Genome-wide insights into development and function of thermogenic adipocytes

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Abstract

Brown and brown-like adipocytes are specialized adipocytes with a high capacity to convert metabolic energy to heat. This function is eminent in supporting organismal thermogenesis but may also have potential in the fight against obesity. The latter has spurred a massive interest in understanding the development and regulation of these thermogenic adipocytes. Here we review how genome-wide studies based on next generation sequencing have provided insight into how the chromatin and transcriptional landscapes are established in thermogenic adipocytes and how thermogenic signals can change the genomic programming of white adipocytes. Furthermore, we discuss how the integration of genomic data can be used to discover novel transcriptional pathways that may be modulated as part of therapeutic strategies for the treatment of obesity.

Studying transcriptional regulation in thermogenic adipocytes at a genome-wide level

The regulation of brown adipose tissue (BAT) function and development has recently received considerable attention. The main reason for this is that this tissue is specialized to convert metabolic energy to heat, and this energy-wasting capacity has been proposed to have potential for the treatment of obesity [1]. Several studies have demonstrated that there is an inverse correlation between the amount of BAT and body mass index in humans [2-4]. Furthermore, activation of human BAT leads to increased glucose uptake and energy expenditure and promotes insulin sensitivity [5-9], suggesting that BAT could improve metabolic health in humans.

Similar to white adipocytes, brown adipocytes store large amounts of metabolic energy in the form of triglycerides. However, rather than releasing this energy as fatty acids to be used in other tissues, brown adipocytes are specialized to combust metabolic energy to generate heat in response to, for example, cold stimulation. This process is termed **non-shivering thermogenesis (NST)** (see glossary) and relies on a high number of mitochondria as well as expression of uncoupling protein 1 (UCP1) in the inner mitochondrial membrane. In addition to the well-established UCP1-dependent uncoupling, thermogenic adipocytes have recently been proposed to contribute to NST through futile cycles of creatine metabolism or endogenous mitochondrial uncoupling by *N*-acyl amino acids [10, 11].

Originally, brown adipocytes in BAT were the only adipocytes to be defined as thermogenic. However, thermogenic adipocytes expressing UCP1 are now also

recognized in white adipose tissue (WAT), where their formation can be induced by cold exposure acting through β-adrenergic signaling [12]. In addition, other signals, such as thiazolidinediones, which, as agonists for peroxisome proliferator-activated receptor γ (PPARγ), may mimic constitutive fatty acid signals, have been shown to induce the emergence of brown-like adipocytes in WAT, as well as in cultures of white adipocytes [13, 14]. These brown-like adipocytes have been termed beige [15] or brite (for brown-in-white) [13] and distinguish themselves from classical brown adipocytes by being inducible and by the expression of distinct markers [16], some of which may be positional [17]. Importantly, however, it appears that the thermogenic gene program is highly similar between brown and brown-like adipocytes [18]. Thus, future studies are required to decipher whether these brown-like adipocytes are functionally distinct from classical brown adipocytes, apart from being inducible. Indeed, it is possible that what distinguishes the brown-like adipocytes in WAT is their cellular plasticity. Here, we refer to these inducible cells as 'brown-like' adipocytes.

Currently, significant resources are being invested in identifying signals and mediators that can activate brown adipocytes or induce browning of WAT to combust excess energy. This involves the identification of transcriptional regulators that are capable of controlling the development and function of brown and brown-like adipocytes by modulation of thermogenic gene programs. With the recent progress in genome-wide sequencing technologies, it has become possible to study such gene regulatory mechanisms at a much more insightful level, and integrative genomic approaches are increasingly often used as a tool to identify novel

thermogenic regulators. In this review, we summarize and discuss how approaches based on genome-wide sequencing have increased our understanding of the transcriptional mechanisms that control brown and brown-like adipocyte differentiation.

Technological advances have increased our understanding of gene regulatory mechanisms

Only one decade ago, the most common way to study gene regulation was to look at how one transcription factor (TF) controlled the expression of one or a few genes. However, the recent advances in genome-wide sequencing technologies have revolutionized the approach to studying as well as the insight into eukaryotic transcriptional regulation (box 1). The extensive mapping of transcriptional activity, TF binding sites, epigenetic modifications, and chromatin structure in multiple mammalian cell types and tissues has revealed that the genome comprises an unexpectedly high number of functional elements that are activated in a highly cell type-specific manner [19]. Furthermore, it is now evident that the gene programs controlling most cellular functions are regulated by coordinated transcriptional events that allow signals from multiple regulatory pathways to become integrated at the level of chromatin (box 2).

Defining the molecular signatures of brown and brown-like adipocytes by transcriptomic analysis

Transcriptomic studies in the form of microarrays and genome-wide sequencing of RNA (RNA-seq) have provided insights into the different transcriptional signatures of

white, brown, and brown-like adipocytes (Table 1, and Tables S1 in the supplemental information online). In particular, there has been significant focus on the identification of different marker genes, which can be used to distinguish adipocytes from different tissues in relation to their thermogenic potential. This includes genes thought to represent markers of "classical" brown adipocytes, such as *ZIC1* and *LHX8* [20, 21], markers of brown-like adipocytes isolated from WAT, such as *TMEM26*, *CD137*, and *CITED1* [16, 22], or white adipocyte markers, such as *TCF21* [20]. Interestingly, a recent study used a genetic mouse model to profile ribosome-associated mRNA specifically in UCP1-positive adipocytes derived from **inguinal WAT (iWAT)**, perigonadal WAT (pgWAT), and BAT [18]. Although the major part of the transcriptional program was similar between UCP1-positive adipocytes from different fat depots, only iWAT-derived UCP1-positive adipocytes displayed a signature of smooth muscle-like cells. Subsequent *in vivo* fate mapping experiments confirmed that at least a subpopulation of brown-like adipocytes in iWAT originated from smooth muscle-like cells.

It is becoming increasingly clear that brown and brown-like adipocyte marker genes vary greatly with anatomical location of the adipose tissue [17, 23], and may not necessarily predict the function of the adipocyte. Some studies have recently shown that, even within a particular depot, there is a high degree of heterogeneity between the adipocytes [24, 25]. In particular, single-cell analysis of gene expression in adipocytes from **interscapular BAT (iBAT)** showed a large variability in the expression of brown marker genes, including UCP1, even though the cells looked morphologically similar [25]. This emphasizes the need to supplement average

population analysis with single-cell analysis, to provide much more detailed information about different cell constituents of adipose tissues.

Transcriptomic insights into the relationship between mouse and human thermogenic adipocytes

Several studies have focused on the comparison of human BAT with brown and brown-like adipose depots from mice to determine whether human BAT is most closely related to classical brown fat or inducible brown-like fat from mice. While most of these studies only investigate a subset of previously described marker genes, a recent transcriptomic study compared *in vitro* differentiated clonal preadipocytes originating from human supraclavicular BAT with clonally derived brown-like and classical brown adipocytes from mice [26]. Clustering analysis showed that the immortalized human brown adipocytes were closer to brown-like adipocytes than to brown adipocytes from mice [26], which supported previous findings based on a smaller panel of genes [16, 22]. By contrast, comparison of a limited number of genes in human neck fat with five different mouse depots showed that the closest relation of human BAT was to classical mouse iBAT [27]. Furthermore, a mixed expression of both brown and brown-like mouse adipocyte marker genes has been reported in human supraclavicular BAT [28]. Based on these observations, it appears likely that several subpopulations of brown adipocytes exist, the signature of which is dependent on the anatomical location of the adipose depot and even the position within a given depot, as suggested by single-cell analysis. In addition, gene expression in WAT and BAT can also vary according to the species of, for example, mice [29, 30]. Thus, more extensive transcriptomic characterizations are needed in

order to better elucidate the comparability between mouse and human adipose depots.

Identification of novel regulators of thermogenesis by comparative transcriptomic analysis

Comparative transcriptomic analysis of RNA from different adipose tissues has also proven useful in the identification of important regulators of thermogenic gene expression (Figure 1). An example is PR (PRD1-BF1-RIZ1 homologous)-domain-containing protein 16 (PRDM16), which was identified in a qPCR-based expression screen of transcription-related genes, as a putative transcriptional regulator with higher expression in BAT compared with WAT [21]. PRDM16 has subsequently been shown to be important for the expression of thermogenic genes in both brown and brown-like mouse adipocytes [21, 31]. A similar primer array approach identified zinc-finger protein 423 (ZFP423) as a TF important for the activation of the *PPARG* gene and adipogenic differentiation, in both WAT and BAT [32]. Interestingly, in addition to its general role in adipogenesis, ZFP423 was recently shown to suppress thermogenic gene expression in mature white adipocytes through the repression of early B cell factor 2 (EBF2) activity [33]. Thus, overexpression of ZFP423 in mice led to “whitening” of BAT, whereas depletion of ZFP423 promoted browning of WAT. In another search for regulators of adipogenesis, screening a genome-sized cDNA library for activation of the aP2 promoter revealed transducin-like enhancer protein 3 (TLE3) as an interesting candidate [34]. Further analysis showed that TLE3 enhances the ability of PPARγ to stimulate adipogenesis, and that TLE3 disrupts the interaction of PPARγ with PRDM16, thereby inhibiting thermogenic gene expression

[35]. A different reporter-based screening was recently used to identify zinc-finger protein 516 (ZFP516) as a TF capable of activating a UCP1 promoter-enhancer-driven reporter [36]. Subsequent studies showed that ZFP516 promoted both brown adipocyte adipogenesis and browning of WAT, and indicated that it might do so by interacting with PRDM16 [36]. Furthermore, in a recent transcriptomic-based study, the homeobox protein C10 (HOXC10) was identified as a TF highly expressed in iWAT, compared with **epididymal WAT (eWAT)** and BAT, in different mouse strains. The physiological importance of this differential expression remains to be shown, but overexpression of HOXC10 in cell culture inhibited browning of white adipocytes [30]. Finally, using transcriptomic analysis to identify putative transcriptional regulators induced upon thermogenic activation of BAT in mice, zinc finger and BTB domain-containing 16 (ZBTB16) was identified as a cold-inducible factor that activates brown adipocyte-characteristic gene programs [37].

Non-coding RNAs are important regulators of thermogenic gene expression

In addition to TFs, transcriptomic analysis has also begun to provide insight into non-coding RNA involved in the regulation of thermogenic gene expression (Figure 1, and Table S2 in the supplemental information online). Thus, comparative global microRNA (miRNA) expression profiling has identified miRNAs that are functionally important for brown and brown-like adipocytes [38-41]. In mice, it was recently shown that the miRNA-processing enzyme Dicer1 is downregulated upon high-fat feeding, leading to a global reduction in miRNA levels. Interestingly, downregulation of miR-328 led to decreased the expression of BAT-characteristic genes through increased levels of β-secretase 1 (Bace1) [42]. Accordingly, overexpression of miR-

328 or inhibition of Bace1 increased expression of BAT-characteristic genes, and Bace1 inhibition counteracted weight gain in mice.

Recent miRNA profiling in human adipocyte cultures identified the miRNAs Let-7i-5p and miR-125b-5p as negative regulators of browning and mitochondrial biogenesis and function, respectively [43, 44]. Interestingly, miR-125b-5p was expressed to a lesser extent in human subcutaneous biopsies from lean, compared with overweight subjects. However, their targets for regulation are still unknown. Another miRNA, miR-92a, is secreted from brown adipocytes to the serum, and could be used as a new diagnostic tool to evaluate BAT activity [45].

Besides miRNAs, long non-coding RNAs (lncRNAs) have been found to have regulatory roles in thermogenic gene expression. A global search for lncRNAs expressed in thermogenic adipocytes found brown fat lncRNA 1 (Blnc1) to be enriched in and induced during brown adipogenesis [46]. Subsequently, Blnc1 was found to interact with EBF2 to induce the expression of thermogenic genes in brown and brown-like adipocytes. Another study that combined global expression of lncRNA in BAT with publically available binding profiles of chromatin marks and adipocyte TFs, identified lnc-BATE1 as an important regulator of both the differentiation and function of brown adipocytes [47].

A genome-wide view of epigenetic modulation in thermogenic adipocytes

Several lines of evidence from genome-wide studies support the notion that histone modifications and epigenetic modulators have an important role in cell fate specification in adipocytes (Figure 2, and Table 2). In embryonic stem cells, lineage-

specific genes are occupied by the bivalent histone marks histone H3 lysine 4 trimethylation (H3K4me3) and H3K27me3, which keep these genes silenced, but poised for future activation [48]. During the commitment phase to the preadipocyte lineage, adipocyte-specific genes gain an alternative set of bivalent marks, namely H3K4me3 and H3K9me3, where the H3K9me3 mark is subsequently lost during differentiation, allowing a high expression of the adipocyte genes [49]. Strikingly, brown adipocyte-specific genes are demarcated by the repressive H3K27me3 mark in both BAT- and WAT-derived preadipocytes, whereas white adipocyte-specific or common adipocyte genes are not [50]. During brown adipocyte differentiation, a jumonji domain-containing protein 3 (JMJD3)-guided removal of the H3K27me3 mark is involved in the selective activation of the brown fat genes [50]. Furthermore, the ubiquitously transcribed X chromosome tetratricopeptide repeat protein (UTX) has been shown to catalyze the demethylation of H3K27me3 on brown fat-specific genes in response to β-adrenergic stimulation, thereby allowing further activation [51]. It remains to be investigated why only brown, but not white or common fat genes retain the bivalent H3K27me3/H3K4me3 signature; however, it is possible that the special combination of histone marks, including H3K27me3, serves to recruit a specific transcriptional machinery to the brown fat genes, allowing these to become activated only during brown adipocyte differentiation. Future studies should investigate the molecular basis of the specific demarcation of brown adipocyte genes in WAT and BAT.

In addition to the H3K27me3 mark, the methylation and demethylation of H3K9 also appear to have an an important role in the development and function of brown

adipocytes. Euchromatic histone-lysine N-methyltransferase 1 (EHMT1) promotes brown adipocyte lineage over muscle lineage specification by maintaining high levels of H3K9 methylation at the promoters of muscle-specific genes [52]. Similarly, G9a (EHMT2) inhibits brown adipocyte differentiation by keeping high levels of H3K9me2 on the PPARγ promoter [53]. Another H3K9 demethylase, jumonji domain-containing protein 1A (JMJD1A), is recruited to thermogenic target genes upon β-adrenergic stimulation of brown adipocytes, where it appears to serve a dual function by removing the repressive H3K9me3 mark [54] and facilitating long-range thermogenic enhancer-promoter interactions [55].

Another histone methyltransferase with reported functions in brown adipocyte development is the H3K4me1/2 methyltransferase mixed-lineage leukemia 4 (MLL4). During brown adipocyte differentiation, MLL4 co-localizes at adipocyte-specific enhancers with cell type-specific TFs. Deletion of MLL4 leads to a marked decrease in H3K4me1/2 at these enhancers, as well as the loss of other signatures of active enhancers, thereby resulting in compromised brown adipocyte differentiation [56].

In several recent studies, the lysine-specific demethylase 1 (LSD1) was found to be important for the thermogenic functions of WAT and BAT via different mechanisms [57-60], reflecting the previously reported dual coactivator and corepressor functions of LSD1 [61]. LSD1 and PRDM16 were shown to colocalize at a large subset of promoters of white adipocyte-selective genes, leading to the demethylation of H3K4me1/2, which results in repression of these genes in BAT [60]. This might also involve the action of the repressive CoREST complex, which is specifically targeted to

LSD1-occupied white adipocyte-selective genes in BAT [59]. On the other hand, LSD1 has been reported to have direct coactivating effects on a larger number of genes associated with brown adipocyte function [57-59]. In this context, LSD1 interacts with ZFP516 at the promoter of UCP1, resulting in a demethylation of H3K9me1/2, which activates the UCP1 gene [58]. Furthermore, **motif analysis** in LSD1 promoter binding sites in BAT revealed enrichment of a binding motif for nuclear respiratory factor 1 (NRF1), which is likely to contribute to LSD1-mediated activation of BAT-selective genes [59]. These findings clearly illustrate that the epigenetic regulators operate in a highly context-dependent manner to modulate the specialized gene programs of thermogenic adipocytes.

Diversity in the genomic landscapes of white, brown and brown-like adipocytes reveals novel thermogenic regulators

Recent genome-wide studies have demonstrated marked differences in TF occupancy between white, brown and brown-like adipocytes (Table 2). ChIP-seq profiling of PPARγ in *in vitro* differentiated adipocytes obtained from different white and brown mouse adipose depots, revealed that approximately 5% of all identified PPARγ-binding sites are highly selective to brown adipocytes [62]. Comparable fractions of brown/brown-like-selective PPARγ binding sites were observed when comparing WAT and BAT from mice [63], as well as white and brown-like human multipotent adipose-derived stem (hMADS) adipocytes [64]. These findings indicate that PPARγ regulates distinct gene programs in white and brown/brown-like adipocytes, and that lineage-specific binding sites are likely to be dependent on different cooperating TFs. Thus, based on motif analysis of BAT-selective PPARγ-

binding sites, EBF2 was identified as a brown-selective TF that facilitates PPARγ recruitment and cooperates with PPARγ in activating the brown adipocyte-selective gene program [63]. BAT-selective TFs, such as EBF2, might be especially important for the establishment of BAT **super-enhancers**, which were recently shown to be present in the vicinity of several brown adipocyte genes in mice BAT, including *Ucp1, Cidea* and *Prdm16* [65]. Interestingly, PRDM16 itself is required for the recruitment of MED1 to BAT super-enhancers, suggesting that the brown adipocyte gene program is regulated by interconnected autoregulatory feedback mechanisms (Figure 3).

Molecular mechanisms involved in thermogenic activation and maintenance of brown-like adipocytes in WAT

During the recent years, several studies have focused on how brown-like adipocytes form in WAT, in response to thermogenic stimuli [66-70]; however, it still remains an open question to what extent brown-like adipocytes primarily arise by transdifferentiation of mature white adipocytes, or by *de novo* differentiation of a distinct pool of inducible brown adipocyte precursor cells within WAT. Pulse-chase fate-mapping studies suggested that *de novo* differentiation is the predominant mechanism of the recruitment of brown-like adipocytes after stimulation by cold or β-adrenergic receptor (AR) agonists, in subcutaneous WAT [67]. By contrast, using similar strategies, another group showed that unilocular white adipocytes in transdifferentiate into UCP1-positive adipocytes upon cold treatment [68]. This would suggest that transdifferentiation as well as de novo differentiation have a role in browning of WAT. In either case, it remains unknown whether this special ability

to undergo browning is limited to a distinct pool of cells in WAT, and to which extent this ability is intrinsically versus extrinsically programmed. In further support of the adipocyte plasticity, UCP1-positive adipocytes induced by cold were able to convert into UCP1-negative white adipocytes following subsequent warm acclimation [69]. Furthermore, when re-exposed to cold, the same cells again become UCP1 positive, demonstrating a high degree of interconvertibility between white and brown-like adipocytes. A recent study investigated the molecular mechanisms underlying "whitening" of brown-like adipocytes following the removal of thermogenic stimuli in mouse iWAT [71]. Here, it was shown that that removal of the stimulus (β-adrenergic agonist or cold) induced the expression of genes related to autophagy, which was accompanied by an increase in mitochondrial clearance. Interestingly, the binding motif for the mitophagy-activated TF family MiT/TFE was highly enriched in the vicinity of the upregulated autophagy genes, and genetic ablation of autophagy-related protein 12 (ATG12), an autophagy regulator downstream of the MiT/TFEs, resulted in prolonged maintenance of brown-like adipocytes, and protected mice from diet-induced obesity [71]. Future studies will need to clarify the direct role of MiT/TFEs in this process.

A couple of recent studies have shed light on the genomic and transcriptional changes that occur during adipocyte browning induced by PPARγ agonists. In iWAT-derived adipocytes from mice, treatment with rosiglitazone led to extensive changes in the transcriptional landscape, including an induction of the thermogenic gene program [14]. Similarly, treatment of human adipocyte cultures with PPARγ or PPARα agonists induced a major transcriptional reprogramming, with induction of

thermogenic, as well as fatty acid anabolic and catabolic pathways [72]. Mechanistically, rosiglitazone has been suggested to facilitate stabilization of the PRDM16 protein [14], as well as induce a sirtuin 1 (SIRT1)-dependent deacetylation of PPARγ [73], which subsequently triggers the white-to-brown adipocyte phenotypic switch. At the chromatin level, rosiglitazone-mediated browning of human adipocytes is associated with changes in the binding of PPARγ and cofactors, resulting in the formation of a set of PPARγ super-enhancers selective for brown-like adipocytes that are located in the vicinity of key thermogenic genes (Figure 3). Interestingly, this reprogramming of adipocyte super-enhancers was recently used as a discovery tool to predict novel browning factors, including Kruppel-like factor 11 (KLF11), which directly activates a large set of brown-like adipocyte genes in human adipocytes [64]. Thus, it is likely that KLF11 is a part of a larger network of TFs that collectively drives the genomic programming during browning via their action on brown-like adipocyte super-enhancers [74].

Integrative system and –omics approaches reveal key features of thermogenic adipocytes

As for the genomic area, other -omics approaches (e.g., lipidomics, metabolomics, and proteomics) have undergone major technological advances over the past decade. The exploratory power of integrating data from these different areas has just begun to unfold and will likely result in a more coherent understanding of the regulatory networks that control thermogenic adipocyte functions. A recent study combined transcriptomics with lipidomics in iBAT of mice exposed to cold and showed that cold increases both thermogenic and glycerolipid gene programs. This is

accompanied by extensive remodeling of triacylglycerols and glycerophospholipids in iBAT, thereby indicating that the remodeling of glycerophospholipids is at least in part transcriptionally regulated [75]. Consistent with this, proteomic analysis has shown that enzymes from the glycerophospholipid pathway are upregulated in iBAT of mice exposed to cold [76]. Interestingly, proteomic analysis of BAT and WAT from humans is now available [77] and could be compared with transcriptomic data of WAT and BAT from the same anatomical regions.

A recent **genome-wide association study (GWAS)** made elegant use of an integrative approach and reported a novel putative link between adipocyte browning and obesity in humans [78]. By investigating chromatin states in 127 reference epigenomes from multiple primary cells and tissue types [79], the authors showed that a region harboring the high-risk allelic variant rs1421085 in the first intron of the fat mass and obesity-associated protein (FTO) gene overlapped with a putative super-enhancer in adipose-derived mesenchymal stem cells. Using previously generated **HiC** data to define the putative target genes of this super-enhancer, iroquois-class homeodomain protein (IRX) 3 and IRX5 was identified as candidate targets. Subsequently, it was shown that the high-risk **single-nucleotide polymorphism (SNP)** variant rs1421085 leads to derepression of IRX3 and -5 expression in adipocytes, by disrupting the binding of a repressor. This in turn leads to a developmental shift from browning to whitening programs in adipocytes, which might explain the increased risk of obesity caused by this SNP variant [78]. These recent examples clearly demonstrate the power of integrative system approaches to understand the genomic basis for cellular function, physiology, and disease.

Concluding Remarks and Future Perspectives

The remarkable new insights gained from the use of genome-wide technologies have greatly increased our understanding of the transcriptional networks that control the development and functions of brown and brown-like adipocytes. The realization that TFs and epigenetic regulators cooperatively program the genomic and transcriptional settings to activate thermogenic gene programs in adipocytes not only provides major leaps in our basic understanding of these processes, but also improves the possibilities for designing therapeutic strategies that target these pathways (see Outstanding Questions).

Even with these major advances in genomic technologies, several challenges still remain. First of all, most genomic data are based on population-based data (i.e., are averages of cells in a population). Due to the heterogeneity of (adipose) tissues, this poses a major challenge in data interpretations. Here, single-cell analysis now offers a way to investigate the cellular heterogeneity within adipose tissues in a genome-wide manner [80]. For example, this would allow examining the epigenetic basis of the variable susceptibility to thermogenic stimuli in adipose tissues. Secondly, most genomic data are primarily based on correlations rather than causality. The recent advances in genome editing techniques make it possible to manipulate the DNA-sequence in a cellular or even *in vivo* context, to test causality. Thus, TF binding and interaction maps of adipocytes can now be functionally validated, and the activity of regulatory regions can be modulated in order to target key transcriptional pathways in thermogenic adipocytes. Third, so far, only very few genomic and transcriptomic

data sets exist from human BAT. Notably, several recent studies have reported *in vivo* browning of WAT in response to different stimulations or diseases (e.g., in patients with pheochromocytoma [81], cancer cachexia [82], or burn trauma [83]). Genome-wide insight into the transcriptional and epigenetic changes during these processes is needed to increase our understanding of how mature white adipocytes can be redirected to a thermogenic cell fate in humans, which could be of therapeutic interest. Finally, data management, and the integration of different types of data, constitutes a major challenge that will require the continuous development and improvements of bioinformatic tools. Thus, the questions and challenges are many; however, given the current technological developments, there is great hope that many of these will be overcome in the near future, and that this will provide a much more accurate insight into the biology and therapeutic potential of thermogenic adipocytes.

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Box 1: Predicting transcription factor action from genome-wide sequencing data

Using genome-wide sequencing-based techniques, a few key experiments can provide a very informative overview of the genomic landscape in a given cell type or tissue. First of all, one should obtain a global overview of the location and activity of genomic action points (i.e., enhancers and promoters). The position of such genomic action points can be derived from techniques that allow identification of accessible chromatin regions, such as **DNase-seq** [84, 85] and **assay for transposase-accessible chromatin (ATAC-seq)** [86] (Figure I). Furthermore, **chromatin Immunoprecipitation (ChIP-seq)** profiling of highly dynamic transcriptional coactivators, such the mediator subunit (MED1) [87] as well as enhancer-associated histone marks, such as histone H3 lysine 27 acetylation (H3K27ac) [88], has been successfully applied as a proxy for enhancer activity, and is used to show differences in enhancer activity between cell types and changes in activity, in response to a given cellular stimulus. Recently, MED1 binding and other enhancer marks were used to define a special class of enhancers, so-called super-enhancers [89], which constitute powerful regulatory regions controlling the expression of cell identity genes. Another surrogate for enhancer activity is the degree of RNA transcription from enhancers (i.e., eRNA), which can be quantified by **global run-on (GRO-seq)** [90] or related techniques [91]. Gene expression data can be obtained by evaluating steady-state RNA levels using RNA-seq or determining acute transcriptional regulation by RNA polymerase II ChIP-seq [92], GRO-seq, or **intron RNA-seq (iRNA-seq)** [93].

By coupling genomic activity profiles to gene expression data, one can predict the genomic elements that are involved in shaping the regulatory landscape. **Motif**

analysis in key regulatory sites in the vicinity of genes that change expression in response to a given signal has proven to be a valuable method to predict TFs that are involved in mediating the signal (Figure I) [94, 95]. During the recent years, more automated methods for the detection of TF motif enrichment have been developed, such as the integrated system for motif activity response analysis (ISMARA) [96]). An alternative approach to identify key TFs involved in a given transcriptional response is to use super-enhancers as a predictive tool, since super-enhancers regulate genes encoding lineage-determining TFs in many different cell types [97]. Once the candidate TF is predicted, binding profiles should be obtained, and, by correlating the change in binding of this particular factor with changes in histone marks or cofactor binding, as well as changes in RNA-seq signal, one can address whether this factor has activating or repressing effects on specific enhancers and associated genes, in response to a given signal.

Box 2: Transcription factor cooperativity in hotspots and super-enhancers controls core gene programs

Several studies have demonstrated that TFs expressed in a given cell type tend to co-localize within defined genomic regions, which have been termed TF hotspots [98, 99]. Interestingly, the number of TFs bound to a given site correlates with the activity level of the enhancer, as well as the transcription of nearby genes [100], indicating that the establishment and activation of the enhancer landscape in a particular cell type are accomplished through a highly cooperative and dynamic effort between multiple TFs, cofactors, and chromatin remodeling complexes. In addition to the clustering of TFs in enhancer hotspots, it is now evident that individual enhancers at a genome-wide level tend to cluster into larger enhancer domains. These enhancer clusters, which have been termed super-enhancers [89] or '**stretch-enhancers'** [101], are powerful interconnected regulatory regions, that are often found near cell type-specific genes that define the identity of a given cell type. This includes genes encoding master lineage-determining TFs, which often control the expression of their own genes as well as genes encoding other key TFs, and cell type-defining metabolic effectors [89, 97]. Furthermore, these special classes of enhancers show a strong enrichment of disease-associated non-coding single nucleotide polymorphisms (SNP) risk-variants [97, 101] and are highly responsive to perturbations of the signaling pathways converging on these [102]. This underlines the strong regulatory potential of these enhancers and supports their key important function in controlling lineage determination and cellular function.

Glossary box

Assay for transposase-accessible chromatin-sequencing (ATAC-seq): a technique used to measure chromatin accessibility by applying an engineered Tn5 transposase that catalyzes the insertion of sequencing adaptors into native chromatin.

Chromatin immunoprecipitation sequencing (ChIP-seq): a technique used to investigate the genome-wide binding of TFs, cofactors or histone marks, based on immunoprecipitation of chromatin-associated proteins followed by deep sequencing of the associated DNA.

DNase-seq: a technique used to determine "open", i.e. nucleosome depleted, regions in chromatin based on limited DNase I digestion of chromatin and high throughput sequencing of liberated fragments.

Epididymal white adipose tissue (eWAT): the largest visceral white fat depot associated with the epididymis in male mice.

Genome-wide association study (GWAS): an approach that compares a set of DNA markers across the genome in a larger group of human subjects to identify genetic variants associated with a particular disease.

Global run-on sequencing (GRO-seq): a technique used to measure the amount and orientation of transcriptionally engaged RNA-polymerase II at a genome-wide level, based on isolation of labeled *in vitro* produced transcripts followed by deep sequencing. This technique has been successfully applied to determine short-lived transcripts such as those produced at enhancers.

HiC: a technique used to analyze genome-wide chromatin organization by capturing interactions between all genomic regions.

Inguinal white adipose tissue (iWAT): the largest subcutaneous white fat depot in mice located at the thigh, where brown-like adipocytes are easily formed upon thermogenic stimulation.

Interscapular brown adipose tissue (iBAT): the largest brown fat depot in mice that is found between the shoulder blades.

Intron RNA-seq (iRNA-seq): a computational method that determines genome-wide transcriptional activity based on the signal in intronic regions obtained from total RNA-seq data.

Motif analysis: the analysis of TF binding motifs in genomic sequences. If a binding motif is enriched in a defined set of regulatory sites over random background sites, it is likely that the TFs recognizing this motif will bind to many of these regulatory sites.

Non-shivering thermogenesis (NST): the production of heat, mostly from brown adipose tissue, that is not caused by muscle shivering.

Single nucleotide polymorphism (SNP): a genetic variation in a single nucleotide at a specific position in the genome.

Super-enhancers and stretch-enhancers: large clusters (>tens of kilobases in length) of individual enhancers in close proximity that can be identified by their ultra-high levels of coactivators, active histone marks, and lineage-specific transcription factors. Super-enhancers and stretch-enhancers are often found near cell type-specific genes that define the identity of a given cell type.

Trends Box

- The development and thermogenic functions of brown adipocytes are regulated by coordinated actions of many different transcription factors, cofactors, non-coding RNAs, and histone modifiers.

- Activation of thermogenic transcriptional regulators can change the genomic programming of white adipocytes and lead to the formation of brown-like adipocytes.

- Integrative genomic approaches allow deciphering of the transcriptional networks that control the development and specialized functions of thermogenic adipocytes.

- Integrative genomic approaches may reveal novel transcriptional pathways that can be targeted in order to increase the thermogenic capacity of adipocytes.

Outstanding Questions Box

- What is the molecular basis for the differential preprogramming of white and brown adipocyte progenitor cells?

- Do differences in the relative brown/white preprogramming of adipocyte progenitor cells between subjects have a role in human health?

- Are there fundamental differences between brown and brown-like adipocytes, or do brown-like adipocytes represent shades of brown?

- How well can genomic and transcriptomic data sets from thermogenic adipose tissues in mice be used to predict the regulatory landscape and function of human BAT?

- What is the molecular basis for the context-dependent and site-specific functions of transcription factors and cofactors, and can this be exploited therapeutically?

- Can specific targeting of regulatory hubs in chromatin (e.g., hotspots or super-enhancers) in thermogenic adipocytes be used as a future treatment strategy for obesity and obesity-related complications?

Figures and legends

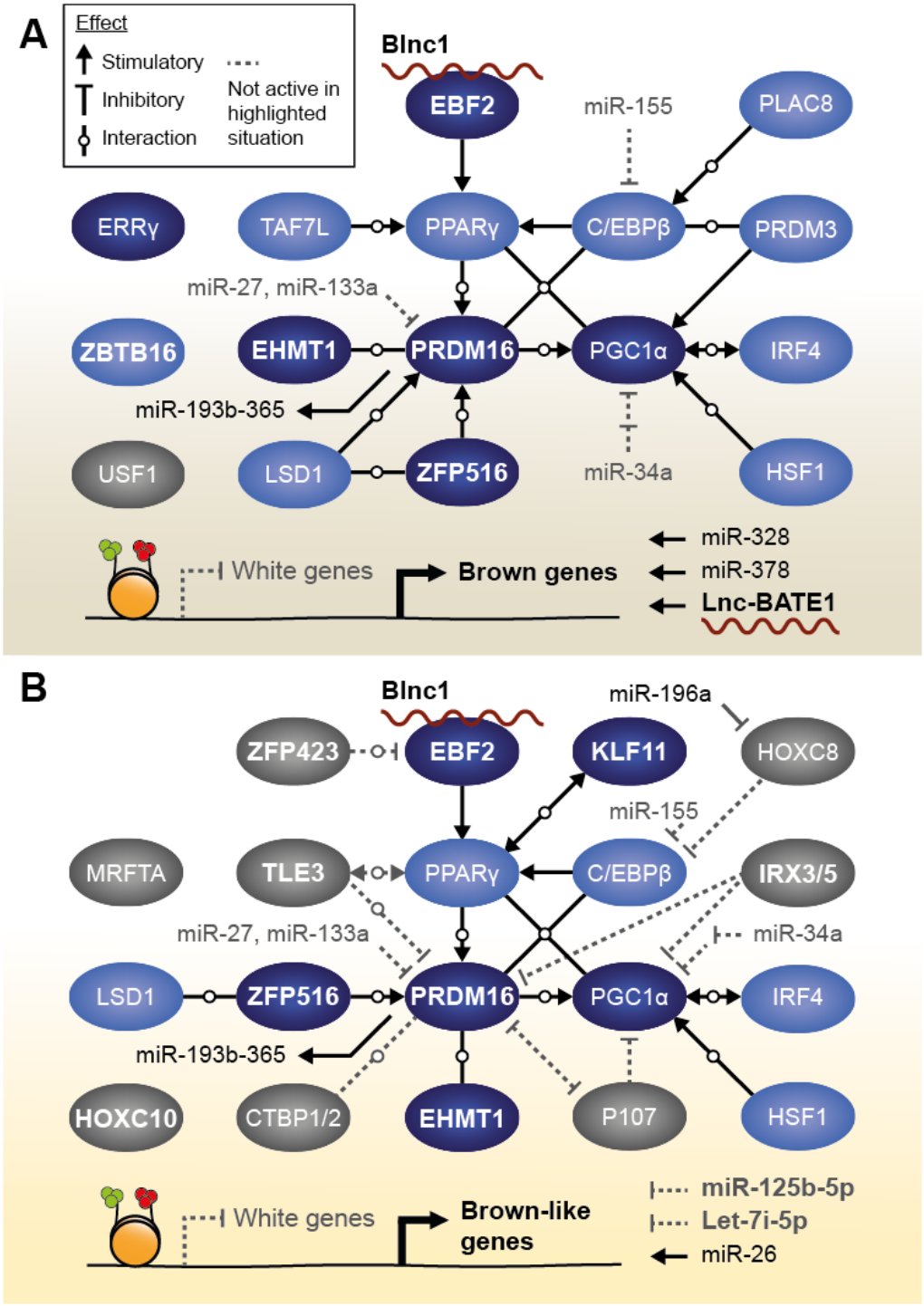

Figure 1: Transcription factor networks of brown and brown-like adipocytes.

Transcriptional regulators that have been shown to stimulate (blue) or inhibit (gray) brown (A) and brown-like (B) adipocyte development and/or function are shown.

Stimulatory factors that are not specific for brown/brown-like versus white adipocytes are marked in light blue. Positive regulation (e.g., induction of expression) is indicated as an arrow, negative regulation as a bar-headed arrow, and reported protein-protein interactions or cooperativity is indicated by a line with a circle. Dashed lines illustrate regulation that is not occurring in brown/brown-like adipocytes, but is important in white adipocytes. Factors marked in bold have been identified in a thermogenic context by genome-wide approaches. Several non-coding RNAs are highlighted ([103]; see also Table S2 in the supplemental information online); long non-coding RNAs are illustrated in red. Abbreviations: C/EBPβ [104], CCAAT/enhancer-binding protein β; CTBP1/2 [105], carboxy-terminal-binding protein 1/2; EBF2, early B-cell factor 2; EHMT1, euchromatic histone-lysine N-methyltransferase 1; ERRγ, estrogen-related receptor γ [106]; HOXC8 [107], homeobox protein C8; HOXC10, homeobox protein C10; HSF1 [108], heat shock factor 1; IRF4 [109], interferon regulatory factor 4; IRX3/5, iroquois-class homeodomain protein 3 and 5; KLF11, kruppel-like factor 11; LSD1, lysine-specific demethylase 1, MRFTA, myocardin-related transcription factor A [110]; P107 [111], retinoblastoma-like 1; PGC1α [112], PPARγ coactivator 1 α; PLAC8 [113], placenta-specific gene 8 protein; PPARγ, peroxisome proliferator-activated receptor γ; PRDM3 [114], PR-domain-containing protein 3; PRDM16, PR-domain-containing protein 16; TAF7L [115], TATA-binding protein associated factor 7L; TLE3, transducin-like enhancer protein 3; USF1 [116], upstream stimulatory factor 1; ZBTB16, zinc finger and BTB domain containing 16; ZFP423, zinc-finger protein 423; ZFP516, zinc-finger protein 516. See main text for references to factors discussed in this review.

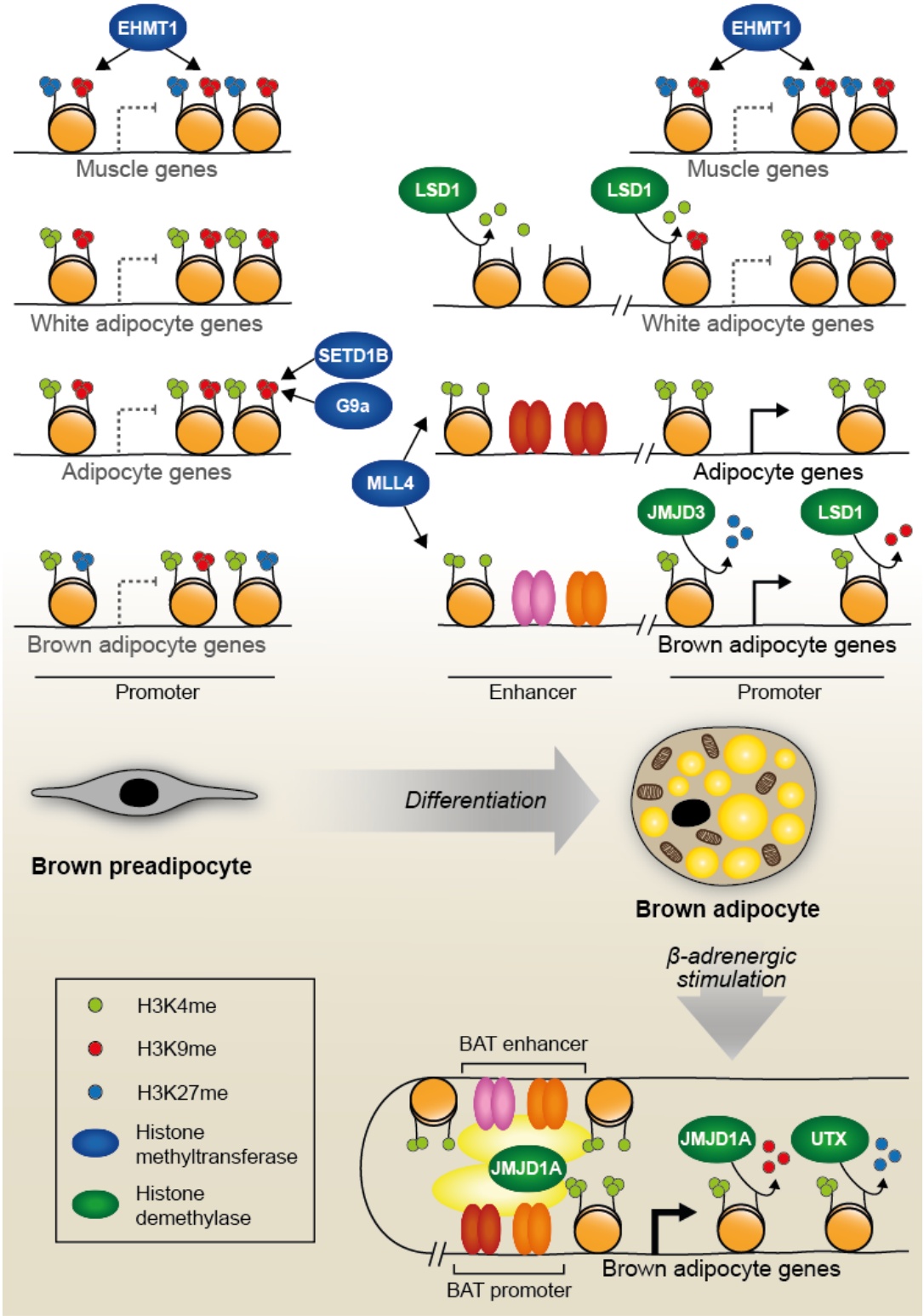

Figure 2: Epigenetic regulation of brown adipocytes.

In the brown preadipocyte state (left), muscle genes are kept silenced by the presence of repressive marks (e.g., by EHMT1-mediated methylation of histone H3

Lys9 (H3K9)). Furthermore, adipocyte lineage-specific genes are inactive, but poised for activation by the presence of bivalent H3K4 and H3K9 trimethyl marks, which involves the action of the H3K9 methyltransferase SETD1B. These genes become gradually activated during differentiation via removal of the repressive H3K9me3 mark. White adipocyte-specific genes are kept silenced in the mature brown adipocyte state (right) at least in part via LSD1-mediated removal of H3K4me1 and -2 at nearby regulatory sites. Brown adipocyte-specific genes are selectively marked by H3K27me3 in brown preadipocytes compared with common and white adipocyte genes, and this mark is removed via JMJD3 during differentiation, allowing for a partial activation of these genes. Furthermore, MLL4-guided methylation of H3K4 at enhancers as well as LSD1-mediated removal of H3K9me1/2 at promoters contributes to the activation of the brown adipocyte gene program. During β-adrenergic stimulation of mature brown adipocytes, JMJD1A promotes further activation of brown adipocyte-specific genes by removing the repressive H3K9me3 mark from promoters, and by acting as a scaffold protein facilitating long-range enhancer-promoter interactions. Thermogenic stimulation also activates UTX-guided removal of H3K9me3 from BAT-selective promoters. Abbreviations: EHMT1, euchromatic histone-lysine N-methyltransferase 1; LSD1, lysine-specific demethylase 1; JMJD1A, jumonji domain-containing protein 1A; JMJD3, jumonji domain-containing protein 3; MLL4, mixed-lineage leukemia; SETD1B [49], SET domain containing protein 1B; UTX, ubiquitously transcribed X chromosome tetratricopeptide repeat protein. See main text for reference to factors discussed in this review.

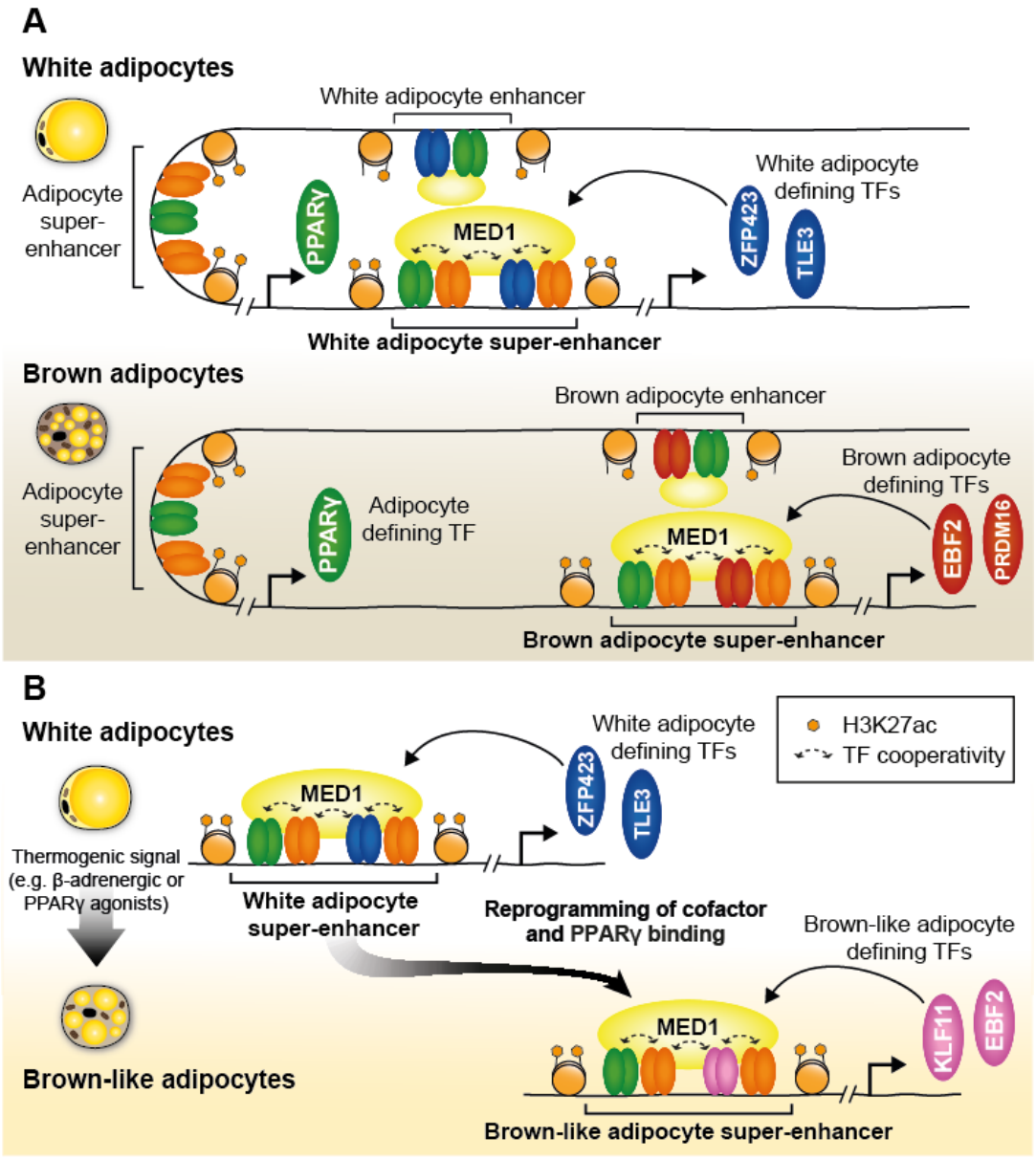

Figure 3: Genomic landscapes of white, brown and brown-like adipocytes.

(A) The genomic landscapes of white and brown adipocytes are similar overall, with many shared regulatory elements controlling core adipocyte gene programs in both cell types. However, brown and white adipocyte-selective gene expression is driven by enhancers and super-enhancers that are specific for brown or white adipocytes. Brown adipocyte super-enhancers regulate the expression of genes that promote the brown adipocyte identity, such as the lineage-determining TFs EBF2 and PRDM16, whereas white adipocyte super-enhancers are found near genes encoding

white adipocyte-specifying factors (e.g. ZFP423). (B) During browning of white adipocytes in response to thermogenic stimuli, such as β-adrenergic or PPARγ agonists, there is redistribution of the transcriptional machinery, including general adipocyte regulators and cofactors from white adipocyte super-enhancers to brown-like adipocyte super-enhancers. This leads to specific activation of a brown-like adipocyte gene program, in particular through the activation of genes associated with brown-like adipocyte super-enhancers. Some of these genes encode brown-like adipocyte-defining TFs, such as KLF11. Abbreviations: EBF2, early B-cell factor 2; KLF11, kruppel-like factor 11; MED1, mediator complex subunit 1; PPARγ, peroxisome proliferator-activated receptor γ; PRDM16, PR-domain-containing protein 16; TLE3, transducin-like enhancer protein 3; ZFP423, zinc-finger protein 423. See text for reference to factors discussed in this review.

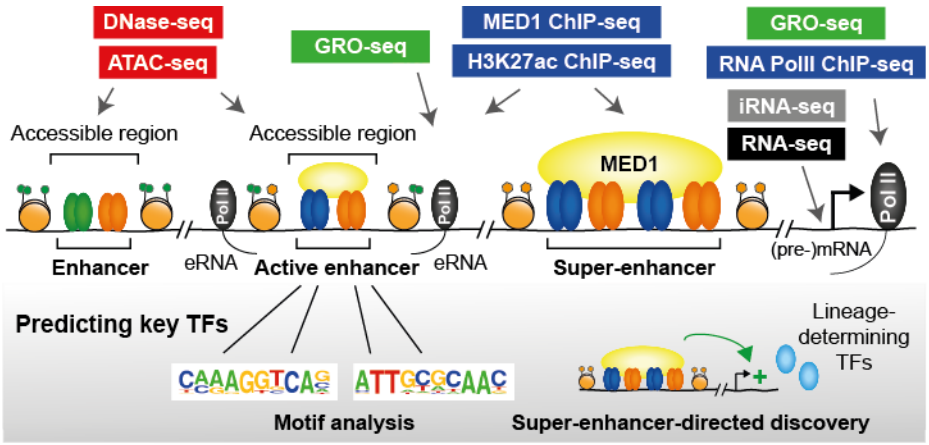

Figure I: Next generation sequencing-based technologies and strategies for uncovering key TFs involved in mediating a particular transcriptional response.

A series of different techniques can be used to investigate the location and activity of regulatory genomic regions and predict the transcriptional regulators that act on these to mediate a given transcriptional response. DNase-seq and ATAC-seq are used to pinpoint nucleosome-depleted accessible regions. ChIP-seq of dynamic cofactors (MED1), and enhancer-associated histone marks (H3K27ac) can further be used to determine the activity of enhancers, which also can be accomplished by GRO-seq. The transcriptome can be explored by the use of RNA-seq, whereas on-going transcription can be determined by RNA Polymerase II ChIP-seq (Pol II), GRO-seq, or iRNA-seq. To predict TFs that define the cellular identity or mediate a particular response, one can use use bioinformatic tools to analyze the DNA sequence of key enhancers and identify TF-binding motifs that are specifically enriched. In addition, the position of super-enhancers can be used to predict lineage-determining TFs. Abbreviations: ATAC-seq, assay for transposase-accessible chromatin with high throughput sequencing; GRO-seq, global run-on sequencing; H3K27ac, Histone H3 lysine 27 acetylation; iRNA-seq, intron RNA sequencing; MED1, mediator complex subunit 1.

Table 1: Key transcriptomic studies in thermogenic adipose tissue or cells

Samples	Key finding	Treatment	Accession	Ref
Mouse				
Adipocytes from BAT and eWAT	Different marker genes of BAT (*Zic1, Tbx15, Lhx8*) and eWAT (*Hoxc8, Hoxc9, Tcf21)*	Primary stromal vascular (SV) cells from NMRI mice *in vitro* diff. for 4 or 7d	GSE7032 Microarray	[117]
Clonal adipocytes from iWAT and BAT	Distinct brown-like fat cells are found in murine and human fat depots	Immortalized and clonally-selected SV cells from 129/Sv mice, *in vitro* diff., 4h forskolin	GSE39562 Microarray	[16]
Rosiglitazone-treated adipocytes from iWAT	Extensive PRDM16-dependent transcriptional changes upon rosiglitazone treatment	Primary SV cells from C57BL/6J mice *in vitro* diff. for 7d, in the presence or absence of rosiglitazone	GSE35011 Microarray	[14]
Pre- and adipocytes from iBAT, iWAT and eWAT	UCP1-positive adipocytes in iWAT convert into UCP1-negative adipocytes following a subsequent warm period	Primary SV cells and mature adipocytes from C57BL/6J mice	GSE44059 Microarray	[69]
Brown preadipose cells	Ebf2 is a specific marker of brown/brown-like adipocyte progenitor cells	FACS-sorted cells from Myf5(GFP+) and Ebf2(GFP)-/+ embryos	GSE60443 RNA-seq Microarray	[118]
UCP1-positive adipocytes from BAT, iWAT and pgWAT	Brown-like but not brown adipocytes expresses a smooth muscle-like signature	UCP1-TRAP mice subjected to 2w of cold treatment	GSE56248 RNA-seq	[18]
Cold-treated iBAT, iWAT and eWAT	Glucose metabolism is comprehensively regulated in BAT upon cold treatment	C57BL/6J mice subjected to 2 or 4d of cold treatment	GSE63031 DGE profiling	[119]
Cold-treated iBAT	Glycerophoslipid pathway is extensively reprogrammed in BAT upon cold treatment	C57BL/6J mice subjected to 3d of cold treatment	GSE70437 RNA-seq	[75]
BAT, iWAT and eWAT, adipocytes from BAT	lnc-BATE1 is a long non-coding RNA regulating BAT development	Primary SV cells from BALB/c mice *in vitro* diff. for 3d or 5d	GSE66686 RNA-seq	[47]
13 adipocytes (single cells) from iBAT	Large transcriptome variability between brown adipocytes from BAT	Tissues from CD-1 mice were cultured for 1d	GSE56638 RNA-seq	[24, 25]
Adipocytes from iWAT and BAT	KCNK3 and MTUS1 are required for thermogenic adipocyte function	Primary SV cells from C57BL/6J mice *in vitro* diff. for 6-7d. Some cells rosiglitazone-treated.	E-MTAB-2624 RNA-seq	[26]
iWAT and eWAT	Browning capacity of different white fat depots is dependent of genetic background	Tissues from C57BL/6J, 129/Sv, FVB/NJ mice	NA RNA-seq	[30]

Adipocytes from CL316,243-treated iWAT	Brown-like-to-white adipocyte transition is controlled by autophagy-mediated mitochondrial clearance	GFP-reporter mice treated with CL316,243 for 7d, adipocytes taken 1, 5, 10, 15, and 30d post b3-AR agonist	E-MTAB-3978 RNA-seq	[71]
Human				
BAT and sWAT from thyroid region	*CKMT1, HMGCS2*, and *KCNK3* higher expressed in human BAT compared to WAT	9 patients undergoing surgery	GSE27657 Microarray	[120]
Clonal pre- and adipocytes from supraclavicular BAT and sWAT	UCP1-positive human adipocytes are more similar to mouse brown-like adipocytes compared to classical brown	Immortalized and clonally-selected SV cells from two patients diff. for 21d, some cells treated with forskolin for 4h	E-MTAB-2602 RNA-seq	[26]
Clonal preadipocytes from BAT and WAT in neck	PREX1 and EDNRB are required for human brown adipocyte differentiation	Immortalized and clonally-selected SV cells from 4 patients undergoing surgery	GSE68544 Microarray	[121]
Human white and brown-like adipocytes	Human white-to-brown-like adipocyte formation induces both fatty acid anabolic and catabolic pathways	Human adipose-derived multipotent stem cells diff. for 14d, treated 4d with PPARγ or PPARα agonist	GSE71293 Microarray	[72]

Table 2: Genome-wide profiling of TFs, histone marks, and chromatin accessibility in thermogenic adipose tissue or cells

Factor/mark	Key finding	Samples	Treatment	Accession	Ref
Mouse					
PPARγ	Preadipocytes from distinct fat depots are differently programmed to permit depot-specific recruitment of PPARγ	BAT, iWAT and eWAT	Primary stromal vascular (SV) cells from NMRI mice *in vitro* diff. for 7d	GSE41481 ChIP-seq	[62]
PPARγ	EBF2 is a regulator of brown adipocyte cell fate that specifically recruits PPARγ to target sites in brown fat cells	BAT and eWAT	Tissue from 129/Sv mice	GSE43763 ChIP-seq	[63]
MLL4, PPARγ, C/EBPβ/α, MyoD, H3K27ac, H3K4me1/2/3	MLL4 is required for enhancer activation during brown adipocyte differentiation	Pre- and adipocytes from BAT	Immortalized preadipocytes from 129/Sv mice, *in vitro* diff. for 0, 2, 7d	GSE50466 ChIP-seq RNA-seq	[56]
H3K4me1/3, and H3K27ac	ENCODE mouse data set	BAT	Tissue from C57BL/6 mice	GSE49847 RNA-seq ChIP-seq	[122]
H3K27me3	JMJD3-mediated removal of H3K27me3 specifically activates BAT-specific genes during brown adipocyte differentiation	Pre- and adipocytes from BAT	Immortalized preadipocytes from C57BL/6J mice, *in vitro* diff.	GSE55469 ChIP-seq	[50]
H3K4me3, H3K27Ac, BRG1, PPARγ ARID1A, JMJD1A	JMJD1A promotes long-range chromatin interactions and induces thermogenic gene expression	Adipocytes from BAT	Immortalized preadipcoytes from C57BL/6 mice, in vitro diff., 2h isoproterenol	GSE67586/ GSE58936 ChIP-seq FAIRE-seq Microarray	[55]
PRDM16, MED1, RNA pol II, H3, H3K4me3, H3K27ac	PRDM16 specifically recruits MED1 to super-enhancers near BAT-selective genes	BAT and eWAT	Tissue from C57BL/6 mice	GSE63964/ GSE63965 ChIP-seq	[65]
LSD1, PRDM16, H3K4me1/2	LSD1 associates with PRDM16 to repress expression of white fat-selective genes in BAT	BAT	Tissue from C57BL/6 mice	NA ChIP-seq	[60]
LSD1	LSD1 represses WAT-selective and activates BAT-selective genes in BAT by a dual mechanism	BAT	Tissue from C57BL/6N mice	GSE81557 ChIP-seq RNA-seq	[59]
GR, H3K4me1, H3K27ac, CBP C/EBPβ, P-CREB	GR is largely dispensable for adipogenesis in culture and BAT development in mice.	Pre- and adipocytes from BAT	GR f/f brown preadipcoytes from C57BL/6 mice, *in vitro* diff.	GSE76619 ChIP-seq RNA-seq	[123]

DNA methylation	Promoter methylation of several Hox genes differs between white and brown adipocytes	Pre- and adipocytes from iBAT and iWAT	Primary SV cells from C57BL/6 mice in vitro diff. for 0, 4 or 6d, some treated with norepinephrine	GSE80961; Bisulfite-seq RNA-seq	[124]
Human					
PPARγ, KLF11, H3K27ac, CBP, MED1	Browning of human adipocytes reprograms PPARγ super-enhancers and KLF11 is required for the browning process	Human pre- and white/ brown-like adipocytes	Human adipose-derived stem cells diff. for 16/19d, with or without PPARγ agonist for 3d	GSE59703 ChIP-seq DHS-seq RNA-seq	[64]