Review Article

Fubiao Shi[1] / Sheila Collins[2]

Second messenger signaling mechanisms of the brown adipocyte thermogenic program: an integrative perspective

[1] Integrative Metabolism Program, Sanford Burnham Prebys Medical Discovery Institute, Orlando, FL 32827, USA
[2] Integrative Metabolism Program, Sanford Burnham Prebys Medical Discovery Institute, 6400 Sanger Road, Orlando, FL 32827, USA, E-mail: scollins@sbpdiscovery.org

Abstract:
β-adrenergic receptors (βARs) are well established for conveying the signal from catecholamines to adipocytes. Acting through the second messenger cyclic adenosine monophosphate (cAMP) they stimulate lipolysis and also increase the activity of brown adipocytes and the 'browning' of adipocytes within white fat depots (so-called 'brite' or 'beige' adipocytes). Brown adipose tissue mitochondria are enriched with uncoupling protein 1 (UCP1), which is a regulated proton channel that allows the dissipation of chemical energy in the form of heat. The discovery of functional brown adipocytes in humans and inducible brown-like ('beige' or 'brite') adipocytes in rodents have suggested that recruitment and activation of these thermogenic adipocytes could be a promising strategy to increase energy expenditure for obesity therapy. More recently, the cardiac natriuretic peptides and their second messenger cyclic guanosine monophosphate (cGMP) have gained attention as a parallel signaling pathway in adipocytes, with some unique features. In this review, we begin with some important historical work that touches upon the regulation of brown adipocyte development and physiology. We then provide a synopsis of some recent advances in the signaling cascades from β-adrenergic agonists and natriuretic peptides to drive thermogenic gene expression in the adipocytes and how these two pathways converge at a number of unexpected points. Finally, moving from the physiologic hormonal signaling, we discuss yet another level of control downstream of these signals: the growing appreciation of the emerging roles of non-coding RNAs as important regulators of brown adipocyte formation and function. In this review, we discuss new developments in our understanding of the signaling mechanisms and factors including new secreted proteins and novel non-coding RNAs that control the function as well as the plasticity of the brown/beige adipose tissue as it responds to the energy needs and environmental conditions of the organism.

Keywords: β-adrenergic receptor, beige adipocyte, brown adipocyte, long non-coding RNA, microRNA, natriuretic peptide, thermogenesis, uncoupling

DOI: 10.1515/hmbci-2017-0062

Received: August 18, 2017; **Accepted:** August 29, 2017

Introduction

White adipose tissue (WAT) evolved to store excess nutrient energy in the form of triglycerides that could then be accessed in times of food scarcity. And while this storage bank is incredibly important, there were also other challenges to early mammals including humans. Brown adipose tissue (BAT) evolved as a means of generating heat from stored calories as a special adaptation termed non-shivering thermogenesis (NST). In early humans NST was particularly important before the advent of houses and clothing. In order to understand the importance of this evolutionary advance, it is important to know that brown adipocytes are highly enriched in mitochondria and express uncoupling protein-1 (UCP1), a unique protein that serves to 'uncouple' the mitochondrial proton gradient from ATP production. These cells are avid consumers of glucose and fatty acids, the net result being energy expenditure. Active brown fat is now appreciated to be present in humans throughout their lifespan [1], [2], [3], [4], [5]. Moreover, its amount, as in rodents, correlates with lower percentage of body fat and greater insulin sensitivity [6], [7], [8], [9]. Therefore, an increase of brown fat cells and their metabolic activity in humans could target obesity and its comorbidities.

Sheila Collins is the corresponding author.

The existence of brown adipose tissue (BAT) and its function has had a meandering history (see Box 1). It was first described anatomically over 300 years ago, but not until the early 1960s was it realized that with its rich vasculature and innervation, BAT was a major site of heat production in small mammals exposed to cold [10], [11]. Maintaining body temperature in a cold environment has been crucial for eutherian mammals. Shivering thermogenesis results from increased muscular activity, which results in the generation of heat as a by-product. But NST in brown fat evolved as a more efficient mechanism for generating heat. When exposed to cold temperatures, the sensory nerves in peripheral tissues transduce the signal to the hypothalamus, which controls the activity of the sympathetic nervous system (SNS) by triggering the SNS outflow and the release of the catecholamine norepinephrine (NE) from the neural terminals in adipose tissues [12], [13]. Surgical or chemical denervation of the adipose depots blocks this response [14], illustrating that the SNS is the central regulator of the brown fat response to cold. NE acts on β-adrenergic receptors (βAR) to drive the thermogenesis program in adipocytes. There are three βAR subtypes β_1, β_2 and β_3. β_3AR is highly expressed in rodent adipose tissue, but is significantly lower in humans. That said, human subjects treated with a selective β_3AR agonist could nevertheless elicit glucose uptake and imaging of brown fat throughout the body [15]. Binding of NE to β_1AR and β_3AR stimulates BAT for adaptive thermogenesis; meanwhile it also increases lipolysis, which produces fatty acids serving as substrates and activators of thermogenesis. There is also uptake of fatty acids from circulating lipoproteins. In the past few years there have been reports suggesting that M2 macrophages accumulate in the adipose tissue and produce catecholamines [16], [17]. However, this mechanism has recently been called into question [18]. In addition to the activation of brown fat (as in the homogeneous interscapular depot of rodents), SNS activation by cold exposure or selective β_3AR agonists can induce the appearance of UCP1-positive brown-like cells in specific white adipose depots, usually higher in inguinal WAT (iWAT) and retroperitoneal WAT but much lower in others. We also know that there are significant strain- and gender-specific differences in the occurrence of these cold-exposure-induced brown-like adipocytes [19], [20], [21].

Box 1: Adapted from Afzelius, BA in: Brown Adipose Tissue O. Lindberg, Ed. 1970, Elsevier Pub., New York.

Brief history of brown adipose tissue

1670–1817:	Brown adipose tissue is regarded as a part of the thymus.
1817–1863:	Brown adipose tissue is believed to be an endocrine gland, and by some authors an organ active in the formation of blood.
1863–1902:	Brown adipose tissue is considered a modified form of fat tissue serving as a reservoir for food substances.
1902 -----:	Brown adipose tissue is again believed to be an endocrine organ.
1961 -----:	Brown adipose tissue is regarded as a thermogenic effector; a tissue with the function of heating the blood passing through it.

Adapted from Afzelius, BA in: Brown Adipose Tissue O. Lindberg, Ed. 1970, Elsevier Pub., New York.

Intracellular signaling mechanisms from the SNS

It has been known for decades that catecholamine-stimulated thermogenesis in BATs depends on βAR dependent elevation of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)-activity [22]. In addition to activation of βARs, the cAMP produced can be hydrolyzed to adenosine, which can further modulate this signaling by either inhibiting (via A1 adenosine receptors) or further stimulating (via A2 adenosine receptors) cAMP production [23]. Adenosine can increase blood flow to BAT [24], and more recently adenosine released from BAT upon sympathetic nerve stimulation was reported to act via binding to adenosine A2A receptor to promote beige adipocyte recruitment and energy expenditure [25]. Thus the breakdown of ATP to adenosine following SNS activation of brown fat via βARs may have an augmenting effect through the A2A receptors.

In addition to PKA, activation of βAR signaling triggers a series of downstream signaling events that ultimately promote transcription of the *Ucp1* gene and a suite of components for mitochondrial biogenesis and fatty acid oxidation. We identified p38 mitogen-activated protein kinase (MAPK) and MAP Kinase Kinase 3 (MKK3) as necessary components downstream of βARs and PKA to elicit the transcription of classic brown adipocyte genes such as the *Ucp1* and the *Pgc1α* [26], [27], [28] (Figure 1). Specifically, p38 MAPK phosphorylates and activates the transcription factor ATF2 as well as the co-regulator PGC-1α. Actually a number of other

signaling molecules that impact brown adipocytes and BAT also require this p38 MAPK module. These include BMP7/BMP4, BMP8b, orexin, irisin and cardiac natriuretic peptides [29], [30], [31], [32], [33].

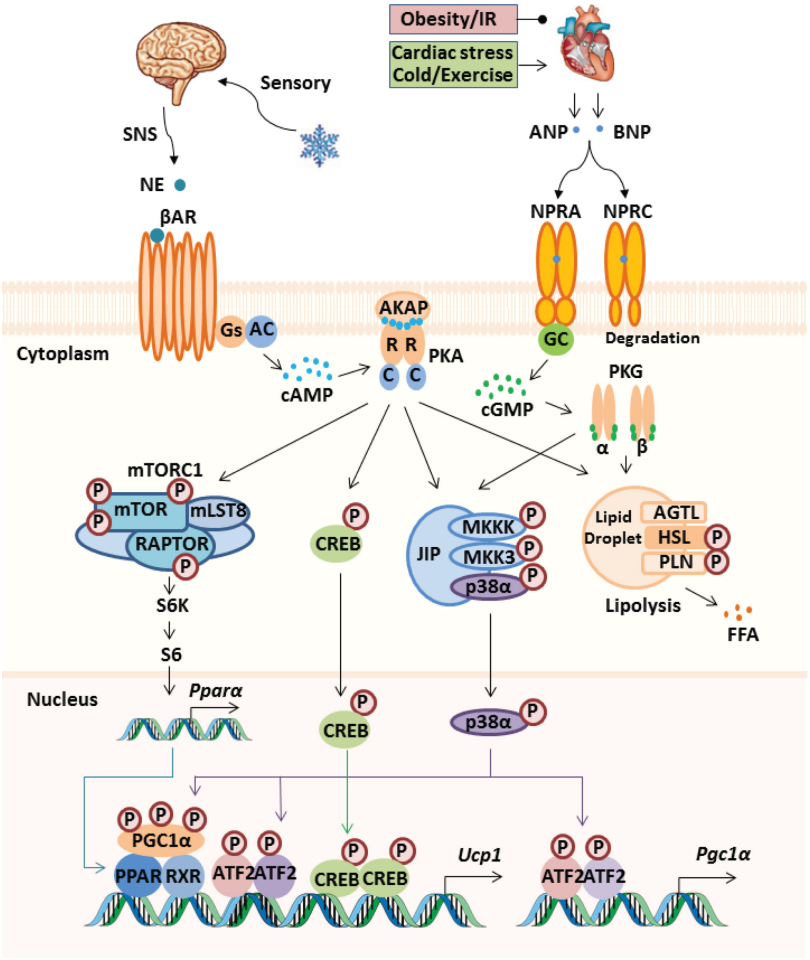

Figure 1: β-adrenergic receptor and natriuretic signaling pathways of adipocyte thermogenic program.
(A) When exposed to cold temperature, the sensory nerves in peripheral tissues transduce the signal to the hypothalamus, which controls the activity of the sympathetic nervous system (SNS) to trigger the release of the catecholamine norepinephrine (NE) from the nerve terminals in adipose tissues. Binding of NE to β-adrenergic receptor (βAR) activates the Gs protein (Gs)-coupled adenylyl cyclase (AC), which produces cyclic AMP (cAMP). cAMP binds to the regulatory subunit (R) of protein kinase A (PKA) and results in the release and activation of the catalytic subunits (C). PKA-mediated phosphorylation cascades increase lipolysis, activate the transcription factor cAMP response element binding protein (CREB), and lead, through a still poorly defined pathway, activation of a p38 mitogen-activated protein kinase (MAPK) module. We recently showed that PKA also activates the mammalian target of rapamycin complex 1 (mTORC1) to drive the brown adipocyte thermogenic program. (B) Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are produced in the heart. The levels of circulating NPs are reduced with obesity and insulin resistance (IR), but increased with cardiac wall stress, cold exposure and exercise. Binding of NP with NPRA activates its intracellular guanylyl cyclase (GC) for cyclic GMP (cGMP) production, which in turn activates protein kinase G (PKG). Binding of NP with NPRC causes peptide internalization and degradation. In parallel to PKA, PKG-mediated phosphorylation cascades also enhance lipolysis and activate p38 MAPK to drive the brown adipocyte thermogenic program. (C) p38 mitogen-activated protein kinases phosphorylate and activate the transcription factor ATF2 and co-regulator PGC1α, which together induce the transcription of downstream thermogenic genes, including the *Ucp1*. ATF2 also increases the transcription of the *Pgc1a* gene itself. PGC1α binds to DNA through interactions with peroxisome proliferator-activated receptor PPARγ/α and retinoid X receptors (RXR). Phosphorylated CREB binds to the CREB-response element in proximal promoter region of the *Ucp1* gene. Activation of mTORC1 increases the expression of Pparα, which further controls the expression of *Ucp1* and other genes whose products are enriched in brown adipocytes.

Natriuretic peptide signaling in adipose tissue

The cardiac atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are hormones secreted by the heart. They were first discovered as pivotal factors controlling blood pressure [34]. The physiological actions

of ANP and BNP are mediated by binding to NP receptor A [NPRA], which activates its guanylyl cyclase (GC) domain to produce cyclic guanosine monophosphate (cGMP), leading to activation of cGMP dependent protein kinase G (PKG) [35], [36] (Figure 1). The other receptor for these cardiac NPs, NP receptor C (NPRC), does not possess an intracellular domain with GC activity and functions to clear the NPs from circulation through receptor-mediated internalization [37], thus reducing the NP-NPRA signaling [38]. That said, there have been reports from time to time that NPRC could couple to the heterotrimeric Gi complex to inhibit adenylyl cyclase [39], [40], [41]. However, it seems that as yet this has not gained general recognition as a bona fide aspect of its function and may deserve further investigation.

Two decades ago NP receptors were shown to be expressed in adipose tissue [42]. Lafontan and colleagues [43] subsequently demonstrated that ANP could stimulate lipolysis in cultured human adipocytes with a potency similar to catecholamine. Consequent clinical studies in humans reported that NPs could increase energy expenditure and fat oxidation independent of the β-adrenergic axis [44]. More recently, we discovered that ANP and BNP can induce the browning program in adipocytes, representing a heart-adipose connection for the regulation of energy metabolism [33], [45]. This aspect of NPRA-cGMP-PKG signaling cascade also depends on the activation of p38 MAPK [33] (Figure 1).

There is growing clinical evidence suggesting that NPs are central cardiometabolic regulators. Obese human subjects show lower circulating BNP levels and a blunted blood pressure response to NPs [46], [47]. The level of NPRC in the adipose rises with obesity; this decreased NPRA/NPRC ratio (NPRR) is also related to glucose intolerance and insulin resistance, with pharmacological improvement in insulin sensitivity in diabetic subjects associated with increased NPRRs and higher levels of thermogenic gene expressions such as the *UCP1* and the *PGC1α* [48]. While the increase in NPRC in adipose tissue has been posited to contribute to the lower circulating BNP concentrations observed in obese subjects, due to increased peptide clearance and degradation [49]. However, obesity is reported to be associated with lower levels of the incompletely processed form – N-terminal-proBNP (NT-proBNP) – and weight reduction significantly increased these indicators of ANP and BNP output [50], [51]. As NT-proBNP is not cleared by NPRC [52], it is possible that there may also be reduced secretion of NPs in obesity. In any event, obesity poses a severe cardiometabolic disease risk.

NPRC plays a critical role in modulating the metabolic effect of NPs, particularly in the adipose tissues [33]. The ability of NP to stimulate lipolysis was reported to be primate-specific and to not occur in rodent adipose tissues at physiological conditions [53]. While this species difference was suggested to be due to the fact that the level of NPRC in rodents is 100-fold higher [53], [54], there is nevertheless a very dynamic regulation and activity of the NP system in rodents. For example, the expression of *Nprc* in mouse and rat adipose tissues is significantly induced by high-fat diet feeding conditions, but repressed by cold exposure or β-agonist treatment. *Nprc*-deficient adipocytes clearly show enhanced PKG activity and lipolytic response to ANP treatment. $Nprc^{-/-}$ mice have markedly reduced body weight, fat mass and higher expression of UCP1 in their white adipose depots. Furthermore, our recent studies show that deletion of *Nprc* in adipose tissues, but not in skeletal muscle, promotes fuel consumptions and protects against diet-induced obesity and insulin resistance, suggesting that the adipose tissue plays a major role for NP-mediated metabolism [55].

A novel route from PKA and PKG to mammalian target of rapamycin complex 1 (mTORC1) is essential for adipose browning

The control of fuel storage and mobilization in the adipocyte is controlled by insulin and the catecholamines, respectively. As already discussed, adipose tissue responds to catecholamines and natriuretic peptides by increasing not only lipolysis, but also the net amount of UCP1-expressing 'thermogenic' adipocytes, sometimes referred to as 'browning' or 'beige adipocytes'. On the other hand, a major signaling node for the anabolic actions of insulin that strongly promotes lipogenesis and protein synthesis involves activation of AKT, leading to the mammalian target of rapamycin (mTOR). mTOR is a 250 kDa conserved Ser/Thr kinase that regulates cell growth and metabolism in response to environmental cues such as growth factors and nutrients. There are two protein complexes containing mTOR. mTORC1 contains the partner protein RAPTOR (regulatory-associated protein of mTOR), and mTORC1 is inhibited by the drug rapamycin; one of the best-characterized downstream targets of mTORC1 is p70 ribosomal S6 kinase (S6K1) (see review by [56]). Surprisingly, our lab recently discovered that the activation of mTORC1 is also required for the βAR stimulation of adipose browning. While catecholamines are involved in the breakdown of energy stores for their consumption, their role in expanding these UCP1-expressing cells within WAT nevertheless entails a significant elevation in biosynthetic capacity, including enhanced mitochondrial and cell protein mass. βAR activation of S6K1 through mTORC1 is triggered by PKA in both mouse and human adipocytes. As shown in Figure 1, PKA phosphorylates mTOR at three

sites and RAPTOR at one site. Importantly, this is independent of the well-known insulin-AKT axis, and is also independent of ERK and p38 MAPK [57].

The mechanisms by which mTORC1 activation drives the thermogenic gene expression program need to be further explored. Considering that TOR was first described as a regulator of protein translation [58], it would not be surprising to find a mTOR-dependent layer of translational regulation in the thermogenic program. In addition to the PKA-dependent pathway just described, a more recent study showed that the tumor suppressor folliculin (FLCN) functionally interacts with mTOR and regulates adipose tissue browning via mTOR and the transcription factor TFE3 [59]. Adipose-specific deletion of FLCN relieves mTOR-dependent cytoplasmic retention of TFE3, leading to direct induction of the PGC-1 transcriptional coactivators, drivers of mitochondrial biogenesis and the browning program [59]. The cytoplasmic retention of TFE3 by mTOR is separate from the canonical mTOR signaling to S6K [59]. This represent an alternative FLCN-mTOR-TFE3-PGC-1β pathway, separate to mTOR-S6K, in the regulation of adipose browning. Taken together, mTORC1 plays a crucial role in the integration of various signals to direct the adipose browning program.

Non-coding RNAs (ncRNAs) as regulators of brown/beige adipocyte program

MicroRNAs (miRNA) are small ncRNAs, which usually contain 22 nucleotides and function in RNA silencing and post-transcriptional regulation of gene expression. miRNAs are first transcribed as primary miRNA (pri-miRNA) in the nucleus and processed to generate precursor miRNAs (pre-miRNA) by a protein complex composed of DiGeorge syndrome critical region 8 (DGRC8) and RNase III enzyme Drosha. The pre-miRNAs are exported to the cytoplasm and further processed by RNase III enzyme Dicer to produce the miRNA duplex about 22 nucleotides in length. Fat-specific ablation of Dicer, results in 'whitening' of interscapular brown fat and lipodystrophy [60]. Adipose tissue-specific deletion of Dgcr8 impairs interscapular brown fat function and browning of subcutaneous WAT [61].

Further studies identified several critical functional miRNAs in the brown/beige adipocytes, such as miR-133, miR-196a, miR-155 and miR-378. The muscle-enriched miR-133 is regulated by myocyte enhancer factor-2C (MEF2C) and directly targets and negatively regulates PR domain-containing-16 (PRDM16), the key regulator of brown fat. Cold temperature exposure reduced miR-133 expression in BAT and iWAT, as a result of the decreased MEF2C expression, and further induced PRDM16 expression and promoted brown adipocyte program [62]. In addition, miR-133 controls the choice of myogenic vs. brown adipocyte determination in adult satellite cells. Antagonism of miR-133 in muscle regeneration increases uncoupled respiration, glucose uptake, and thermogenesis in local treated muscle and augments whole-body energy expenditure, improves glucose tolerance and impedes the development of diet-induced obesity [63]. Furthermore, genetic deletion of miR-133 leads to elevation of the brown and thermogenic gene programs in subcutaneous WAT and increased insulin sensitivity, glucose tolerance and cold temperature-induced thermogenesis [64]. Cold temperature-induced miR-196a activates C/EBPβ by down-regulating the repressor gene *Hoxc8*, and promotes brown fat program [65]. The brown fat-enriched miR-155 and its target C/EBPβ form a bistable feedback loop integrating transforming growth factor-β1 (TGFβ1)/small-mothers against decapentaplegic (SMAD) signaling to maintain the balance between proliferation and differentiation of brown pre-adipocytes [66]. miR-378 increases classical brown fat (BAT) mass but suppresses formation of beige adipocytes in subcutaneous WAT, potentially by targeting phosphodiesterase (Pde)1b in BAT but not in WAT [67]. Taken together, these studies suggested that miRNA plays an essential role for brown fat development, function and recruitment.

Long ncRNAs (lncRNAs) are transcripts generally larger than 200 nucleotides in length and devoid of open reading frame (ORF). lncRNAs can either be or not be poly-adenylated, and locates either in the nuclear or cytoplasmic fractions. lncRNAs exert their gene regulation function by various mechanisms, including chromatin remodeling, epigenetic modification, transcriptional and post-transcriptional regulation [68]. The application of deep-sequencing technology has greatly advanced the discovery of novel lncRNA and annotation of their biological functions.

Several studies suggested that lncRNAs are important regulators of adipogenesis and brown/beige differentiation (Table 1). By profiling the transcriptome of the various adipocyte cell lines, researchers have identified 175 lncRNAs that are specifically regulated during adipogenesis [69]. Among these lncRNAs, the lncRNA Firre interacts with the nuclear-matrix factor heterogeneous nuclear ribonucleoprotein U (hnRNPU) and localizes across five distinct chromosome loci, acting as an important regulator of nuclear architectures [70]. Through global profiling of lncRNA gene expression during thermogenic adipocyte formation, a recent study identified brown fat lncRNA 1 (Blnc1) as a nuclear lncRNA that promotes brown and beige adipocyte differentiation and function, potentially by forming a ribonucleoprotein complex with transcription factor 'early B cell factor 2' (EBF2) to stimulate the thermogenic gene program [71]. In another study, researchers used RNA-Seq to profile

the transcriptome of mouse brown, inguinal white and epididymal white fat and identified ~1500 lncRNAs, among which 127 BAT-restricted loci were induced during differentiation and often targeted by key regulators PPARγ, CCAAT/enhancer binding protein (C/EBP)α and C/EBPβ [72]. One of these lncRNA, lnc-BATE1, binds to nuclear-matrix factor hnRNPU and is required for establishment and maintenance of BAT identity and thermogenic capacity [72]. Altogether, ncRNAs including miRNAs and lncRNAs appear to play important roles for brown and beige adipocyte development, differentiation and function. In the years to come this exciting area of investigation will likely yield important new components and mechanisms that will be integrated into the growing literature on the control of adipose tissue development and physiology.

Table 1: Non-coding RNA regulators of brown adipocyte thermogenic program.

Genes	Category	Pathways	Function	Phenotypes	Refs.
Dicer	miRNA	miRNA biogenesis	Process of pre-miRNA into miRNA duplex	Fat-specific deficiency results in 'whitening' of interscapular brown fat and lipodystrophy	[62]
Dgcr8	miRNA	miRNA biogenesis	Process of pri-miRNA into pre-miRNA	Adipose tissue-specific deletion impairs interscapular brown fat function and browning of subcutaneous WAT	[63]
miR-133	miRNA	Repressed by cold temperature via MEF2C	Negatively regulates PRDM16 and brown adipocyte program	Genetic deletion leads to elevations of the brown and thermogenic gene programs in subcutaneous WAT and increased insulin sensitivity, glucose tolerance and cold temperature-induced thermogenesis	[65], [66]
miR-196a	miRNA	Induced by cold temperature	Activates C/EBPβ via targeting the repressor gene *Hoxc8* and promote brown fat program	Fat-specific transgene in mice induces the recruitment of brown adipocyte-like cells in WAT, enhances energy expenditure and resistance to obesity	[67]
miR-155	miRNA	Targets C/EBPβ	Forms bistable feedback loop to integrate TGFβ1/Smad signaling	miR-155-deficient mice exhibit increased brown adipose tissue function and 'browning' of white fat tissue. Transgenic overexpression in mice causes a reduction of brown adipose tissue mass and impairment of brown adipose tissue function	[68]
miR-378	miRNA	Targets Pde1b	Regulates brown adipogenesis in vitro	Transgene in adipose tissue specifically increases classical brown fat (BAT) mass, but not white fat (WAT) mass, and prevents both genetic and high-fat diet-induced obesity	[69]
Firre	lncRNA	Interacts with hnRNPU	Acts as important regulator of nuclear architectures	Genetic deletion results in loss of colocalization of these trans-chromosomal interacting loci	[72]
Blnc1	lncRNA	Interacts with EBF2	Promotes brown and beige fat differentiation and function	RNAi knockdown in brown preadipocytes severely impaired adipogenesis and the expression of brown fat markers	[73]
lnc-BATE1	lncRNA	Interacts with hnRNPU	Required for BAT thermogenic capacity	lnc-BATE1 inhibition impairs concurrent activation of brown fat and repression of white fat genes	[74]

Conclusion remarks and outlook

Obesity is one of the major risk factors of many metabolic disorders, including type 2 diabetes (T2D), cardiovascular diseases and several forms of cancer [73], [74], [75], [76]. Even modestly increasing energy expenditure by brown/beige adipocyte activation and recruitment is a promising strategy of obesity therapy. Recent progress

in the field has advanced our understanding of the signaling mechanisms of brown adipocyte thermogenic program and provides valuable insight for the strategy to promote energy expenditure and combat obesity and related metabolic disorders.

Author Statement

Research funding: Authors state no funding involved.

Conflict of interest: Authors state no conflict of interest.

Informed consent: Informed consent is not applicable.

Ethical approval: The conducted research is not related to either human or animals use.

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