82). Further, Ebf1 is induced by both CCAAT/enhancer binding protein delta (C/EBPd) and CCAAT/enhancer binding protein beta (C/EBPβ) and then directly activates both Pparg (via binding to the Pparg1 promoter) and Cebpα, which positively feeds back to Cebpβ (84). A previous study demonstrated EBF2 to regulate PPARg binding to determine brown versus white fat fate, leading to its classification as a brown-specific transcriptional regulator (85). It was further suggested that BAT-specific target genes of PPARg were categorically bound by EBF2 that then served as a mark for the later recruitment of PPARg at sites regulating the expression of brown fat selective genes such as Prdm16 and Ucp1 (85).

**PR domain containing 16**

The 140 kDa PRD1-BF-1-RIZ1 homologous domain containing protein-16, PRDM16, is a core transcriptional co-regulator in both brown and beige fat, showing selective expression in brown over white fat (13). In vitro cell culture studies in classical brown fat cells demonstrated the requirement of PRDM16 to develop and maintain a thermogenic gene program. However, in vivo deletion of Prdm16 did not affect the function of classical brown fat but had a significant impact on the development of beige fat cells. Additionally, deletion of Prdm16 along with its closest homolog Prdm3 led to an early and extreme loss of brown fat thermogenic gene expression (86). Most importantly, PRDM16 has been demonstrated to function as a molecular switch between white and brown programs by coupling with diverse protein interaction partners simultaneously, mediating both activating and repressive gene expression programs. These dichotomous activities are executed through the use of chromatin remodellers, C-terminal binding proteins (CtBPs) that function via the recruitment of histone deacetylases (87). PRDM16 interacts with either PGC1α or CtBPs to, respectively, activate the brown or suppress the white gene expression program. During brown fat activation, recruitment of PGC1α displaces CtBPs from the complex to activate brown fat-specific genes. Additionally, it has been suggested that PRDM16 binds to specific TFs linked to the promoters/enhancers of target genes, further guiding the recruitment of PGC1α or CtBPs to initiate the formation of a co-activator or a co-repressor complex (87). The TFs that guide PRDM16 to its respective target genes and their molecular underpinnings have not been mapped yet, and it is quite likely that numerous factors are involved in this regulatory process.

**Placenta-specific 8**

Placenta-specific 8 (PLAC8), a key upstream molecule in the brown adipogenic regulatory network, functions by associating with Cebpβ and binding to its promoter to induce transcription (88). In vivo genetic deletion of Plac8 results in the loss of expression of several brown fat-specific components such as Prdm16 and Pgc1α. However, general adipocyte differentiation factors such as PPARg and AP2 are not downregulated, indicating the importance of PLAC8 in recruiting BAT during thermogenic challenges. Consequently, in vivo genetic inactivation of Plac8 leads to errors in BAT differentiation and thermogenic programs, causing cold intolerance and late onset obesity (88). However, the upstream signaling activators of Plac8 induction and those that promote PLAC8 binding to C/EBPβ are as yet uncharacterized and merit further investigation.

**CCAAT/enhancer binding protein beta and CCAAT/enhancer binding protein delta**

PPARg and C/EBPβ, the key molecular players of adipogenesis, vary in their molecular function in brown versus white fat programs. In response to pro-adipogenic stimuli, C/EBPβ and C/EBPδ transition from a repressive complex, involving the nuclear co-repressors NCoR1 and SMRT (89), into an active one. This in turn allows these TFs to induce expression of PPARg and C/EBPβ in cooperation.
with the epithelial–mesenchymal transition TF ZEB1 (90), which drives terminal fat cell differentiation. C/EBPβ is important for adipogenic differentiation in immortalized adipocytes with slightly inconspicuous effect in mouse embryonic fibroblasts (MEFs). While it has been demonstrated that C/EBPβ-deficient mice have reduced adiposity, this might be attributed to impairment in lipogenic and not adipogenic programs (54,91). It is quite likely that C/EBPδ may take over for C/EBPβ in a knock out model, as a double knockout mouse model for C/EBPb and C/EBPd shows a further decline in adipose tissue mass (91). Moreover, C/EBPβ has been shown to work in collaboration with PPARγ to induce the expression of C/ebpa, as PPARγ is required to release the inhibitory chromatin remodeler HDAC1 from the C/ebpα promoter (92). Thus, C/EBP -b and -d work synergistically with other members of the core differentiation circuit to execute downstream gene regulatory programs.

Core differentiation circuit

**Peroxisome proliferator-activated receptor gamma**

The ligand-dependent nuclear receptor PPARγ expressed primarily in adipocytes is induced during adipogenesis and is central to the adipogenic regulatory network. It has two different isoforms, PPARγ1 and PPARγ2, of which PPARγ2 is exclusively present in the adipose tissue in contrast to PPARγ1, which is ubiquitously expressed. PPARγ functions as a regulator in adipogenic programs common to both white and brown fat differentiation, and no other factor has so far been found that can promote adipogenesis in the absence of PPARγ. All pro-adipogenic transcriptional regulators such as C/EBPαs and some KLFs such as KLF5, KLF15 converge on one of the two Pparg promoters while anti-adipogenic transcriptional regulators such as the GATA factors function by repressing Pparg expression (93–95). Additionally, key adipogenic signaling pathways also converge on Pparg expression and activity. Upon activation by ligands, PPARγ forms a heterodimer with RXR and binds to a Ppar response element (PPRE) in cooperative fashion (96,97), enabling it to control the expression of a specific subset of PPRE-containing genes such as Ap2, Fabp4 and Lpl in white adipocytes (98). Studies showed that stimulation of white adipocytes in vitro or in vivo by strong PPARγ agonists such as rosiglitazone leads to ‘browning’ of the white cells (99). The latter triggers an upregulation of mitochondrial genes such as Ucp1, Cox7a1 and Cox8b, subsequently causing an increase in mitochondrial mass, enhanced oxygen consumption and lipid oxidation (100,101). This browning effect is characterized by a suppression of adipokine production such as Resistin, Alpha1-acidglycoprotein and Haptoglobin (102–104). The phenotypic outcome of supplementing white adipocytes already known to express Pparg and its endogenous ligands with additional PPARγ agonists suggests that brown adipocytes produce a distinct repertoire of PPARγ ligands that differ in activity from those produced in white adipocytes (105). It is likely that this regulatory mechanism is then executed via the actions of PRDM16/PGC1α and PRDM16/CtBP complexes as they are known to mediate brown versus white programs.

**CCAAT/enhancer binding protein alpha**

C/EBPα plays a crucial role in differentiated adipocytes and works in concert with PPARγ to constitute the core differentiation circuit. The molecular function of C/EBPα is central to the acquisition of insulin sensitivity as opposed to accumulation of lipids or expression of adipocyte genes (106).

**Nuclear Factor IA**

The Nuclear Factor I (NFI) family of TFs including NFIA, NFIB, NFIC and NFIX are widely expressed, with each TF regulating a distinct set of target genes as evidenced by different phenotypes yielded by mouse knockout models (107). Previously, both NFIA and NFIB have been identified as positive regulators of white adipocyte differentiation via in vitro cell culture studies in the 3T3L1 cell line (108). Using an integrative genomics approach, we discovered that NFIA is also involved in the brown adipogenesis gene regulatory network (109). Notably, we observed that Nfia is differentially expressed during brown fat cell differentiation and has a strong positive correlation with Ucp1 expression levels in the mouse genetic reference panel (BXD) (110) and human clonal BAT cell lines (111). Further, genome-wide localization analyses based on ChIP-seq revealed that, while NFIA does not directly bind to Ucp1, it does, however, bind to general adipogenic marker genes such as Zfp423, core adipogenic regulators such as Pparg and Rxrg, early brown specific TFs such as Ebf2 and finally drivers of the thermogenic module such as Ppara and Pgc1a. A follow-up on the mechanistic details of NFIA action in brown versus white is necessary, especially to delineate its mode of regulatory action in these tissue types.

Apart from the core members of this differentiation circuit, additional adipogenic regulators include positive regulators such as ZEB1 (90), ASLX2 (112), Kruppel like factors (KLF)-5 (93), -15 (113) and 6 (114), STAT5α (115,116), while negative regulators include GATA transcription factors (94,95), ASLX1 (112), KLF-2 (117). These transcriptional details have been reviewed extensively elsewhere (54,103).

**Thermogenic circuit**

**Peroxisome proliferator-activated receptor alpha**

The peroxisome proliferator-activated receptor alpha (PPARα), a protein from the nuclear receptor family comprising PPARγ/d/b, is a fatty acid-activated TF that executes transcriptional regulation of genes involved in
cellular metabolism (118). It is particularly detected in tissues such as brown fat, liver or heart, known to have high rates of fatty acid oxidation and peroxisomal metabolism. High expression of Ppara distinguishes brown from white fat and is related to the lipid oxidation capacity of brown fat. It was shown that PPARα activators stimulate Ucp1 gene expression both in brown adipocytes and mature BAT, acting through a PPRE element situated in the upstream enhancer of the Ucp1 gene that is also responsible for PPARγ-dependent regulation (119). PPARα interacts with CBP and PGC1α, forming a tight regulatory complex to activate Ucp1 gene expression. An interesting regulatory mechanism exists between the two PPARs, PPARα and PPARγ, during brown adipogenesis. While it was demonstrated that PPARα and PPARγ bind and activate Ucp1 gene transcription through the same Ucp1-PPRE element, the predominant molecular function of any particular subtype depends on the relative amount of each subtype. For example, crosstalk with other signaling pathways such as regulation of PPAR transcriptional activity by MAPK-dependent phosphorylation enhances PPARα (120) but decreases PPARγ activity (121), as well as ligand availability and interaction with co-regulators such as PGC1α. The interaction of PGC1α with PPARα is ligand dependent, whereas the one with PPARγ is not (122). Therefore, these and possibly other uncharacterized events may determine which PPAR subtype activates Ucp1 transcription in response to brown adipocyte-inducing conditions. In other words, it could be PPARγ in association with differentiation-dependent events, or PPARα in concert with increased lipid catabolism in active BAT, highlighting the importance of understanding this regulatory mechanism. Further, it was observed that acute administration of the PPARα ligand Wy 14,643 upregulates Ucp1 mRNA expression in those physiological situations in which endogenous PPARα ligands are expected to be low. This is in agreement with previous findings demonstrating that PPARα sensitivity in vivo depends upon the status of lipid metabolism (123).

**Carbohydrate response element binding protein**

Yet another interesting mechanism is the differential regulation of PPARα and carbohydrate response element binding protein (ChREBP), a TF that is responsive to glucose signaling and is more highly expressed in brown compared to white fat tissue, influencing lipid storage and lipolysis in brown adipocytes. It was demonstrated that ChREBP and PPARα regulate each other via an inhibitory feedback mechanism that is activated during conditions of high and low glucose, respectively (124). As such, ChREBP and PPARα coordinate establishment of a feedback loop between lipogenesis and lipolysis in brown adipocytes. The molecular mechanism underlying this crosstalk remains largely unexplored and may serve as an avenue for further research to develop novel therapeutics.

**Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha**

Given that PPARγ alone is insufficient to induce browning, extensive investigation into other putative regulators led to the identification of PGC1α. The involvement of PGC1α was first demonstrated in the thermogenic circuit when a dramatic increase in Pgc1α expression was observed in mice exposed to cold in both skeletal and brown fat (122). PGC1α is also triggered in brown fat cell lines upon treatment with the beta-adrenergic receptor agonist isoproterenol (122). It interacts with multiple nuclear hormone receptors that bind to the Ucp1 gene enhancer in either a ligand-dependent or -independent manner as in the case of Retinoic Acid Receptor (RAR) and Thyroid Receptor (TR) or PPARγ, respectively. PGC1α not only boosts the efficiency of transcription but also plays a key role in specifying the genes that are targeted by its partner TFs. This is illustrated by overexpression of PGC1α in white fat cells leading to the induction of Ucp1 through the PPARγ binding sites but a failure to express the PPARγ target gene Ap2. As PGC1α is a co-activator and is dependent on the binding of particular TFs to implement different gene expression programs, repression of binding events could affect different biological responses. Thus, targeting singular binding events between PGC1α and particular TFs might offer new therapeutic opportunities.

However, while PGC1α is a critical regulator of adaptive thermogenesis, it is not the master regulator of brown differentiation. This is evidenced by the fact that reduction in PGC1α levels during brown fat cell differentiation has no significant impact on brown adipogenesis. However, it has a strong impact on the induction of thermogenic genes by CAMP (105). Apart from its molecular involvement in regulating thermogenesis, it also aids in inducing angiogenesis via the co-activation of the orphan nuclear receptor ERRA on the Vegf promoter. PGC1α was stimulated by an inadequacy in nutrients as well as oxygen leading to increased Vegf expression and subsequently angiogenesis in BAT and skeletal muscle in vivo (125). Thus, the transcriptional functions of PGC1α are key to the development and thermogenic activity of brown adipocytes.

**Forkhead box C2**

Forkhead box protein C2 (FOXC2), a member of the Forkhead box family of TFs, regulates different aspects of adipocyte metabolism by activating PPARγ and C/EBPs as well as by enhancing cAMP-mediated PKA signaling (126). Most importantly, FOXC2 responds strongly to high caloric load via strong induction of cAMP signaling as demonstrated by high fat feeding experiments in which FOXC2-overexpressing mice showed reduced weight gain in response to the amount of food consumed. This categorizes FOXC2 as a putative therapeutic target based on its molecular function as a metabolic regulator and a factor that can induce adaptive thermogenesis (126). Additionally,
enhanced expression of Foxc2 has also been implicated in mitochondrial biogenesis via trans-activation of the nuclear encoded mitochondrial transcription factor A as well as induction of mitochondrial fusion via activation of Mitofusin 1 and 2, and Optic atrophy 1 homolog (127). This correlation was observed in humans, making FOXC2 an attractive target for therapy. Foxc2 expression was also shown to increase in response to high caloric feeding, whilst repressed by cold (127). This suggests that FOXC2 has a distinct role in regulating mitochondrial function from that observed in cold-adapted thermogenesis, known as metabolo-regulatory thermogenesis. This is different from thermo-regulatory thermogenesis, which is categorically induced by cold and is dependent on expression of Pgc1a and Ucp1 (8).

In addition to the key factors described above, new factors have recently been implicated in early brown adipogenesis. These include the Kruppel like factor 11 that binds to PPARγ and activates brown fat genes in rosiglitazone-treated human adipocytes (128) as well as the EWS/YBX1/PPARγ and activates brown fat genes in rosiglitazone-esis. These include the Kruppel like factor 11 that binds to its binding site (133). It has also been demonstrated that pRb functions as a PPARγ co-repressor, thereby suppressing its transcriptional activity (141).

Anti-adipogenic regulators affecting the PGC1α–UCP1 axis

Given the central function of PGC1α and UCP1 in the thermogenic program, their expression and activity are tightly regulated by a multitude of factors that serve as negative regulators. This mode of negative regulation most likely functions to restrain thermogenesis, whose prolonged activation may lead to adverse conditions such as hyperthermia, which in turn has the potential to cause organ failure and death (133).

Nuclear receptor interacting protein 140

Nuclear receptor (NR) interaction protein 140 (Rip140) is a ligand-dependent transcriptional repressor of nuclear receptors including estrogen receptor and PPARs and also functions as a transcriptional co-repressor that shares a number of downstream targets with PGC1α and inhibits PGC1α’s transcriptional activity by binding to it directly (134). Along with suppressing PGC1α target genes, Rip140 also represses Ucp1 expression by recruiting inhibitory histone modifying and DNA methylation enzymes to the promoter of Ucp1 (135). Lack of Rip140 increases Ucp1 expression by de-repression of PPARα, PPARγ and PGC1α. Studies suggest that, given Rip140’s high expression in WAT compared to BAT, it functions to suppress the brown phenotype. This is evidenced by significant enhancement of brown-related genes upon knockdown of Rip140 in white fat cells (136). Further, observations from in vivo studies demonstrated that Rip140 null mice exhibit expenditure rather than storage of the consumed fat (137). However, in-depth investigation into the molecular mechanisms underlying Rip140-mediated ‘browning’ suppression is pending.

Liver X receptor alpha

The Liver X receptor alpha (LXRα) does not directly interfere with PGC1α’s transcriptional activity or binding, rather, when ligand-activated, this TF interferes with the induction of Ucp1 expression by displacing PPARγ from the Ucp1 enhancer and by drafting RIP140 as a corepressor to its binding site (133). It has also been demonstrated that mice lacking LXRα show increased Ucp1 expression in both BAT and WAT with no significant change in the expression of Pgc1a, thus manifesting a lean phenotype (133,138).

Retinoblastoma protein

pRb acts as a molecular toggle between the white and brown adipocyte differentiation programs (139) by direct binding to the Pgc1a promoter leading to transcriptional repression (140). Additionally, pRb functions as a PPARγ co-repressor, thereby suppressing its transcriptional activity (141).

Twist basic helix–loop-helix TF 1

Twist basic helix–loop-helix TF 1 (TWIST1) functions as an antagonist of brown fat-mediated thermogenesis by binding directly to PGC1α and suppressing its transcriptional activity. This is executed by Twist1-mediated recruitment of the histone deacetylase HDAC5 to the promoters of PGC1α target genes, causing transcriptional repression (142). Other key anti-adipogenic factors suppressing the PGC1α and UCP1 axis include unliganded Vitamin D receptor that directly suppresses Ucp1 expression via binding to a Vitamin D receptor element in the proximal region of the Ucp1 promoter (133), steroid receptor co-activators such as SRC2 and SRC3 that repress the transcriptional activity of PGC1α, TRPV4 (a transient receptor potential cation channel 4) that represses PGC1α via the ERK1/2 protein kinases (143) and SHP (orphan nuclear receptor) that inhibits the ERR-γ mediated promoter transactivation of PGC1α (133).
Outlook

While the white adipocyte differentiation network is well characterized, the brown adipogenic signaling and gene regulatory network remains rather sparse, warranting further investigation to annotate missing nodes and interactions. The latter include additional regulators that are capable of inducing browning in concert with key molecular players, thereby providing alternative points of safe therapeutic intervention. Most notably, through a comprehensive literature-based reconstruction of the brown signaling and gene regulatory network, we illustrate the requirement to focus on delineating implicated signaling pathways and on how they converge on the transcriptional components. This will enable a more thorough understanding of key molecular events and guide better drug discovery efforts. An additional focus should be on elucidating mechanisms that govern the white to brown switch, aiding in the development of drugs that can safely and specifically mediate browning.

Recent studies have also elucidated alternative modes of thermogenesis that function without the need for UCP1 activation, wherein beige and white adipocytes can directly respond to cool temperature (27–33°C) and activate a thermogenic program in a cell-autonomous manner. This particular type of thermogenic program was determined to be independent of the canonical cAMP/protein kinase/CREB pathway downstream of beta-adrenergic signaling (144). Furthermore, in a study conducted on human subjects, it was observed that elevated thermogenesis upon cold exposure is not inhibited by the chemical blockade of beta-adrenergic signaling alone (145). This suggests the existence of alternative pathways for induction of a thermogenic program through a cell-autonomous effect of temperature on certain fat depots. Recently, a futile cycle of creatine metabolism has been identified as an alternative pathway for energy expenditure and thermal homeostasis (146). The implicated proteins Creatine Kinase Mitochondrial 1 and 2 (CKMT1 and CKMT2) were enriched in human BAT, raising the possibility of exploiting this pathway in manipulating energy consumption in patients with metabolic diseases, particularly via dietary supplementation and/or administration of novel drugs.

One of the main challenges in the field of translational research is the design of safe therapeutic interventions. Several treatment options targeting BAT are being explored, but more often than not, they suffer from potential systemic side effects. We believe that a systems perspective summarizing various input stimuli, downstream signaling and transcriptional components will provide a strong framework to aid in the development of targeted drug design. Furthermore, the field’s focus should not only be on identifying specific druggable pathways but also strategies for drug delivery such as the development of controllable microenvironments, scaffolds, biomaterials for transplantation of energy expending cells as well as focused pharmacological intervention to stimulate the activity of resident progenitor cells in situ by triggering endogenous brown adipogenesis.

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Conflict of interest statement

All authors declare no conflict of interest.

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