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Transcriptional Regulatory Circuits Controlling Brown Fat Development and Activation

Brown and beige adipose tissue is specialized for heat production and can be activated to reduce obesity and metabolic dysfunction in animals. Recent studies also have indicated that human brown fat activity levels correlate with leanness. This has revitalized interest in brown fat biology and has driven the discovery of many new regulators of brown fat development and function. This review summarizes recent advances in our understanding of the transcriptional mechanisms that control brown and beige fat cell development.

Brown and beige adipocytes burn chemical energy to produce heat and have garnered much attention because of their capacity to counteract obesity and metabolic disease (1). Brown adipocytes develop in discrete and relatively homogenous deposits of brown adipose tissue (BAT), whereas beige adipocytes arise in white adipose tissue (WAT) in response to various stimuli, most notably cold exposure. Both brown and beige adipocytes are packed with mitochondria that contain uncoupling protein 1 (UCP1) (2). When activated by cold/β-adrenergic signaling, UCP1 allows protons to leak across the inner mitochondrial membrane, resulting in increased oxygen consumption along with the production of heat (2,3).

The heat produced by BAT and beige adipose tissue is essential for protecting mice against hypothermia (4,5). Brown and beige adipose cell activity also significantly affects energy balance and systemic metabolism. In particular, mice engineered to have high levels of brown and/or beige fat activity resist weight gain and have a healthy metabolic profile (1,6–9). Conversely, animals with reduced brown and/or beige fat function are more susceptible to obesity under certain environmental conditions (4,5). Of note, brown fat activity can be increased in humans and

likely has beneficial metabolic effects (10,11). For example, people who underwent cold exposure for only 2 h/day for 6 weeks lost fat mass in proportion to the amount of BAT activity they gained (11). Altogether, it thus seems likely that increasing brown and beige fat activity could be used therapeutically to reduce obesity.

Developing and implementing methods to enhance brown and beige fat activity requires a detailed understanding of the mechanisms that control the differentiation and function of these cell types. Although brown and beige fat cells have distinct developmental origins and express some distinct marker genes, the evidence to date suggests that a common set of transcription factors operates in both cell types to control thermogenic potential.

GENERAL ADIPOGENIC NETWORK

The general program of fat cell differentiation is coordinated by peroxisome proliferator–activated receptor (PPAR)-γ and members of the c/EBP family of transcription factors. c/EBPβ is induced early during the process of adipogenesis and plays a critical role in activating PPARγ expression. PPARγ, in collaboration with c/EBPα, binds and regulates the expression of most adipocyte-related genes in fat cells. This core transcriptional network that operates in all types of fat cells has been reviewed extensively by others (12,13). The mechanisms that determine brown, beige, and white fat cell fate are built on top of the general adipogenesis program. Indeed, many of the transcriptional factors that direct brown versus white fat identity act through modulating the core adipogenic transcriptional machinery.

PPARγ

PPARγ is the undisputed master regulator of adipocyte differentiation. Although not sufficient to drive brown fat

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programming of adipocytes, PPARγ is intimately involved in regulating brown adipocyte–selective characteristics of adipocytes. It has been known for some time that PPARγ participates in the activation of brown fat genes, including *Ucp1* (14). Genome-wide binding analyses show that PPARγ binds to many brown (vs. white) fat–specific genes in brown fat cells and tissue (15,16). Synthetic PPARγ activators, especially those in the thiazolidinedione (TZD) class, are particularly potent activators of mitochondrial biogenesis and brown fat–selective genes in adipocytes, including *Ucp1* (17–26). TZD treatment is associated with an increased capacity for UCP1-mediated uncoupled respiration (19,25). However, treatment of animals with TZDs does not increase energy expenditure. This is likely due to the effects of TZDs in promoting lipogenesis and in dampening the β-adrenergic–mediated activation of adipocytes in vivo (27–29).

The mechanism by which TZDs induce the transcription of brown fat genes involves the activation of SIRT1, an NAD-dependent deacetylase that deacetylates two residues in PPARγ (30). The deacetylated form of PPARγ more efficiently binds to PRDM16 (see PR DOMAIN–CONTAINING PROTEIN-16), a powerful transcriptional coactivator of brown fat genes (Fig. 1). Increasing SIRT1 activity levels in adipose tissue promotes WAT browning and alleviates obesity. Through an apparently unrelated mechanism, TZDs also stabilize PRDM16 protein to increase its levels in adipocytes (31). Consistent with these findings, *Prdm16* is required for the browning effects of TZDs (31). The extent to which natural PPARγ agonists regulate the development of beige or brown fat cells in vivo remains an important question.

PPARα, a related family member, is expressed at much higher levels in brown relative to white fat cells and is considered to be a reliable marker gene/protein of brown fat cells. Pharmacological activation of PPARα stimulates WAT browning, and *Pparα* is required for the browning effects of irisin and erythropoetin (32–34). PPARα is well-known for its function in controlling mitochondrial β-oxidation in various cell types, including brown adipocytes. However, PPARα also directly activates critical brown fat–selective genes, including *Ucp1* (using the same element as PPARγ in the −2.5-kb enhancer), *Prdm16*, and *Pgc1α*, which are key drivers of brown fat differentiation (35,36). The extent to which PPARα and PPARγ bind and regulate overlapping and distinct sets of genes in brown adipocytes remains to be studied.

KRÜPPEL-LIKE FACTOR 11

Recent studies by Loft et al. (37) of the Mandrup laboratory identified Krüppel-like factor 11 (KLF11) as an important mediator of TZD-induced browning of human adipocytes. They discovered that KLF11 expression is induced by the TZD rosiglitazone (rosi) and that KLF11 is required for the rosi-mediated activation of brown fat genes. KLF11 and PPARγ bind together and activate brown fat–selective target genes in rosi-treated adipocytes. Of note, unlike in mouse cells, rosi is only required to establish brown fat gene programming in human subcutaneous adipocytes and is dispensable thereafter for maintaining brown fat identity. Loft et al. proposed that once induced, KLF11 stabilizes the beige and brown fat gene program, making it no longer dependent on rosi. KLF11 may thus alter the conformation of the PPARγ complex to keep it in an activated state at brown genes. Future gain-of-function studies will be needed to test this hypothesis.

PR DOMAIN–CONTAINING PROTEIN-16

Many of the transcriptional regulators of brown fat identity have also been shown to physically interact
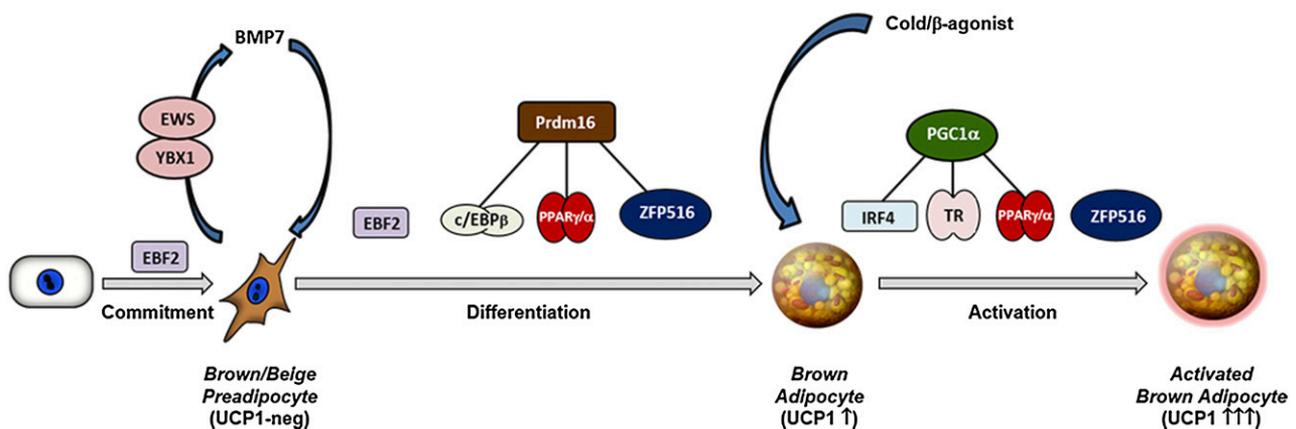
Figure 1—Transcriptional regulation of brown fat cell differentiation and activation. EBF2 marks committed brown preadipocytes and may regulate the commitment process from upstream stem cells. EWS/YBX1 regulates BMP7 production, which then acts in an autocrine manner to induce brown adipogenesis. EBF2, PRDM16, and ZFP516 specifically regulate the induction of brown fat–specific genes during the differentiation process. PRDM16 coactivates c/EBPβ, PPARγ, PPARα, thyroid receptor (TR), and ZFP516. Upon cold exposure/β-adrenergic agonist treatment, brown fat cells are activated to undergo thermogenesis and increase their expression of thermogenic genes. IRF4 plays a major role in this process through recruiting the PGC-1α coactivator. PGC-1α can also coactivate PPARs and TR to activate the transcription of thermogenic genes.

with the general adipogenic machinery. In a search for transcription factors that regulate brown fat development, we identified *Prdm16* as a gene expressed at higher levels in brown relative to white fat tissue (38). Ectopic expression of PRDM16, a histone methyltransferase and zinc-finger–containing protein, into white fat precursor cells drives a brown fat–selective transcriptional program, including the repression of certain white fat–selective genes (38–40). Mechanistic studies indicate that PRDM16 binds and enhances the transcriptional function of PPARγ, PPARα, thyroid receptor, PGC-1α, and c/EBP proteins (Fig. 1) (14,35,41,42). PRDM16 binds to chromatin at many brown fat genes and stimulates transcription through recruitment of MED1 and the Mediator complex, a coactivator of many RNA polymerase II (Pol II)–dependent genes (42,43). PRDM16 also regulates promoter/enhancer looping interactions at brown fat genes, which is likely to be important for efficient transcription (Fig. 2). Using a biochemically defined system, the Roeder laboratory elegantly demonstrated that PRDM16 binds to MED1/Mediator complex and enhances the transcriptional function of PPARγ and the thyroid receptor in activating the transcription of *Ucp1* (42).

A critical role for PRDM16 in brown and beige fat development and function has been demonstrated through a variety of genetic mouse models. Transgenic expression of PRDM16 in adipose tissue stimulates a robust

Figure 2—Model for transcriptional regulation of brown fat–selective genes through EBF2 and PRDM16. *A*: EBF2 binds to enhancer regions of brown fat genes at early time points during brown fat cell differentiation. *B*: Once bound, EBF2 facilitates the binding of PPARγ and other transcription factors to these enhancer regions. *C*: PRDM16 is recruited to these sites indirectly, likely through c/EBPβ, PPARγ, and/or ZFP516. PRDM16 binds and recruits the MED1/Mediator complex to facilitate enhancer/promoter looping and preinitiation complex (PIC) assembly. This action of PRDM16 is required to promote the efficient transcription of brown fat genes.

conversion of subcutaneous WAT, particularly in the inguinal depot, into UCP1+ brown-like (beige) tissue (9). This brown-like remodeling of the inguinal WAT is associated with reduced obesity and improved insulin action in animals fed a high-fat diet. Conversely, deletion of *Prdm16* in adipocytes makes animals more susceptible to high-fat diet–induced weight gain and insulin resistance (44). However, the loss of *Prdm16* in adipocytes only compromises beige fat differentiation while leaving the classical BAT intact (44).

By contrast, knockout (KO) of *Prdm16* using *Myf5-Cre*, which is active in the embryonic mesoderm before brown fat lineage commitment, causes a striking aging-associated loss of brown fat identity and function (45). The brown fat in mutant animals is morphologically indistinguishable from that in their wild-type counterparts at birth and up until ~3 months of age. However, starting at ~3 months, there is a progressive loss in the brown adipose–specific features of the KO BAT. Specifically, *Prdm16* KO tissue adopts white fat–like characteristics, including large unilocular lipid droplets and reduced levels of UCP1 and mitochondria. As a result, BAT-mediated thermogenesis is severely reduced in older but not young *Myf5-Cre; Prdm16* KO mice.

We have speculated that the systemic or tissue environment of embryonic/young animals activates compensatory pathways that bypass the requirement for *Prdm16* in brown adipocyte differentiation. An obvious candidate for this role is PRDM3, the most highly related PRDM family member to PRDM16. However, although concurrent loss of *Prdm3* and *Prdm16* accelerates the postnatal deterioration of brown fat fate compared with loss of *Prdm16* alone, double KO animals still have relatively normal embryonic BAT (45). Thus, there may be additional redundancy that supports embryonic BAT development in the absence of both *Prdm3* and *Prdm16*. In support of this idea, mice lacking the histone methyltransferase EHMT1, an important partner of PRDM16 in regulating brown fat differentiation, display a profound deficit in brown fat development (46). Moreover, the residual EHMT1-deficient BAT ectopically expresses muscle-specific transcripts, suggesting that another factor can participate in recruiting EHMT1 to its critical target genes in brown fat. This putative factor may activate brown fat genes and repress muscle genes during embryonic BAT development in the absence of PRDM16. An alternative hypothesis is that adult and embryonic precursors for brown adipocytes are distinct cell types and that only adult cells require the PRDM16/PRDM3 pathway.

Although PRDM16 is not essential for the induction of brown fat–specific genes in embryos/young animals, PRDM16 is required to suppress the expression of many white fat genes, such as resistin and angiotensinogen, at all ages studied (45). Thus, the repression of these genes is a nonredundant function of PRDM16. Of note, the groucho-related protein TLE3 was previously shown to antagonize PRDM16 function and drive a white fat gene

profile in adipose tissue (47). Specifically, TLE3 disrupts the binding of PPARγ to PRDM16 and cooperates with PPARγ to activate a white fat gene profile. Loss of PRDM16 in BAT may thus enhance the binding of TLE3 with PPARγ, which leads to enhanced activation of white fat genes. Additionally, PRDM16 is believed to directly bind to certain white fat genes and repress their expression through recruiting repressive complexes containing CtBP1,2 and/or EHMT1 (40,45).

An important question is how ectopic overexpression of white fat genes affect BAT function. BAT thermogenesis surprisingly was not affected in young *Myf5-Cre*; *Prdm16* KO mice, which have normal BAT morphology and *Ucp1* expression but elevated white gene levels. Thus, whether these white genes affect other functions of BAT and whether the repression of these white genes is more important in beige fat remain to be determined.

PRDM16 expression/activity in fat cells is controlled by a variety of mechanisms. In particular, several studies have found that miR-133 targets and reduces PRDM16 levels (48–50). Of note, miR-133 expression in muscle satellite cells is required to repress PRDM16 expression (50). Blocking miR-133 activity in regenerating muscle increases PRDM16 levels and leads to ectopic development of metabolically active brown adipocytes. miR-133 also inhibits brown fat cell differentiation, and genetic loss of miR-133 in mice increases WAT browning and promotes insulin sensitivity (48,49). Increasing PRDM16 function and brown fat activity through pharmacological blockade of miR-133 may therefore be possible.

EARLY B-CELL FACTOR-2

PPARγ is a master regulator of white and brown/beige fat differentiation, but how PPARγ (and for that matter, PRDM16) is recruited to brown genes in BAT has been unclear. Through chromatin immunoprecipitation analyses, brown fat–specific binding sites of PPARγ (and PRDM16-binding sites in BAT) were found to be highly enriched with a DNA motif for early B-cell factor (EBF) (15). EBF family members play an important role in promoting adipogenesis acting upstream of PPARγ (51,52). Of note, *Ebf2* is a selective marker of brown and beige adipogenic precursor cells in BAT and WAT, respectively (53). *Ebf2*-expressing adipogenic cells are competent to induce brown fat genes like *Ucp1*, whereas the *Ebf2*-negative cells undergo adipocyte differentiation but do not activate the brown fat program under defined culture conditions (53). In addition to marking brown preadipocytes, *Ebf2* also regulates the brown fat precursor–selective gene signature, suggesting that EBF2 may play a major role in brown preadipose cell commitment.

EBF2 drives a robust program of brown fat differentiation when expressed in muscle or white fat precursors (Fig. 1). Conversely, BAT from *Ebf2* KO mice displays a near-complete loss of brown fat–specific characteristics and increased expression of white fat–selective genes (15). Mechanistic studies have suggested that EBF2 binds

to enhancers in brown fat–specific genes and facilitates the binding of other transcription factors, including PPARγ (Fig. 2). EBF2 also cooperates more directly with PPARγ to stimulate the transcription of brown fat genes. Recent studies demonstrated that a brown fat–enriched long noncoding RNA named Brown fat lncRNA 1 (*Blinc1*) binds and enhances EBF2 transcriptional function (54). EBF2 also directly increases *Blinc1* expression, and *Blinc1* is required in brown fat cells for the expression of brown fat–specific genes such as *Ucp1*, *Cidea*, *Pparα*, and mitochondrial components. Altogether, these studies suggest that EBF2 and *Blinc1* play a critical role in establishing brown fat precursor identity and for setting up the brown fat–specific transcriptional program in adipocytes.

EWS/YBX1/BMP7 AXIS

The RNA-binding protein EWS was recently discovered as a key regulator of early BAT development (55). KO of EWS in mice led to a near-complete loss of BAT formation. Unlike EBF2 and PRDM16, which are only required for induction of brown fat–specific genes, EWS is also required for general adipocyte differentiation. This requirement appears to be fat cell autonomous because *Ews*-null brown preadipocytes fail to undergo adipogenesis at all (55). Of note, residual *Ews* KO BAT and isolated KO brown preadipocytes ectopically express muscle-specific genes, suggesting that EWS controls the cell fate choice between skeletal muscle and BAT development. Mechanistically, EWS interacts with its partner YBX1 to induce the transcriptional activation and production of BMP7, a secreted morphogen that regulates BAT and beige fat development (Fig. 1) (56). Consistent with this model, *Bmp7* KO animals display a similarly severe defect in BAT development (56). Treatment of EWS-null cells with recombinant BMP7 leads to a full rescue of brown adipogenic differentiation capacity (55), implying that autocrine BMP7 signaling in preadipose cells is critical for BAT development. As with most other brown fat differentiation factors, EWS also regulates beige fat development in adult mice. Specifically, haploinsufficient $Ews^{+/-}$ mice have impaired browning responses to rosi or β3-adrenergic agonists (55). Altogether, these results show that EWS and YBX1 stimulate BMP7 production to regulate brown fat differentiation. It will now be interesting and important to determine how EWS/YBX1 expression/activity is regulated during brown/beige adipogenesis.

THERMOGENIC GENE ACTIVATION IN RESPONSE TO COLD/β-ADRENERGIC AGONISTS

Cold exposure directly activates thermogenesis in brown fat cells through the sympathetic nervous system. Briefly, cold, sensed mainly through sensory nerves in peripheral tissues, activates central sympathetic outflow and catecholamine secretion in brown fat. This stimulates β-adrenergic receptors on brown fat cells and elicits a signaling cascade that acutely activates lipolysis and UCP1. The immediate thermogenic effect of this pathway cannot be explained by changes in gene expression. However, the

expression of genes involved in thermogenesis, such as *Ucp1*, *Dio2*, and *Pgc1α*, is also increased in brown fat by catecholamines, and this ramps up the thermogenic capacity of the tissue. In fact, increases in *Ucp1* expression levels in response to various stimuli often is used as a surrogate measure of BAT activity.

In general, the acute catecholamine-linked activation of thermogenic genes occurring in mature brown fat cells seems to be largely independent from the differentiation-linked mechanisms that induce the brown fat program developmentally. Related to this, the brown fat–specific differentiation gene program and the β-agonist–induced activated gene program are quite distinct. Although developmental factors like PRDM16, EBF2, and EWS/YBX1 are required to set the stage for thermogenic activation, there is little evidence that they are directly involved in the activation process. Instead, several other transcription factors have been shown to play a particularly critical role in driving β-agonist–induced gene activation in brown fat cells.

PPARγ COACTIVATOR-1α

The best example of such a factor is the transcriptional coactivator protein PPARγ coactivator-1α (PGC-1α). Puigserver et al. (57) originally isolated and cloned PGC-1α from brown fat cells as an interacting partner of PPARγ. In gain-of-function experiments, expression of PGC-1α into white adipocytes turns on many characteristics of brown fat cells, including UCP1 expression and increased mitochondria (57,58). Over the past 15 years or so, PGC-1α has emerged as a central regulator of mitochondrial biology in many cell types (59). PGC-1α also drives adaptive metabolic gene programs in various tissues, typically acting in response to environmental stimuli. In brown fat cells, PGC-1α controls thermogenic gene activation in response to cold/β-adrenergic agonists, but it is not required for brown fat cell differentiation per se (Fig. 1) (60,61). PGC-1α is however required for the expression of UCP1 in WAT and for the browning effect of FGF21 (62,63).

Elegant studies by Cao et al. (64) showed that PGC-1α is phosphorylated and directly activated by p38 mitogen-activated protein kinase following β-adrenergic stimulation of brown fat cells. PGC-1α then binds to various transcription factors to induce the expression of thermogenic genes, like *Ucp1*. *Pgc-1α* (and *Ucp1*) transcription is also increased in activated brown fat cells by ATF2 and CREB. Until recently, however, the key transcriptional partners for PGC-1α in mediating thermogenic gene induction were unclear.

INTERFERON REGULATORY FACTOR-4

Interferon regulatory factor-4 (IRF4) was recently identified as an important transcription factor partner of PGC-1α and a critical activator of the thermogenic gene program in brown fat cells (65). IRF4 expression is highly induced in WAT and BAT by cold exposure or β-agonist treatment of mice. Targeted overexpression of IRF4 in mature *Ucp1*⁺ brown fat cells further elevates UCP1 levels and enhances BAT activity. This is associated with increased energy expenditure and a reduction in fat mass in animals fed a chow or high-fat diet. In this model, the increased thermogenesis observed is almost certainly due to increases in classic BAT activity because there is very little effect of the transgene on UCP1 expression in WAT. Conversely, deletion of *Irf4* specifically in brown and beige fat cells is associated with depressed energy expenditure and increased susceptibility to obesity and insulin resistance, especially under high-fat diet conditions (65). Mechanistic studies have indicated that IRF4 induces the expression of PGC-1α and physically interacts with PGC-1α to stimulate target gene transcription (Fig. 1). PGC-1α is not able to activate thermogenic genes in the absence of IRF4, suggesting that IRF4 recruits PGC-1α to thermogenic genes. It will now be important to determine the overlap between IRF4 and PGC-1α binding to the genome in cold-activated brown fat.

ZINC-FINGER PROTEIN 516

Zinc-finger protein 516 (ZFP516) is a newly identified zinc-finger–containing transcription factor that plays a critical role in both the differentiation and the activation of brown and beige fat cells (Fig. 1). Dempersmier et al. (66) identified ZFP516 in an unbiased search for transcription factors that could activate *Ucp1* transcription. Virtually nothing is known about this factor. Of note, ZFP516 binds at a previously uncharacterized site in proximity to the *Ucp1* promoter to activate *Ucp1*-driven reporter genes. ZFP516 is expressed at higher levels in BAT relative to WAT and is enriched in adipocytes relative to precursor cells. ZFP516 levels are also increased by cold exposure or β-agonist treatment of mice, probably through activation of CREB, which binds to the *Zfp516* promoter under these conditions. There is a remarkable induction of ZFP516 protein levels, especially in WAT, after only 6 h of cold exposure, suggesting that this factor may also be subject to posttranslational regulation.

Transgenic overexpression of ZFP516 in adipose tissue leads to a dramatic increase in WAT browning in the inguinal depot and suppresses obesity. This is reminiscent of the phenotype observed in fat-specific PRDM16-overexpressing mice (9). In this regard, ZFP516 binds directly to PRDM16, suggesting that PRDM16 may be an important coactivator of ZFP516 in fat cells. KO of *Zfp516* in mice, in contrast to *Prdm16* deficiency however, causes a striking defect in BAT development (66). *Zfp516* mutant BAT depots are small and pale and have poorly structured mitochondria. Molecular analyses show that the residual *Zfp516* KO tissue expresses reduced levels of *Ucp1* and increased levels of muscle genes. Similar to EWS, cell culture studies have suggested that ZFP516 is required for general adipogenesis as well as elaboration of the brown fat gene program. The more severe loss-of-function phenotype for *Zfp516* compared with *Prdm16* suggests that ZFP516 may be coactivated by other cofactors, possibly redundant to PRDM16, during embryonic BAT

development. Altogether, the results so far suggest that ZFP516 functions in a distinctive way to coordinate the differentiation and cold-mediated activation of brown fat cells.

SUMMARY AND PERSPECTIVES

Activating brown and beige fat cells to expend energy is a promising approach to reduce obesity, insulin resistance, and other metabolic diseases. The identification and implementation of brown fat–based therapies will require a detailed understanding of how brown and beige fat cells develop and function. Over the past few years, we have witnessed a renewed focus on brown fat development and biology. As part of this, many novel factors that influence brown fat activity have been identified, including several transcriptional factors. More such factors are likely to be identified as evidenced by the recent discoveries of ZFP516 and IRF4. However, in addition to identifying new factors, the challenging task of integrating the function of known regulators into a cohesive network will be critically important. Along with this, major gaps exist in our understanding of how microenvironmental or systemic cues interact with the brown fat transcriptional network to regulate cell fate and function. In this regard, Farmer and colleagues (67) just reported that beige fat versus smooth muscle gene programs are specifically regulated in WAT through a g-actin/myocardin-related transcription factor-A (MRTF-A) pathway. Studying such beige- versus brown-specific regulatory pathways will be important for defining physiological functions and therapeutically targeting both of these cell types.

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