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REVIEW

Nature's fat-burning machine: brown adipose tissue in a hibernating mammal

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ABSTRACT

Brown adipose tissue (BAT) is a unique thermogenic tissue in mammals that rapidly produces heat via nonshivering thermogenesis. Small mammalian hibernators have evolved the greatest capacity for BAT because they use it to rewarm from hypothermic torpor numerous times throughout the hibernation season. Although hibernator BAT physiology has been investigated for decades, recent efforts have been directed toward understanding the molecular underpinnings of BAT regulation and function using a variety of methods, from mitochondrial functional assays to 'omics' approaches. As a result, the inner-workings of hibernator BAT are now being illuminated. In this Review, we discuss recent research progress that has identified players and pathways involved in brown adipocyte differentiation and maturation, as well as those involved in metabolic regulation. The unique phenotype of hibernation, and its reliance on BAT to generate heat to arouse mammals from torpor, has uncovered new molecular mechanisms and potential strategies for biomedical applications.

KEY WORDS: Brown adipose tissue, Gene expression, Hibernation, Mitochondria, Thermogenesis

Introduction

Brown adipose tissue (BAT) specializes in the burning of fat and is responsible for adaptive, nonshivering thermogenesis (NST) in mammals (as reviewed by Cannon and Nedergaard, 2004). With multiple mitochondria that uncouple the electron transport chain (ETC) from adenosine triphosphate (ATP) synthesis, and a high density of capillaries to deliver oxygen, BAT has evolved to maximize the combustion of fat to generate heat in a short amount of time. Over the past decade, brown adipocytes have been discovered in humans (as reviewed by Nedergaard et al., 2007), sparking interest in developing therapeutic strategies that promote brown adipocyte recruitment and activation in order to combat human obesity and metabolic disorders (as reviewed by Nedergaard and Cannon, 2010; Lidell et al., 2014; Matsushita et al., 2014).

Lipid catabolism by BAT in natural hibernators is likely the highest capacity oxidation of fat (per mass of tissue) that occurs in mammals. As such, hibernating mammals represent an underutilized resource for identifying mechanisms involved in BAT recruitment and metabolic control. In circannual hibernators, such as the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*), BAT mass increases in the autumn primarily owing to an increased lipid content (Burlington et al., 1969). Over 5–6 months of hibernation, these animals do not eat and use white adipose tissue (WAT) as their primary source of fuel. At specific points during the hibernation season, stored lipid is consumed at high rates by BAT, which generates heat and initiates arousal from hypothermic torpor bouts. These regular re-warming events occur in all mammalian hibernators and are referred to as interbout arousals (IBAs). IBAs typically occur every 1–2 weeks and are characterized by rapid whole-body re-warming and a resumption of normothermic metabolic activity for 12–24 h (Fig. 1). To initiate IBAs via NST, lipid in BAT is catabolized and lipid content decreases (Burlington et al., 1969; Carneheim et al., 1989; Nedergaard and Cannon, 1984). In light of the dynamic seasonal regulation of BAT, its proliferation before the hibernation season and its crucial role in endogenous rewarming from torpor, hibernating mammals provide a unique model system for unravelling the molecular mechanisms underlying BAT recruitment and metabolic activity.

Recent efforts have been directed toward dissecting the molecular underpinnings of BAT regulation and function through a variety of strategies, from functional assays to multi-omic techniques. Although hibernation research at the molecular level has intensified over the past 20 years, the genetic mechanisms controlling BAT activity in hibernation have only recently been explored. Recent research findings outlined in this Review have identified players and pathways involved in adipocyte differentiation, maturation and metabolic regulation. The unique phenotype of hibernation and its reliance on BAT can shed light on molecular mechanisms and new strategies for biomedical applications.

Brief history and overall function of BAT in hibernators

BAT was first described by anatomical inspection in hibernating marmots almost 500 years ago (Gessner, 1551) and was later referred to as 'the hibernation gland' (Rasmussen, 1923). Since the early 1900s, BAT has been extensively investigated using a range of mammalian hibernators, such as ground squirrels, hamsters, marmots, bats and dormice (e.g. Barger et al., 2006; Burlington et al., 1969; Chaffee et al., 1964, 1966; Hook and Barron, 1941; Malatesta et al., 2001; Rémillard, 1958; Sheldon, 1924; Smalley and Dryer, 1963; Smith, 1964; Smith and Hock, 1963; Smith and Horwitz, 1969; Wells et al., 1965; Yan et al., 2006). BAT function is uniquely regulated in hibernators through the interplay of ambient temperature (T_a) and body temperature (T_b). As the animal enters torpor, a reduction in the thermoregulatory set point (T_{set}) shifts the lower limit of the thermoneutral zone (TNZ) to lower ambient temperatures (Snapp and Heller, 1981). This drop in T_{set} ceases sympathetic activation of BAT, effectively halting thermogenesis in this tissue to permit T_b equilibrium with the environment; however, NST can increase to defend above-freezing T_b if the environmental temperature drops (Boyer and Barnes, 1999). During arousal from torpor, T_{set} increases toward euthermic levels (Heller and Hammel, 1972), initiating BAT activation and thermogenesis, which results in large increases in the whole-body metabolic rate before T_b increases

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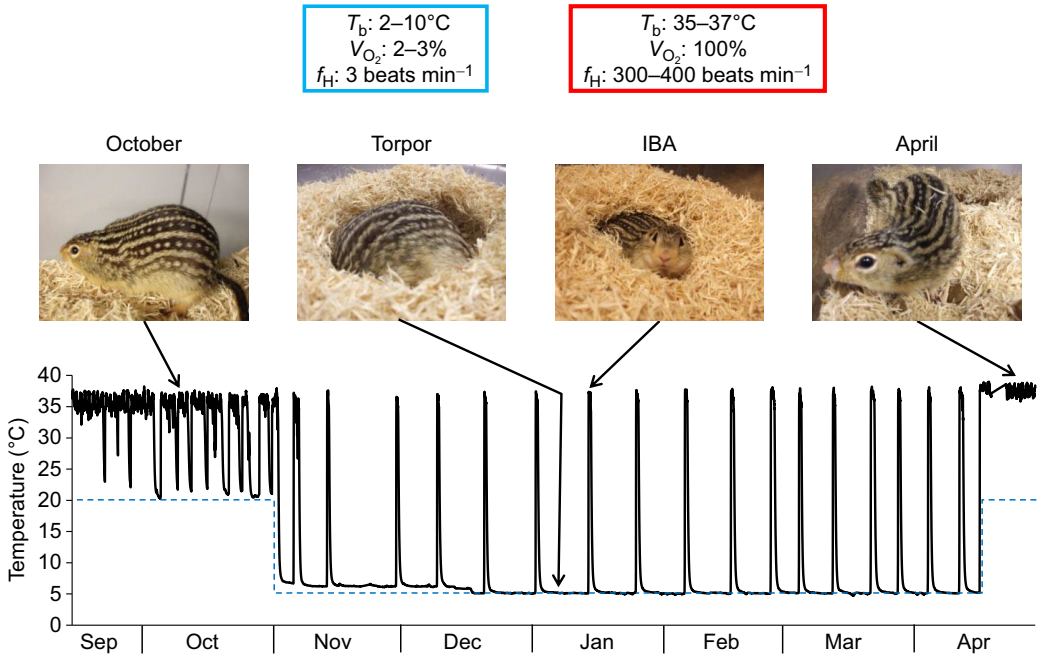

Fig. 1. Graph showing body temperature tracings (solid line) between September and April of a thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) inside an environmental chamber. Body temperature (T_b) was measured using a surgically implanted transmitter. The dashed blue line represents ambient (environmental) temperature, which is lowered to 5°C on 1 November and raised back to 23°C in March or April depending on the experiment. Periodic interbout arousals (IBAs) are seen as regular spikes in body temperature despite a constant ambient temperature of 5°C. Photographs of animals at four different points during the year are indicated and shown above the graph. Characteristic measurements of T_b, oxygen consumption (V_{O_2}) and heart rate (f_H) during torpor and IBA are shown above the respective photographs. The figure is duplicated from Hampton et al., 2013 (with permission).

(Tøien et al., 2001). Shivering thermogenesis follows this BAT-initiated rewarming only after T_b reaches a given level (as reviewed by Cannon and Nedergaard, 2004).

Activation of BAT thermogenesis occurs through a signaling cascade initiated by noradrenaline (NA; also known as norepinephrine) release from the sympathetic nervous system (as reviewed by Cannon and Nedergaard, 2004). The NA activates β_3-adrenergic G protein-coupled receptors on the surface of the brown adipocyte. This in turn stimulates cyclic adenosine monophosphate production and the activation of cytosolic protein kinase A, subsequently activating hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Breitwieser, 2002; Chaudhry and Granneman, 1999; Lowell and Spiegelman, 2000; as reviewed by Nicholls and Locke, 1984; Silva, 2006). The activation of HSL and ATGL triggers free fatty acid (FFA) release from triglycerides within the brown adipocyte cytosol. These FFAs diffuse across the outer mitochondrial membrane, into the intermembrane space, dissipating the proton motive force and uncoupling oxidative phosphorylation (OXPHOS) via uncoupling protein 1 (UCP1) (as reviewed by Nicholls and Locke, 1984; Cannon and Nedergaard, 2004). As protons leak across the inner mitochondrial membrane, the mitochondrial respiration rate increases and heat is produced as a by-product (as reviewed by Nicholls and Locke, 1984; Cannon and Nedergaard, 2004). Thus, the hibernation phenotype is managed in large part by controlling the thermogenic nature of BAT to successfully execute torpor-arousal cycles (Fig. 2A).

Thirteen-lined ground squirrel as a model organism

The relative importance of BAT is evident when studying the rapid T_b changes in a hibernator. During hibernation, mammals enter a period of heterothermy, characterized by lengthy periods of low T_b and inactivity (as reviewed by Melvin and Andrews, 2009), interspersed with short normothermic IBAs (Fig. 1). During torpor, thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) show a heart rate less than 5% of their normothermic rate, use only 2–3% of their normal oxygen levels, and maintain a T_b of 2–10°C; all physiological characteristics that would result in death for most mammals (as reviewed by Andrews, 2007). The majority of this Review will address findings associated with BAT in the thirteen-lined ground squirrel – a 'model organism' for studying the hibernation phenotype.

Heat generation is especially advantageous during hibernation because it plays a vital role in the endogenous rewarming of animals during arousal from torpor via NST (Fig. 2A). BAT is perfused early during arousal and drives the rewarming process (as reviewed by Cannon and Nedergaard, 2004; Hampton et al., 2010). Hibernators can rewarm to normothermia even though ambient temperatures remain low. A large increase in BAT temperature is evidence of BAT activation during this phase. For example, the temperature of BAT during arousal may exceed the rectal temperature by up to 14°C (Smith, 1964; Smith and Hock, 1963). During the arousal phase, the hibernator will use all available thermogenic mechanisms to reach normothermia. Because shivering cannot occur when the animal is in torpor, BAT-derived heat is essential for initiating arousal. Indeed, BAT thermogenesis reduces the energetic costs of the arousal by 60% by accelerating the rewarming process (Oelkrug et al., 2011). Moreover, the highest rate of BAT activity occurs during IBAs when heat production occurs very quickly. The animal's T_b rises 20°C in less than 1 h as warm blood circulates to rapidly rewarm the animal to a normothermic aroused state in less than 3 h (Hampton et al., 2010; Schwartz et al., 2015b; Fig. 2B).

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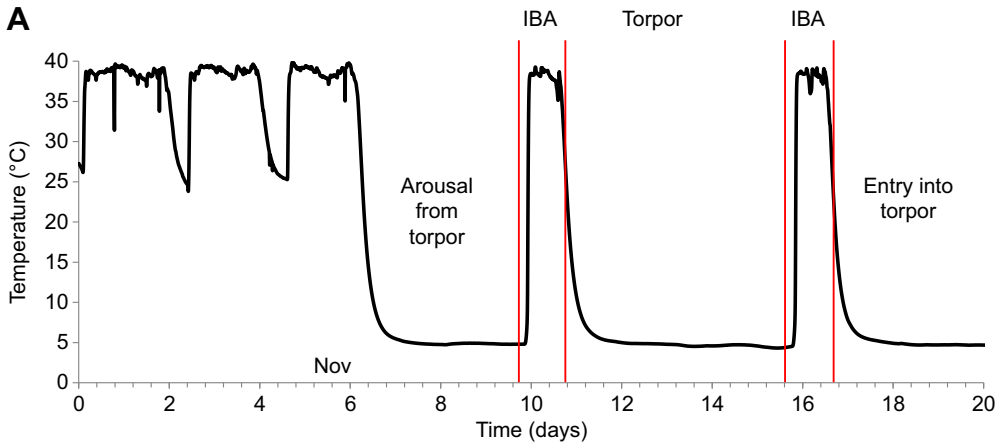

Fig. 2. Examples of torpor bouts and interbout arousal (IBA) cycles in the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). (A) Passive whole-body cooling that occurs during 'entry into torpor' is almost as abrupt as the warming during 'arousal'. Body temperature shows that this abrupt heating and cooling occurs at regular intervals as measured by surgically implanted transmitters. This regular heating and cooling is a regulated process. (B) The highest rate of brown adipose tissue activity occurs during IBAs, where heat production occurs very quickly. The animal's body temperature rises 20°C in less than 1 h and is able to rise to normothermia within 3 h (adapted from Schwartz et al., 2015b).

Brown adipose as a circannual tissue

The overall hibernation phenotype is circannually characterized (Schwartz and Andrews, 2013), with patterns of reproduction, T_b regulation, fattening and food consumption cycling seasonally (Schwartz et al., 2015a). This circannual entrainment is also seen in BAT, where there is a strong switch in morphology between homeothermic and heterothermic phases (Fig. 3). Qualitatively, this is seen through changes in wet mass, quantified via water–fat magnetic resonance imaging techniques (MacCannell et al., 2017) and tissue dissection (Ballinger et al., 2016; Hindle and Martin, 2014). For example, in small rodent hibernators, BAT mass is highest in winter but undergoes atrophy in the spring (Ballinger et al., 2016; Hindle and Martin, 2014). At peak size, BAT equates to ∼5% of body weight in the Djungarian hamster, with lipids composing ∼85% of BAT mass (Rafael et al., 1985). These observations have also been quantified at the cellular level in ground squirrels, with BAT growth accompanied by an increase in mitochondrial abundance (Ballinger et al., 2016; Milner et al., 1989) and replicating cells (Hindle and Martin, 2014). This cycle of atrophy and recruitment is also observed by large proteome differences between the homeothermic and heterothermic time points in BAT compared with other tissues, further speaking to the circannual nature of this tissue (Grabek et al., 2015b; Hindle and Martin, 2014). These changes correspond with the increased expression of several genes and proteins involved in BAT-mediated thermogenesis, such as UCP1 – an indicator of BAT recruitment (Ballinger et al., 2016; Hampton et al., 2013).

Initially, BAT recruitment was thought to occur in response to changes in both temperature and photoperiod (Burlington et al., 1969; Hoffman et al., 1965; Rafael et al., 1985), irrespective of coordinating changes in body mass. However, in thirteen-lined ground squirrels, BAT growth still occurs in the autumn under constant environmental conditions (Ballinger et al., 2016; Hindle and Martin, 2014; MacCannell et al., 2017), suggesting that the endogenous circannual rhythm of BAT recruitment is seasonally programmed in anticipation of winter need (Hindle and Martin, 2014). Although photoperiod has been shown to regulate the seasonal recruitment of BAT (Heldmaier et al., 1981), future studies should identify the molecular regulators controlling BAT recruitment and atrophy in hibernators. Such insights may aid in developing methods to recruit and activate BAT for human therapeutic mechanisms in obese adults (MacCannell et al., 2017).

BAT mitochondria: UCP1 and essential heat production

The peak in BAT recruitment during the autumn and winter months is required to produce the heat needed for the multiple arousals experienced during the hibernation season. Heat production is made possible by the numerous BAT mitochondria, which uniquely contain UCP1. UCP1 is a BAT-specific transport protein of the inner mitochondrial membrane (as reviewed by Nicholls and Locke, 1984; Cannon and Nedergaard, 2004), and is present in most placental mammals (Jastroch and Andersson, 2015; Gaudry et al., 2017). UCP1 is activated by long-chain fatty acids (as reviewed by Klingenberg and Huang, 1999; Fedorenko et al., 2012), and activation of UCP1

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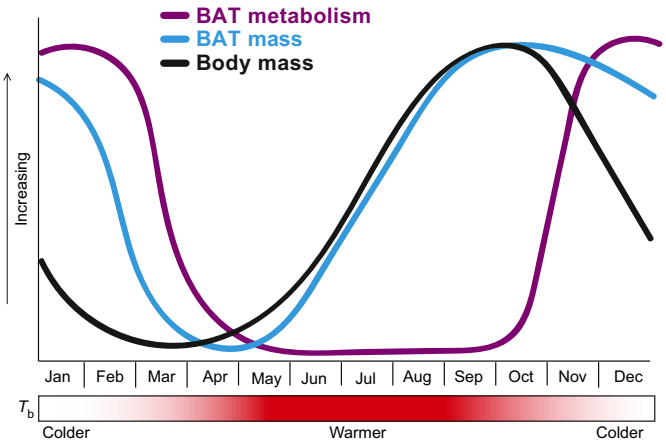

Fig. 3. Circannual cycle of brown adipose tissue (BAT) in hibernators. Colored lines are hypothetically drawn from data simplified from literature (MacCannell et al., 2017; Heim et al., 2017; Ballinger et al., 2016; Hampton et al., 2013; as reviewed by Florant and Healy, 2012). BAT metabolism (purple) is highest during the hibernation season and overall is suppressed during the active season when body temperature (T_b; red gradient) is at a normothermic 37°C. BAT mass (blue) peaks during the autumn preparation phase and at the beginning of the hibernation season when body mass (black) is also highest. Model was adapted from Florant and Healy (2012).

effectively uncouples the ETC from ATP synthesis (as reviewed by Nicholls and Locke, 1984; Klingenberg and Huang, 1999). Because BAT mitochondria express relatively smaller amounts of ATPase (Cannon and Vogel, 1977), virtually all the free energy released by substrate oxidation and the proton motive force is effectively released as heat (as reviewed by Cannon and Nedergaard, 2004).

Overall, the adrenergic regulation of BAT function is well understood (as reviewed by Cannon and Nedergaard, 2004), and the seasonal modifications of BAT function have recently become clearer in hibernators. Recent interest has turned to investigating the functional regulation of BAT mitochondria as hibernators cycle throughout the year and through different phases of a torpor bout (e.g. Ballinger et al., 2016; McFarlane et al., 2017; Heim et al., 2017). Although tissue-specific mitochondrial metabolism is generally suppressed during torpor compared with active seasons (as reviewed by Staples, 2014, 2016; Staples and Brown, 2008), BAT mitochondria have enhanced function during hibernation compared with the active seasons (Ballinger et al., 2016; McFarlane et al., 2017; Heim et al., 2017). Specifically, using isolated mitochondria and various fuels, BAT mitochondrial respiration is not suppressed during torpor compared with IBA or active seasons at low assay temperatures (Ballinger et al., 2016; McFarlane et al., 2017). Moreover, BAT mitochondrial respiration is higher in torpor at various temperatures fueled with various substrates compared with IBA, suggesting reversible suppression in torpor compared with IBA (McFarlane et al., 2017). The temperature sensitivity of BAT adipocyte respiration also does not differ between torpor and IBA (McFarlane et al., 2017), and no differences in respiration are seen in response to NA in BAT adipocyte respiration (McFarlane et al., 2017). Furthermore, there is no suppression of ETC maximal enzyme activity between IBA and torpor (McFarlane et al., 2017), suggesting that temperature sensitivity differences between torpor and IBA in BAT mitochondrial respiration are not the result of ETC enzyme activity suppression but of some other interactions (McFarlane et al., 2017). BAT mitochondria are primed and ready during hibernation to produce heat once the NA signal is present. This suggests an increase in BAT mitochondrial metabolism and efficiency in heat production during hibernation.

This pattern of BAT mitochondrial metabolism is in contrast to liver and other tissues (as reviewed by Dugbartey et al., 2017; Staples, 2014, 2016; Staples and Brown, 2008). For example, isolated liver mitochondria show approximately 70% suppression of respiration rates coupled to ATP synthesis during torpor compared with IBA (as reviewed by Staples, 2014, 2016). This suppression at the organelle level is accompanied by the suppression of maximal activities of ETC complexes (De Meis et al., 2012). The liver contributes greatly to whole animal metabolism, so the suppression of liver mitochondrial metabolism is estimated to contribute 5% to whole animal energy savings, even without any change in T_b (as reviewed by Staples, 2016).

The relatively low level of suppression of BAT mitochondrial respiration or ETC complex activity between torpor and IBA is perhaps not all that surprising given that BAT metabolism is primarily regulated through changes in T_{set} and sympathetic activation (as reviewed by Staples, 2016). During torpor, T_{set} is decreased and sympathetic activation of BAT via FFA release is ceased, thus halting BAT uncoupled mitochondrial respiration (McFarlane et al., 2017; as reviewed by Staples, 2016). Given that uncoupled thermogenesis is likely no longer contributing significantly to the metabolic rate, the suppression of BAT mitochondrial respiration or any further downregulation of substrate oxidation or posttranslational modifications of the ETC enzymes would not conserve significantly more energy (McFarlane et al., 2017; as reviewed by Staples, 2016). During arousal, BAT accounts for a high proportion of whole animal metabolism (Tøien et al., 2001), and in the early stages of arousal, BAT mitochondria begin functioning at very low T_b, and function in a similar way to that seen in normothermia as T_b rises. Thus, because BAT mitochondria need to function at low T_b to initiate arousal, they are primed and ready to function throughout hibernation (Ballinger et al., 2016; McFarlane et al., 2017). This is also suggested by the very few changes in proteins associated with BAT mitochondria between torpor and IBA across the hibernation season (Ballinger et al., 2016; Grabek et al., 2015b; Hindle and Martin, 2014).

Understanding BAT with -omic techniques

With the advent of transcriptomics via ribonucleic acid sequencing (RNA-seq), lipidomics, proteomics and the completed genomes of a handful of hibernators, the molecular biology of the hibernation phenotype is becoming clearer. These new approaches include the use of transgenic strategies in hibernating ground squirrels for the overexpression of proteins *in vivo* (Nelson et al., 2013). Recent efforts have characterized the molecular underpinnings of BAT function both across the circannual cycle and the torpor–arousal phases. Overall, these studies have identified important transcriptional players in the activation and suppression of BAT during both hibernation and across the year. Discovery-based 'omics' approaches have been effective tools in characterizing circannual changes in gene and protein expression (Grabek et al., 2015b), and have identified shared and unique pathways underlying BAT function.

Global gene expression

Early efforts used microarrays to characterize changes in the gene expression of BAT. Initially, messenger ribonucleic acids (mRNAs) encoding heart- and adipose-type fatty acid binding proteins promoting fatty acid mobilization were found to be expressed at higher levels in the BAT of hibernating thirteen-lined ground squirrels compared with normothermic thirteen-lined ground squirrels (Hittel and Storey, 2001). Another study observed that *ADFP*, which encodes perilipin-2 (PLIN2) and is considered to be important in adipocyte differentiation, was expressed at higher

levels in torpid versus summer active arctic ground squirrels (Yan et al., 2006). Genes involved in nearly every step of the biochemical pathway leading to NST are overexpressed in BAT during hibernation, further reflecting the unique role of BAT as a furnace during hibernation (Yan et al., 2006). Global gene expression in BAT in arctic ground squirrels has also been interrogated via microarrays (Williams et al., 2011; Yan et al., 2008). These studies found mRNA levels of genes supporting the paradigm of energy catabolism shift from carbohydrate metabolism during active time points to lipids during hibernation (Williams et al., 2011; Yan et al., 2008), with T_a having a small effect on patterns of gene expression (Williams et al., 2011). Increased availability of glucose in BAT may be necessary to support increased rates of NST for arousal and during steady-state torpor in arctic ground squirrels when they are defending a gradient between T_b and T_a (Barnes and Buck, 2000).

More recently, a comprehensive RNA-seq analysis of BAT gene expression was performed across the circannual cycle of thirteen-lined ground squirrels using Illumina HiSeq (Hampton et al., 2013). Fig. 4 is a model showing the involvement of highly expressed and/or differentially expressed ground squirrel BAT genes involved in adrenergic signaling, lipolysis and heat generation during hibernation. Of special note is β-adrenergic receptor (*ADRB1*), which is upregulated during hibernation (Fig. 4). The rapid turn-on of BAT is controlled by NA through *ADRB1*, whereas the rapid turn-off (i.e. cooling) of BAT is controlled by adenosine via *ADORA1* (Fig. 4). The elevated levels of *UCP1* transcripts during hibernation is evidence of the interplay between mitochondria and thermogenesis in BAT (Hampton et al., 2013), thus further validating the importance of the thermogenic ability of BAT during hibernation. The differential expression of genes involved in adipose differentiation, substrate transport, structure remodeling and heat generation likely enhance thermogenesis in BAT at low body temperatures (Yan et al., 2006).

Lipokine regulation of BAT

In non-hibernating mammals, BAT activity and BAT mass can be increased by exposing animals to the cold. Despite the overall improvement in fat catabolism and the potential for weight loss, cold exposure is uncomfortable and not well-tolerated by non-hibernators. In March 2017, Tseng and colleagues (Lynes et al., 2017) showed that the lipid 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) stimulates BAT activity in mice. Injection of 12,13-diHOME activates the uptake of fuel into mouse BAT, resulting in lowered levels of serum triglycerides and improved tolerance of cold temperatures. Owing to this hormone-like activity, 12,13-diHOME is considered a lipokine and appears to be a molecular on-switch for BAT growth and function.

12,13-diHOME is produced *in vivo* by a three-step reaction beginning with 18-carbon linoleic acid (C18:2 *n*-6) as the starting material. Studies have shown that dietary linoleic acid is important for successful hibernation in mammals (as reviewed by Ruf and Arnold, 2008). The final two steps required for the conversion of linoleic acid to 12,13-diHOME synthesis are catalyzed by epoxide hydrolase 1 (Ephx1) and epoxide hydrolase 2 (Ephx2). In thirteen-lined ground squirrels, the genes encoding Ephx1 and Ephx2 are expressed at very high levels in BAT based on RNA-seq analysis (Hampton et al., 2013). In a normalized comparison of mRNA levels in seven tissues across the hibernation season, *Ephx1* and *Ephx2* show the highest expression levels in BAT (Fig. 5), with *Ephx2* showing significant upregulation during the hibernation

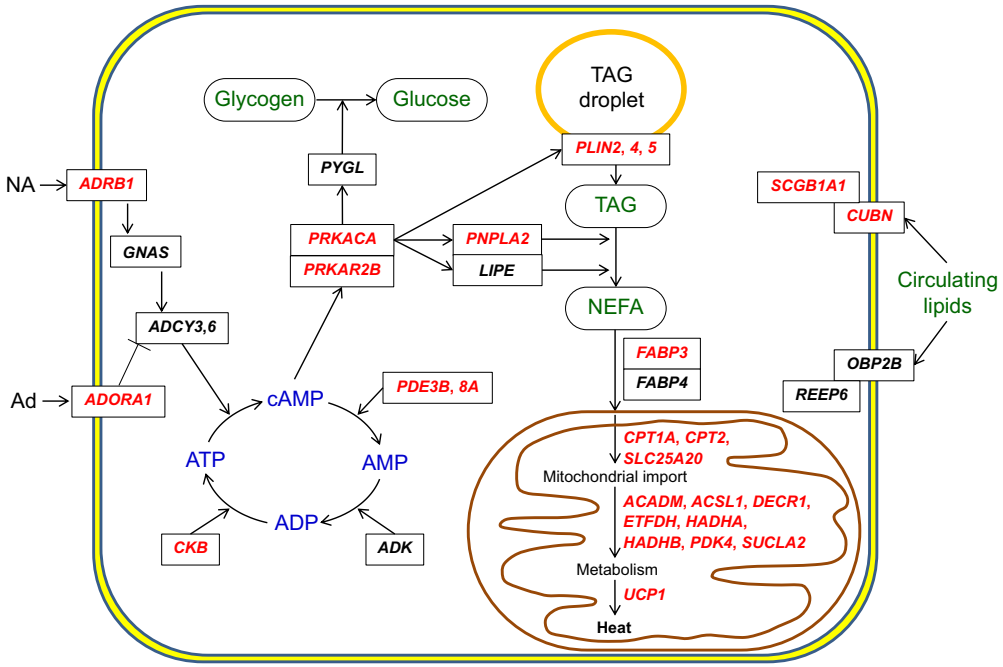

Fig. 4. Model highlighting differentially expressed genes involved in fuel utilization and heat generation in brown adipose tissue (BAT) during hibernation. The role of gene products in various metabolic processes in a brown adipocyte is shown. Genes with abbreviations in red meet the criteria for differential expression, showing highest messenger ribonucleic acid (mRNA) levels during the hibernation phases of torpor or interbout arousal (IBA). Abbreviations of genes that are not differentially expressed but their mRNAs are highly abundant and/or encoded by tissue-specific genes in BAT, are shown in black. Molecules that serve as a source of fuel are labeled in green. Data were taken from a transcriptomic study using RNA sequencing (Hampton et al., 2013). Ad, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; NA, noradrenaline; NEFA, non-esterified fatty acid; TAG, triacylglycerol; UCP1, uncoupling protein 1. Figure is duplicated from Hampton et al., 2013 (with permission).

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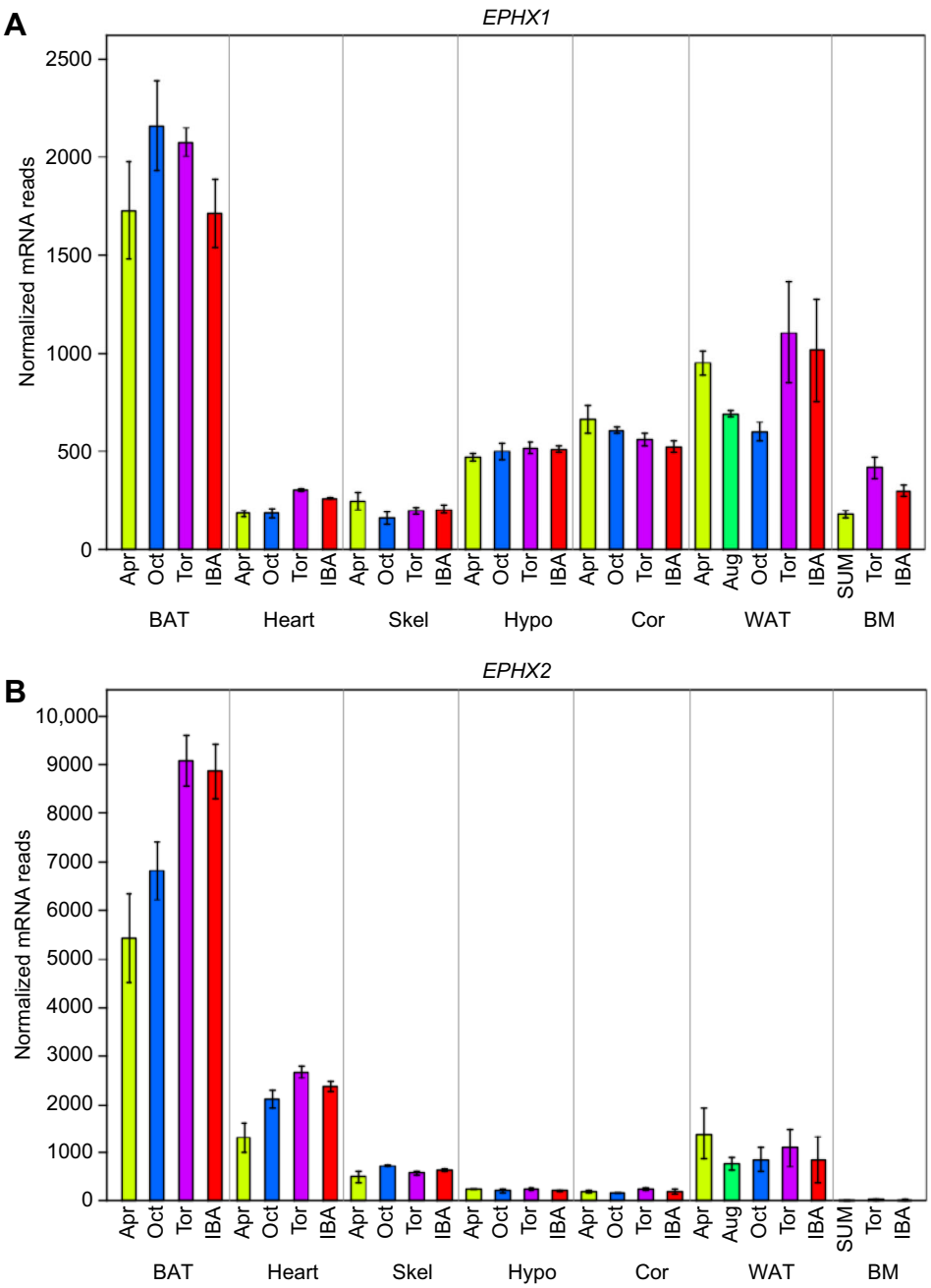

Fig. 5. Seasonal messenger ribonucleic acid (mRNA) expression patterns of *EPHX1* and *EPHX2* across seven tissues in the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). (A) *EPHX1* and (B) *EPHX2* mRNA levels were determined in previous studies of BAT and WAT (Hampton et al., 2013), cortex and hypothalamus regions of the brain (Schwartz et al., 2013), bone marrow (Cooper et al., 2016), and heart and skeletal muscle (Vermillion et al., 2015a). Measurements shown on the *y*-axis are means±s.e.m. of the upper-quartile normalized counts of reads from the four collection points (April, October, torpor and IBA). BAT, brown adipose tissue; BM, bone marrow; Cor, brain cortex; EPHX1, epoxide hydrolase 1; EPHX2, epoxide hydrolase 2; Hypo, hypothalamus; IBA, interbout arousal; Oct, October; Skel, skeletal muscle; Tor, torpor; SUM, summer; WAT, white adipose tissue.

states of torpor and IBA in both BAT and the heart (Fig. 5B). The high levels and strong induction of *Ephx2* mRNA in ground squirrel BAT resembles the cold-induction (1 h at 4°C) of *Ephx2* in mice leading to 12,13-diHOME production and the resulting increase in BAT fatty acid transport (Lynes et al., 2017). In natural hibernators, seasonal induction of *Ephx2* suggests that ground squirrels and possibly other hibernating mammals have the potential to provide additional biochemical clues for increasing BAT size and function.

Post-transcriptional regulation of BAT

Although global suppression of mRNA transcription occurs during torpor (Morin and Storey, 2009; Storey and Storey, 2004; van Breukelen and Martin, 2002; as reviewed by van Breukelen and Martin, 2015), it was initially suggested that BAT might be an exception to the general observation of arrested translation given that polyribosomes appeared to remain intact and protein synthesis may continue during torpor (Hittel and Storey, 2002). However, there is now evidence for general suppression of transcription in the BAT of hibernating thirteen-lined ground squirrels via low T_b during 2-week torpor periods and the action of epigenetic controls (Biggar and Storey, 2014). Specifically, inhibitory transcriptional controls largely remain in place during the arousal phase of the torpor cycle and general reactivation of gene expression can occur during IBA (Biggar and Storey, 2014). Thus, transcription is needed to sustain the thermogenic function of BAT and replenish proteins that are damaged during longer term torpor (Biggar and Storey, 2014; as reviewed by van Breukelen and Martin, 2015). Moreover, BAT microRNAs were assessed during torpor in thirteen-lined ground squirrels and those with altered expression were linked to β-oxidation (Wu et al., 2014).

Recently, BAT mRNA stability was investigated across the torpor–arousal cycle in thirteen-lined ground squirrels and revealed

a subset of transcripts that increase in relative abundance over the torpor bout, likely owing to selective mRNA stabilization (Grabek et al., 2015a). These transcripts were enriched for gene products that are essential for NST, and they are available for immediate use in the early arousal period whereas some cohorts of transcripts increase during torpor at low T_b (Grabek et al., 2015a). Overall, the cycle of transcription, degradation, stabilization and polyadenylation in BAT leads to translation of the correct transcripts at the precise time with minimal energy costs (Grabek et al., 2015a). This mechanism allows transcripts to be prioritized for immediate translation when BAT activity needs to be quickly resumed (Grabek et al., 2015a), which may suggest possible therapeutic strategies for rapid BAT activation in humans (Grabek et al., 2015a).

BAT proteomics

The BAT transcriptome of the thirteen-lined ground squirrel provides inferences on molecular mechanisms within the tissue. However, mRNA levels are not always correlated closely with protein abundance and function in living systems (de Sousa Abreu et al., 2009; Foss et al., 2011; Gry et al., 2009; Nie et al., 2007; Pascal et al., 2008; Yin et al., 2013). Therefore, recent research efforts have involved characterizing global patterns of protein expression of BAT across the circannual cycle in hibernators using various proteomics techniques.

Before the advent of sequencing technology, most protein expression studies were conducted via western blotting. For example, genes encoding AKT, PPARγ and PGC-1 are expressed at higher levels in the BAT of hibernating bats than in the BAT of euthermic bats (Eddy and Storey, 2004). This initial protein expression study supported the substrate switch to lipids during hibernation (as reviewed by Boyer and Barnes, 1999). More recently, a comprehensive study utilizing 2D proteomics characterized the entire BAT proteome across both the circannual cycle and the torpor–arousal cycle (Hindle and Martin, 2014). The most dramatic shift in the BAT proteome is between homeothermic and heterothermic time points (Grabek et al., 2015b; Hindle and Martin, 2014). Despite prolonged metabolic suppression during hibernation, the levels of many BAT proteins increase in the winter. This winter increase of specific BAT proteins includes mitochondrial proteins and likely supports transient but rapid energy production during periods of high metabolic activity. Rather than abundant changes in ETC proteins, components of membrane transporters in BAT were increased in winter. As seen with morphology and copy number variation, the relatively large proteome differences between spring and autumn further supports the circannual observation of how BAT mass is reduced after emergence from hibernation but later proliferates and becomes highly recruited again as animals prepare for the next hibernation season. Changes in depot size occur in conjunction with a strong enrichment of 14-3-3 proteins (Hindle and Martin, 2014).

With proteomic technology rapidly advancing to involve more sensitive methods utilizing mass spectrometry, non-2D gel assessments of protein changes have been utilized. For example, considering the importance of mitochondria during hibernation, we recently analyzed the BAT mitochondrial proteome of active and hibernating thirteen-lined ground squirrels (Ballinger et al., 2016) using isobaric tags for relative and absolute quantitation labeling (Li et al., 2012). Fig. 6 shows a model of the involvement of highly expressed and/or differentially expressed ground squirrel BAT proteins in mitochondrial metabolism, lipolysis and heat generation during hibernation. Of special note are proteins involved in β-oxidation, the ETC and UCP1, which are upregulated during hibernation (Fig. 6). Moreover, a recent study using label-free liquid chromatography mass spectrometry investigated changes in the phosphoproteome of BAT during hibernation in the thirteen-lined ground squirrel (Herinckx et al., 2017). Protein levels of mitochondrial membrane and matrix proteins and respiratory chain complexes in BAT are largely constant between active normothermic squirrels and their hibernating counterparts (Herinckx et al., 2017).

Comparing mRNA and protein expression across the year provides insight into not only BAT biology but also the underlying functional adaptations in BAT in hibernators. The overall findings of these studies support the paradigm shift away from carbohydrate metabolism during normothermic time points toward fatty acid and ketone body oxidation during hibernation in BAT (as reviewed by Carey et al., 2003). This increase in lipid metabolism has also been recently supported by plasma metabolomics and bone marrow transcriptome studies in the thirteen-lined ground squirrel (Cooper et al., 2016; D'Alessandro

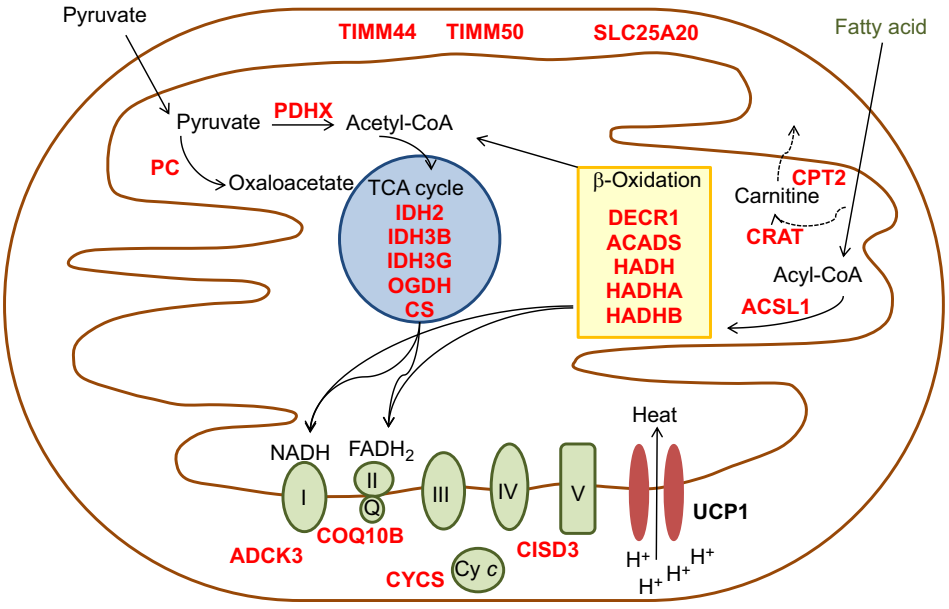

Fig. 6. Model highlighting differentially and highly expressed proteins involved in mitochondrial metabolism and heat generation in brown adipose tissue (BAT) during hibernation. Proteins with abbreviations in red meet the criteria for differential expression, with highest protein levels during the hibernation phases of torpor or interbout arousal. Abbreviations of proteins that are not differentially expressed but are highly expressed, and/or tissue-specific proteins in BAT, are shown in black. Data were taken from recent proteomic (Hindle and Martin, 2014) and mitoproteomic (Ballinger et al., 2016) studies, where similar states were sampled across the year in BAT from thirteen-lined ground squirrels. Cy *c*, cytochrome *c*; FADH, reduced flavin adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; TCA, tricarboxylic acid cycle; UCP1, uncoupling protein 1.

et al., 2017). Furthermore, protein phosphorylation may have an underappreciated role as a regulatory mechanism in BAT given that it is likely involved in causing metabolic changes during hibernation (Herinckx et al., 2017). Overall, these -omic studies have identified essential components for the recruitment and control of BAT activation, which may provide novel targets for effective treatment of human obesity.

Future directions

In addition to the future directions mentioned throughout this Review, we think there are two main avenues researchers should take regarding BAT regulation. One avenue addresses developmental similarities between skeletal muscle and BAT and the other takes advantage of the continued progression of genomic approaches in identifying important regulators of BAT.

Drawing similarities between BAT and skeletal muscle

Almost ten years ago, compelling evidence showed that brown adipocytes have a common origin with muscle cells and not white fat cells (Cannon and Nedergaard, 2008; Cristancho and Lazar, 2011; Kajimura and Saito, 2014; Seale et al., 2007, 2008; Timmons et al., 2007; Tseng et al., 2008). Specifically, young and undifferentiated brown fat cells contain mRNAs that are also found in skeletal muscle, such as Myf5 and myosin (Timmons et al., 2007). Moreover, many proteins found in brown adipocytes (e.g. proteins involved in fat uptake, transport and mitochondrial fat combustion) are more similar to those found in skeletal muscle than in WAT (Cannon and Nedergaard, 2008).

Although the majority of these findings were unmasked in non-hibernating models, other important regulators distinguishing skeletal muscle and BAT may be unmasked by studying these two tissues together more finely in hibernators. It is now possible to analyze the relatedness of the BAT transcriptome (Hampton et al., 2013) with transcriptomes from skeletal muscle (Hampton et al., 2011; Vermillion et al., 2015a) over the course of the hibernation season. Functionally, at the mitochondrial level, skeletal muscle and BAT behave similarly during hibernation (McFarlane et al., 2017; as reviewed by Staples, 2016). Specifically, both tissues have relatively little mitochondrial suppression during torpor, suggesting little selective advantage in terms of energy savings (Ballinger et al., 2016; McFarlane et al., 2017; as reviewed by Staples, 2016). The similarities in mitochondrial function can perhaps be elucidated further through the use of proteogenomics (e.g. Anderson et al., 2016; Vermillion et al., 2015b). Recently, a proteogenomic scan of skeletal muscle across the circannual cycle of thirteen-lined ground squirrels was conducted (Anderson et al., 2016). This scan was able to compare the transcriptomic and proteomic abundance levels to reveal regulators during different phases of hibernation and pathways potentially regulated via post-transcriptional events (Anderson et al., 2016). The proteogenomic approach used in this study revealed numerous peptide sequences that had not been predicted using current gene models, providing a reference point for improving genome annotation and pointing to possible novel proteoforms with a functional role in hibernation (Anderson et al., 2016).

It would be fruitful for a proteogenomic scan to be conducted in BAT across the circannual cycle because not only would this reveal potential novel proteoforms but also it would be useful to compare the proteogenomic scan to the skeletal muscle scan. Through this comparison, potential regulators that underpin skeletal muscle and BAT differentiation may elucidate players that have not been identified before. Altogether, these future scans may provide fertile ground for the generation of hypotheses that can be tested in future studies of mammalian hibernation with the potential for new discoveries relevant to human health. Overall, multi-omics approaches offer a major advantage, especially to the study of non-model systems where genomic information may be sparse or inaccurate. Although the depth of coverage in high-throughput proteomics is not yet to the level of transcriptomics, the disparities between the two and the higher biological relevance of protein data make it a valuable tool not only for testing specific hypotheses but also for the generation of new hypotheses (Anderson et al., 2016).

Regulatory changes of BAT across the year and throughout hibernation

The combination of RNA-seq with the recently completed and annotated thirteen-lined ground squirrel genome project has offered a starting point for illuminating the mechanisms of differential gene expression during hibernation. Although the overall hibernation phenotype is controlled by differential gene expression across the circannual cycle (as reviewed by Carey et al., 2003), the key to hibernation-specific gene expression may well reside in the non-coding regulatory regions that surround those differentially expressed genes that give rise to the hibernation phenotype (as reviewed by Andrews, 2007). Specifically, differences in gene expression can be caused by changes in *cis*-regulation (as reviewed by Romero et al., 2012). The contributions of *cis*-regulatory changes to differential gene expression may shed light on the dynamic cycle and function of BAT across the circannual cycle. By identifying *cis*-regulatory variation across the circannual cycle in BAT, we may be able to elucidate regulatory regions playing roles in both the recruitment–atrophy cycle and heat generation switches.

Moreover, it is possible to assess the contributions of *cis*-acting regulatory mutations to variation in the expression of a given gene by quantifying the relative abundance of allele-specific transcripts (as reviewed by Romero et al., 2012). RNA-seq allows for high-throughput assessment of allele-specific gene expression because it provides a means of quantifying transcript abundance on an allele-specific basis (Storz and Cheviron, 2016). For example, in RNA-seq analyses of BAT samples between heterothermy and homeothermy, metabolic genes that exhibit extreme differences in expression (e.g. *UCP1*) can be targeted for future mitochondrial functional studies. Thus, the results of both RNA-seq and proteomic analyses can generate novel hypotheses that can be experimentally tested (Storz and Cheviron, 2016).

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Competing interests

The authors declare no competing or financial interests.

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