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Review

What puts the heat on thermogenic fat: metabolism of fuel substrates

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Owing to its unique capacity to clear macronutrients from circulation and use them to produce heat, thermogenic fat is capable of regulating glucose, lipids, and branched-chain amino acids (BCAA) circulatory levels. At the same time, its activity yields a higher energy expenditure, thereby conferring protection against cardiometabolic diseases. Our knowledge on the mechanisms of uptake and intracellular metabolism of such energy substrates into thermogenic fat has meaningfully evolved in recent years. This has allowed us to better understand how the thermogenic machinery processes those molecules to utilize them as substrates for heating up the body. Here, we discuss recent advances in the molecular and cellular regulatory process that governs the uptake and metabolism of such substrates within thermogenic fat.

Highlights

The thermogenic fat capacity to clear circulating fatty acids, glucose, and branched-chain amino acids (BCAA) to fuel its own thermogenic machinery is a crucial feature enabling it to improve metabolic health.

Studies in mice indicate that substrate use and fate in thermogenic fat varies with the conditions of thermogenic challenge to meet the timely metabolic demands.

Although thermogenic fat prioritizes fatty acid oxidation for heat generation, glucose and BCAA catabolism are also required in adaptive thermogenesis

Introduction

Thermogenic adipocytes are specialized fat cells that regulate whole-body energy homeostasis. These adipocytes display small multilocular lipid droplets and are mitochondria-rich cells that dissipate energy in the form of heat through uncoupling protein 1 (UCP1)-dependent and -independent mechanisms [1]. Furthermore, several studies also provide evidence that thermogenic adipocytes exert an endocrine role by secreting distinct molecules that regulate thermogenesis and glucose and lipid homeostasis, thus acting at local or distant sites [2,3]. Interestingly, at cold temperatures, **thermogenic fat** (see Glossary) can take up a large amount of energy substrates [i.e., glucose, free fatty acids (FFAs), and **BCAA**] from circulation. The catabolism of these molecules also provides intermediates that fuel the thermogenic machinery of both tissues [4]. The clearance of these circulating energy substrates due to elevated thermogenic fat activity or mass is associated with beneficial cardiometabolic traits [5,6]. Moreover, recent studies have revealed a much more complex view of the mechanisms involved in the fate of energy substrates during thermogenesis. Here we provide a comprehensive overview of the field's current standing, highlighting recent advances in the molecular and cellular regulatory processes that govern the distribution and utilization of such energy substrates within thermogenic fat.

Glucose uptake as an energy source for brown fat

Studies in rodents have consistently demonstrated that active thermogenic fat robustly increases circulating glucose uptake, while ablated brown and beige fat impairs systemic glucose homeostasis [4]. In humans, glucose uptake has been the most commonly used surrogate tool to detect brown adipose tissue (BAT) activity, mainly by F-fluorodeoxyglucose positron emission tomography-computed tomography (**^{18}F-FDG-PET-CT**). Several past studies demonstrated that cold exposure enhances glucose uptake in human BAT [4]. Moreover, pharmacological investigations using β3- [7] or β2- [8] adrenergic receptor (AR) agonists also revealed increased glucose uptake by BAT. Noticeably, enhanced BAT glucose uptake also improves systemic glucose homeostasis in humans. For example, insulin-resistant obese individuals treated with a selective β3-AR agonist (mirabegron) displayed improved glucose tolerance, β-cell function,

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and insulin sensitivity [9]. Interestingly, glucose uptake and glycolytic flux in human BAT is active even at a **thermal comfort state**, suggesting that during warm conditions, glucose is used by BAT for glyceroneogenesis or fatty acid (FA) synthesis to increase triacylglycerol (TAG) stores for further lipolysis when required [10,11]. A study in humans revealed that the ingestion of a mixed carbohydrate-rich meal increases BAT thermogenesis to the same extent as cold stress [12]. Interestingly, in the postprandial state, BAT glucose uptake is higher than FFA, possibly due to the inhibitory effects of insulin on adipose tissue lipolysis. In addition to interventional investigations, a recent retrospective study investigating more than 50 000 ^{18}F-FDG-PET-CT images unequivocally demonstrate that higher BAT mass is associated with lower circulating glucose levels and other metabolic traits. In addition, high BAT content was independently associated with lower prevalence of type 2 diabetes [5]. Of note, those metabolic benefits were particularly prominent in overweight or obese patients, suggesting that thermogenic fat may be an attractive therapeutic target to counteract the derangements in glucose homeostasis.

A key debatable issue is what is the real contribution of glucose to brown and beige fat thermogenesis? In the following section, we highlight the most recent findings linking glucose metabolism to thermogenesis in adipocytes. Although most evidence comes from rodent studies, recent relevant human data is also addressed in this review.

Thermogenic fat catabolism of glucose during thermogenesis

It is well documented that active thermogenic fat takes up circulating glucose through glucose transporters (**GLUT**) 4 and GLUT1 by distinct mechanisms. Glucose is catabolized in cells through either the collaborative action of glycolysis and the tricarboxylic acid cycle (TCA) or by swapping intermediary metabolites to side pathways. A series of recent *in vitro* and *in vivo* studies revealed that the genetic or pharmacological blockage of glycolysis or mitochondrial pyruvate catabolism restrains brown fat thermogenesis. For instance, the inhibition of mouse Glut1 or of glycolytic flux *in vivo* with the hexokinase inhibitor (2-deoxy-D-glucose) **2-DG** impaired BAT thermogenesis [13]. Diminishing glycolytic flux by knockdown of hexokinase 2 and pyruvate kinase in mature brown adipocytes also impaired isoproterenol-stimulated oxygen consumption [13]. *In vivo*, mitochondrial pyruvate carrier inhibition blocks cold-induced glucose oxidation and impairs body temperature maintenance in mice [14].

Although it is often assumed that active thermogenic fat directly metabolizes glucose to provide fuel for thermogenesis, a substantial amount of evidence suggests that distinct side pathways are fueled by glucose [15]. Indeed, a recent study in humans revealed that only a minor fraction of glucose yields substrates that directly fuel cold-induced BAT thermogenesis [10], while other investigations confirmed that most glucose ends up as a carbon source feeding the pentose phosphate pathway (PPP) [16] (Box 1), glycerol synthesis [16,17], and *de novo* lipogenesis (DNL) [16] (Figure 1). Mechanistically, cold stress paradoxically activates BAT DNL by driving carbohydrate response element binding protein (ChREBP) transcriptional activity in a recently revealed serine/threonine kinase 2 (AKT2)-dependent manner [18]. The glycolytic flux in thermogenic fat additionally relies on the cytosolic nicotinamide adenine dinucleotide (NAD) pool, which is critical for sustaining glyceraldehyde-3-phosphate dehydrogenase reactions in the pathway. Accordingly, apoptosis-inducing mitochondrion-associated factor 2 (Aifm2), a lipid droplet-associated protein induced upon adrenergic stimulation, is responsible for providing NAD to support glycolysis and the TCA cycle and ultimately fuel thermogenesis [19].

The fate of glucose in BAT during thermogenesis seems to be distinct in short- or long-term cold stress. For instance, while the catabolism of glucose under chronic cold exposure mostly provides the carbon source for PPP and for feeding the DNL, during acute cold exposure the glucose

Glossary

***N*-Acyl amino acids:** signaling molecules in which an amino acid (amide) bond is covalently linked to a long-chain fatty acid acyl moiety.
BATokine: molecule (lipid, peptide, metabolite, etc.) that is produced and secreted preferentially from brown adipocytes.
BCKDHa: represents the alpha subunit (E1) that is one of the three catalytic components of the branched-chain alpha-keto acid dehydrogenase (BCKD) enzyme.
Branched chain amino acids (BCAA): represents the amino acids leucine, isoleucine, and valine.
Browning: the rise of beige adipocytes from progenitor cells (preadipocytes) in white adipose depots in response to environmental (e.g., cold exposure) or pharmacological (e.g., β-AR agonists) stimulus.
CD36: cluster of differentiation 36 is a ubiquitously expressed scavenger cell surface receptor that mediates the shuttling of fatty acids, lipoproteins, phospholipids, and other ligands into cells.
Cre-driven mice: Cre recombinase protein expression under the control of the UCP1 or adiponectin (Adipoq) promoter. While UCP1-Cre drives the expression of Cre recombinase in brown and beige adipocytes, Adipoq-Cre drives the expression of Cre recombinase in all adipocytes. UCP1-Cre is not restricted to adipose tissue [63]
2-DG: a glucose derivative in which the 2-hydroxyl group gets replaced by hydrogen. This glucose molecule is not able to enter glycolysis.
^{18}F-FDG-PET-CT: positron emission tomography with 2-deoxy-2-[fluorine-18] fluoro-D-glucose integrated with computed tomography. A diagnostic procedure that quantifies glucose mobilization into tissues.
Fatty acid-binding protein (FABP): a family of chaperones that mediates lipid trafficking inside cells.
GLUT: family of glucose transporters that mediate glucose shuttling into the cell.
G-protein coupled receptor (GPCR): heterotrimeric seven-transmembrane receptors that are able to bind with several endogenous and exogenous chemical agents, thereby mediating their intracellular signal transduction that will ultimately result in a biological effect.
High-fat diet (HFD): a diet commonly used for inducing obesity in mice and

Box 1. The pentose phosphate pathway (PPP)

PPP is a key metabolic pathway, downstream to glucose uptake, that is tightly connected to glycolysis. Its primary roles are to yield NADPH and pentoses (five-carbon sugars) that serve as precursors for the synthesis of nucleotides, thus feeding several other metabolic pathways. PPP is conventionally divided in two phases, an oxidative and a nonoxidative. In the first, glucose-6P is dislocated from the glycolysis axis to serve as a substrate for the rate-limiting enzyme G6PDH and further reactions catalyzed by the enzymes 6PGL and 6PGDH, ultimately resulting in the generation of two molecules of NADPH, CO_2, and ribulose 5-phosphate (Ru5P). Although the oxidative phase of PPP does involve G6-P oxidation, its primary role is anabolic rather than catabolic, since its major products NADPH and Ru5P will fuel *de novo* lipogenesis and nucleotide/nucleic acids synthesis, respectively. The nonoxidative branch is fueled simultaneously by the Ru5P from the oxidative branch, and by fructose 6-P and GA3P dislocated from glycolysis. Those substrates fuel a sequence of reactions mediated by the enzymes transketolase (TKT) and transaldolase (TAL), which yields the formation of intermediates that can re-enter glycolysis (fructose 6-P and GA3P) or that will be used for nucleotide and amino acids synthesis (e.g., ribose 5-phosphate and erythrose 4P) [64]. PPP seems to be relevant in adaptive thermogenesis as glucose carbons in brown adipocytes ramify from glycolysis into PPP upon chronic cold challenge [16]. Accordingly, G6PD-deficient mutant mice exhibit compromised cold tolerance and downregulated thermogenic gene expression in BAT [65]. Since there is no evidence of a direct role for PPP intermediates in heat production, it is likely that the aforementioned anabolic processes fueled by PPP intermediates sustain vital cellular processes during cold while the metabolic machinery is driven towards heat production, or can also provide fat substrates for thermogenesis through NADPH-induced *de novo* lipogenesis.

intermediates are mostly oxidized into the mitochondria during thermogenesis [16]. Similarly, the short-term administration of a β3-AR agonist in brown adipocytes enhances glucose oxidation to meet the energy demand resulting from pyruvate dehydrogenase activity [17]. Collectively, these findings indicate that glucose is oxidized to provide fuel during short-term thermogenesis while PPP and its subsequent anabolic processes seem to be the major glucose-induced metabolic reprogramming upon chronic cold exposure [16]. Despite glucose metabolism supporting thermogenesis by yielding different intermediates, there is no mechanistic evidence directly linking those byproducts with UCP1 activity or even with another alternative thermogenic pathway.

A fraction of the glucose entering the brown adipocytes during thermogenesis is metabolized into lactate [16], which is further released into the bloodstream [10]. Interestingly, the pharmacological inhibition of lactate dehydrogenase and genetic deletion of monocarboxylate transporter (MCT) 1 abolish BAT thermogenesis in response to sympathetic stimulus [20]. However, BAT preferentially takes up circulating glucose rather than lactate to feed the TCA cycle upon cold stress. This evidence suggest the existence of coordinated and fine-tuned mechanisms to control intracellular levels of lactate upon thermogenic challenge by optimizing lactate shuttle to the extracellular compartment, and/or by converting it to pyruvate and increasing mitochondrial oxidative activity, and/or by favoring glucose rather than lactate uptake, thereby enriching side pathways. However, the fate of the cold-induced glucose uptake is still imprecise in humans [10]. Recent data suggest that in healthy humans, most glucose is not fully oxidized upon BAT thermogenesis. Instead, lactate and pyruvate release represent a large proportion of glucose uptake during warm and cold conditions [10,21]. Still, a precise flux of glucose into BAT shunt pathways is not quantitatively dissected so far [16]. In addition to its role as a catabolic sink for heat-producing macronutrients, murine thermogenic fat sequesters the circulating TCA cycle intermediate, succinate, upon cold exposure and uses it as a fuel to trigger UCP1-dependent thermogenesis and amelioration of succinate-induced pathological inflammation of the liver *in vivo* [22,23].

A distinct glycolytic beige adipocyte population differentiated in the absence of β-AR signaling through a myogenic progenitor was shown to endure a central role in cold-induced thermogenesis, which might be compelling as a therapeutic tool that does not require β-AR signaling activation [24]. Likewise, asparagine supplementation worked as another alternative strategy for wiring glycolysis through the mammalian target of rapamycin complex (mTORC)1 pathway to nourish thermogenesis [25].

typically displays 60% fat content relative to the total caloric value.
Isotope tracing: an *in vivo* assay to investigate the fate and kinetics of metabolites such as rates of production, appearance, or disappearance. Isotopes are chemically identical elements but different in mass due to differences in the number of neutrons in their nuclei. It can be either radioactive or stable (nonradioactive) isotopes.
Oxidative phosphorylation (OXPHOS): the process by which ATP synthesis is coupled to the movement of electrons through the mitochondrial electron transport chain and the associated consumption of oxygen.
PGE2: prostaglandin E2 is a lipid compound from a diverse group of eicosanoids that is a central mediator of fever (pyrogenic factor) in response to exposure to a pathogen.
SLC25a44: SLC25 family protein that acts by importing BCAAs into the mitochondria.
Thermal comfort state: individuals are usually kept at a subjective self-reported degree of cold perception based on a customized visual analog scale. Thermal comfort represents neither cold nor warm, around 25°C.
Thermogenic fat: general term to designate brown and beige adipose tissue. Both tissues have distinct progenitors and developmental characteristics but share common molecular, morphological (multilocular lipid droplets and high mitochondrial content), and functional characteristics (UCP1-dependent thermogenesis; secretion of adipokines and other molecules).
TRLs: TG-rich lipoproteins are a group of circulating lipoproteins that possess a high content of triglycerides in their core, such as low- and very low-density lipoproteins (LDL and VLDL) and chylomicrons.

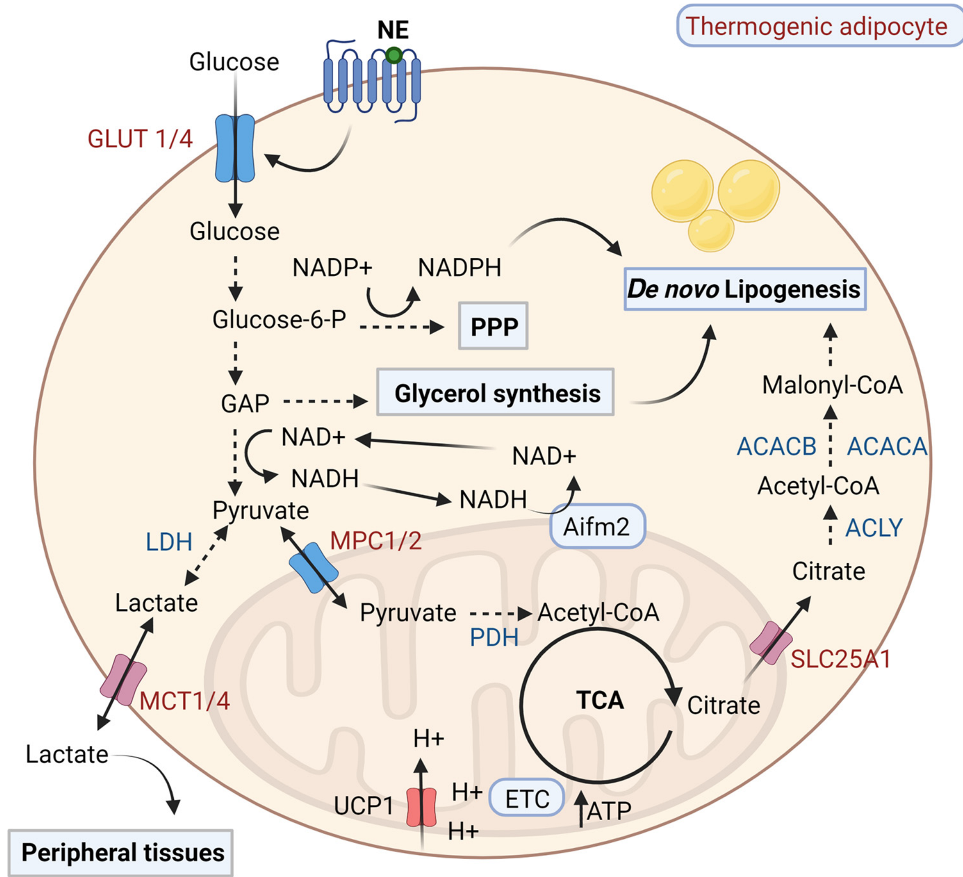

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Figure 1. Glucose metabolism supports heat production in thermogenic fat. Circulating glucose is taken up by GLUT1/4 upon exposure to different stimuli, such as cold or β3-adrenergic signaling, feeding, or insulin signaling. It is initially metabolized through glycolysis until it is converted to pyruvate, which has already been described as a catabolic step that is highly relevant to thermogenesis, such as the regulation of pyruvate mitochondrial entry. As such, Aifm2 is critical in maintaining the cytosolic NAD pool to support the glycolytic flux needed for thermogenesis. In addition to the oxidative pathway throughout the TCA cycle into OXPHOS and uncoupled respiration, glycolysis intermediates can fuel glycogen synthesis, which has a role in controlling UCP1 synthesis through ROS generation, or can also take the PPP pathway, or DNL through DHAP. Lactate, for its part, is taken up by MCT1/4 under incompletely understood controlled conditions. It is then converted into pyruvate to feed the TCA cycle, which may also be fed by circulating succinate. Pyruvate can still be diverted into acetyl-CoA to sustain DNL through PDH or can be converted into lactate, which will be released. Abbreviations: Aifm2, apoptosis-inducing mitochondrion-associated factor 2; AKT, serine/threonine kinase; DHAP, dihydroxyacetone phosphate; DNL, *de novo* lipogenesis; ECT, electron chain transport; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporter; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; LDH, lactate dehydrogenase; MCT, monocarboxylic acid transporter; MPC, mitochondrial pyruvate carrier; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NE, norepinephrine; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PGM, phosphoglucomutase; PPP, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; UCP1, uncoupling protein 1.

Instead of following the route of catabolism, glucose is also stored as glycogen in thermogenic fat. Brown and beige adipocytes store large amounts of glycogen compared with white adipocytes. Surprisingly, glycogen seems to have a key role in beige fat biogenesis and thermogenesis [26]. Glycogen synthesis and degradation are both required for the **browning** of beige fat induced by cold exposure or β3-AR agonists. Increased expression of glycogen-metabolizing genes is observed during thermogenesis in beige fat. In humans, the expression of these genes is negatively correlated with obesity in two distinct cohorts. Mechanistically, glycogen synthesis and

degradation are necessary for the activation of p38 mitogen-activated protein kinases (p38), leading to the glycogen-dependent generation of reactive oxygen species (ROS) [26].

FAs as heat trigger substrates for thermogenic fat

Lipids are essential substrates used by thermogenic adipocytes to support thermogenic machinery activity under cold conditions [27]. Obese individuals possess a lower capacity to clear circulating triglyceride-rich lipoproteins (**TRLs**) through BAT [28], which is in agreement with the fact that obese subjects exhibit a lower BAT activity [29]. Accordingly, a retrospective study reported that individuals with detectable and active BAT displayed a lower prevalence of coronary artery disease and dyslipidemia in comparison to those individuals with undetected BAT [5]. Moreover, TAGs and glucose levels were lower in BAT-positive individuals, while high-density lipoprotein (HDL) levels were higher than in BAT-negative individuals [5]. The intrinsic capacity of BAT to clear circulating TRLs to fuel its thermogenic machinery is a crucial feature enabling it to confer cardioprotection and improve metabolic health.

Who fuels the BAT? Extracellular or intracellular FAs?

Significant progress has been achieved in recent years concerning how FFAs become available to serve as a substrate for metabolism in brown adipocytes. It was long believed that lipolysis into brown adipocytes was the major source of FFAs for UCP1 activation during adaptive thermogenesis [30]. However, independent studies have demonstrated that intracellular lipolysis is dispensable for cold acclimation [31,32]. Specifically, Shin *et al*. [32] showed that while the Adipoq-**Cre-driven** deletion of the rate-limiting lipolytic enzyme *Atgl* impeded cold adaptation in mice, the UCP1-Cre-driven deletion of the same lipase did not. These data imply that FFAs from white adipose tissue (WAT) depots are essential for short-term cold adaptation. Although intracellular lipolysis is not required for cold adaptation, it can regulate the expression and constitutive activity of the **G-protein coupled receptor (GPCR)** GPR3, which can trigger cyclic AMP (cAMP)-dependent thermogenesis, regardless of sympathetic activation [33]. Overexpression of GPR3 protects mice against obesity and glucose intolerance, thereby demonstrating the potential for this intracellular lipolysis-driven pathway to counteract the effects of a **high-fat diet (HFD)**. Curiously, GPR3 knockout mice are not cold intolerant, which agrees with the aforementioned studies, showing that intracellular lipolysis is not essential for adaptive thermogenesis [33]. Of note, lipids exported from the liver may also contribute to thermoregulation as the FFAs released from WAT stimulate beta-oxidation in hepatocytes, thereby boosting the biosynthesis of long-chain acylcarnitines, which in turn are exported to BAT and activate UCP1-dependent heat production [34].

Although DNL is activated during cold in brown adipocytes as a result of enhanced glucose uptake, deletion of genes involved in DNL does not impair cold tolerance in mice [18,35,36], ruling out an essential role for DNL for adaptive thermogenesis. Given that both intracellular lipolysis and DNL are dispensable for cold acclimation, one can infer that the mobilization of intracellular fat does not suffice for heating brown adipocytes up at their total capacity upon cold exposure, perhaps due to the limited local FFA pool within these cells. Consequently, BAT relies on external sources of FFAs to keep the thermogenic machinery fully active.

Mechanisms of FA uptake into thermogenic fat

Although white adipocyte-derived lipids can indeed traffic in the form of FFAs, a major fraction of the circulating lipids are available in TRLs. As brown adipocytes do not take up the entire TRL particle, these lipoproteins need to undergo cell surface lipolysis under hydrolytic activity of the lipoprotein lipase (LPL), resulting in increased FFAs availability prior to its uptake into the cells through either the scavenger receptor **CD36** or the FA transporter FATP1 [37]. In contrast to what was previously believed, TRLs were shown to be not broken down and processed directly

on brown adipocytes [38,39]. Duta-Mare *et al*. [38] demonstrated that lysosomal acid lipase (LAL), an enzyme that hydrolyzes cholesteryl esters and TAGs inside lysosomes, is required for TRL breakdown into FFAs in BAT and that LAL deletion renders mice incapable of maintaining body temperature when in the cold. However, TRL breakdown and processing does not seem to occur directly in adipocytes. Instead, endothelial cells adjacent to adipocytes are the primary site for TRL hydrolysis by LPL to allow its uptake through CD36 and further processing by LAL before the release of FFAs and their further uptake by parenchymal cells in BAT [39,40] (Figure 2). In addition, the FA transporter FATP1 is also required for thermogenesis [37]. More detailed studies are needed to understand whether FATP1 acts only on brown adipocyte surfaces or in other adjacent cells to facilitate FFA entrance (Figure 2).

FFAs can also be chaperoned within the cells by **fatty acid-binding protein (FABP)**. Although these proteins do not mediate FFA internalization at the cell surface level, they facilitate the trafficking of FFAs between organelles [41]. FABP4 is most highly expressed in adipose tissue and is also required for adequate BAT-mediated adaptive thermogenesis. Deletion of FABP isoform 4 (FABP4) renders mice cold-intolerable [42,43], which can also be attributed to the fact that FABP4 mediates the intracellular conversion of thyroxine (T4) to triiodothyronine (T3) and, as a result, can impact thermogenesis independently of its chaperone function [42]. Lipid uptake in brown adipocytes is also facilitated by cold-inducible endogenous ligands. One of the short-term events required for such a purpose is insulin release from beta-cells [44]. FFAs released from WAT due to adrenergic-stimulated lipolysis can promote insulin secretion from the endocrine pancreas. This short-term insulin release leads the hormone to act on BAT to promote LPL- and CD36-mediated TRL breakdown and uptake into the BAT [44]. This step seems to be required for cold acclimation, since insulin gene (Ins1) deletion in beta-cells renders mice unable to maintain body temperature under cold conditions [39].

Upon thermogenic challenge, BAT oxidative machinery seems to be upregulated, as the production of cold-induced oxidized lipokines resulting from the activity of oxygenases and epoxide hydrolases is exacerbated in those conditions [3,45]. The oxylipin 12,13-dihydroxy-9Z-octadecenoic acid (12,13,diHOME) is a **BATokine** that was found to be secreted in both humans and mice under cold exposure to support thermogenesis by shuttling FFAs into BAT through the promotion of CD36 and FATP1 translocation to the cell membrane. In addition, 12,13-diHOME can also stimulate beta-oxidation by a mechanism still unknown [45] (Figure 2).

How do FAs put the heat on fat?

Once FFAs are taken up by brown adipocytes, they can undergo different metabolic reactions, determining their fate in the cell. While in thermoneutrality, FFAs are stored as TAGs; under lower temperatures, FFAs are preferentially used for beta-oxidation and UCP1 activation [46,47]. FFAs stimulate UCP1 by directly binding to the transporter, where it is available as an anion that binds to H^+, thereby bridging it through the channel and allowing for the proton leakage [46]. Long-chain FFA binding to UCP1 is required even for the constitutive and induced functioning of the transporter [48] and for diet-induced thermogenesis [49]. Notably, the FFAs that constitutively activate UCP1 are known to be derived from the phospholipase (PL)-mediated hydrolysis of phospholipids that compound the mitochondrial inner membrane [46]. This may imply that FA uptake under cold conditions serves for energy replenishment and membrane recomposition before phospholipid breakdown by PLs and subsequent FFA binding to UCP1 (Figure 2).

The thermogenic action of FFAs is not limited to UCP1 activation. FFAs may also promote thermogenesis by increasing ROS production and hypoxia-inducible factor 1 (HIF1)-alpha stabilization in endothelial cells, which thus triggers angiogenesis in BAT and browning of WAT [39]

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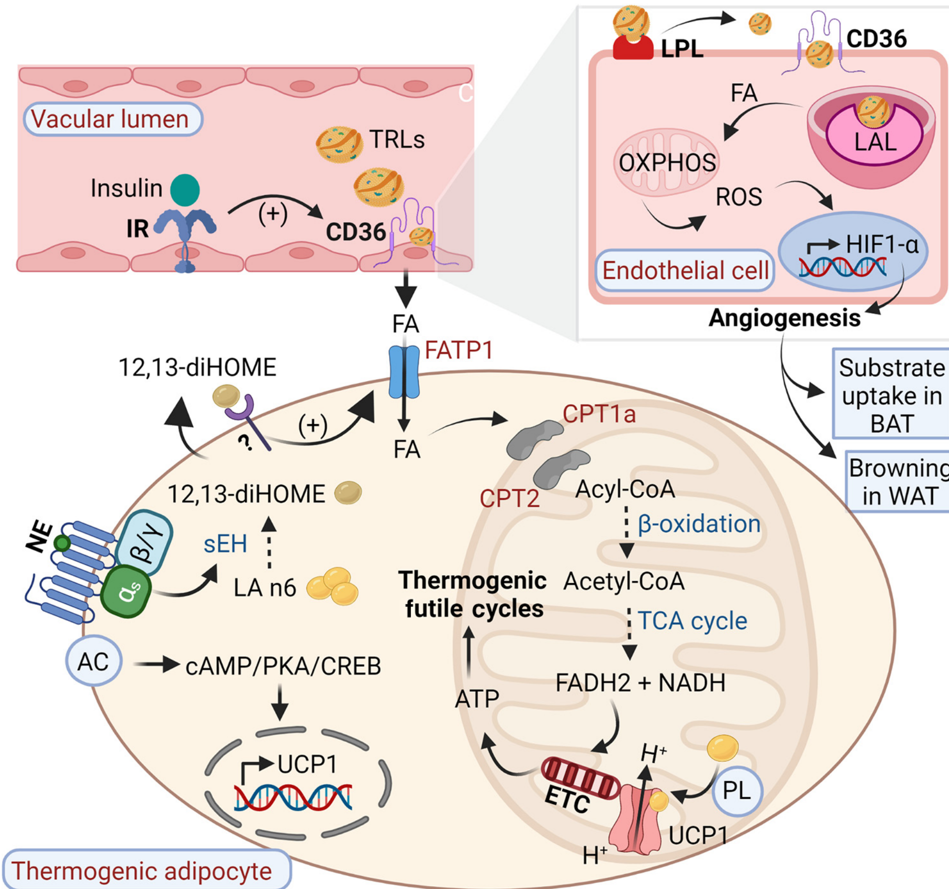

Figure 2. Mechanisms of fatty acid uptake and metabolism for heat production into thermogenic adipocytes. Circulating TRLs are taken up by endothelial cells upon insulin stimulation and after the lipolytic action of extracellular LPL and transport by CD36 at the cell surface. Once internalized in the endothelial cell, the reduced TRLs are broken down by LAL into FFAs that undergo beta-oxidation, thus stimulating OXPHOS and ROS production in the mitochondria. The resulting ROS stabilize HIF1-alpha in the nucleus, triggering angiogenesis. The increased angiogenesis in BAT and subcutaneous fat induces tissue remodeling, optimizing nutrient uptake and promoting browning. The FFAs resulting from endothelial processing are taken up by brown adipocytes through FATP1. FATP1 is translocated in response to the lipid 12,13-diHOME, which is an n-6 lipid product originating from the enzymatic conversion of linoleic acid by sEH, an adrenergic-responsive epoxide hydrolase. Once inside the cell in the free form, the FAs can enter the mitochondria through the cooperation of CPT1a and CPT2 transporter, which delivers it as acyl-CoA in the mitochondrial matrix, where the FA undergoes beta-oxidation, thereby feeding the TCA cycle and generating FADH2 and NADH as substrates for OXPHOS at the ETC. The resulting ATP molecules fuel the ATP-consuming thermogenic futile cycles that ultimately promote heat production and increase energy expenditure independently of UCP1. UCP1 can be transcriptionally upregulated under cold/adrenergic stimuli, but its constitutive and induced activity depends on binding to FFAs originating from the phospholipase-catalyzed hydrolysis of membrane phospholipids. Abbreviations: AC, adenylyl cyclase; ATP, adenine triphosphate; BAT, brown adipose tissue; CD36, cluster of differentiation 36; CPT1, carnitine palmitoyltransferase I; 12,13-diHOME, 12,13-dihydroxy-9Z-octadecenoic acid; ETC, electric chain transport; FA, fatty acids; FADH, reduced flavin adenine dinucleotide; FATP1, fatty acid transport protein 1; FFA, free fatty acid; HIF1-alpha, hypoxia-induced factor 1; IR, insulin receptor; LAL, lysosomal acid lipase; LPL, lipoprotein lipase; NADH, reduced nicotinamide adenine dinucleotide; NE, norepinephrine; OXPHOS, oxidative phosphorylation; PL, phospholipases; ROS, reactive oxygen species; sEH, soluble epoxide hydrolase; TCA, tricarboxylic acid; TRL, triglyceride-rich lipoprotein; UCP1, uncoupling protein 1.

(Figure 2). Moreover, beta-oxidation is also needed for proper BAT function once the deletion of the mitochondrial transporter carnitine *O*-palmitoyltransferase 2 (CPT2) yields an impaired thermogenic capacity in mice [47]. As an efficient source of substrates for the TCA cycle and

oxidative phosphorylation (OXPHOS) at the electron transport chain, beta-oxidation of FFAs ultimately results in ATP production. As ATP synthesis is required for fueling thermogenic futile cycles in brown adipocytes, namely creatine phosphorylation, TAG hydrolysis, and active transport of Ca^{2+} (Box 2), it is likely that those pathways demand efficient beta-oxidation for adequate operation (Figure 2).

BCAA as heat-triggering substrates for thermogenic fat

Thermogenic fat and circulating BCAA levels

BCAA are essential amino acids related to various biological functions. The metabolism of BCAA provides intermediates for mitochondrial oxidation or biosynthesis of molecules (Box 3). From a clinical perspective, studies demonstrate that the circulating BCAA imbalance (i.e., increased circulating levels) is associated with obesity, insulin resistance, and type 2 diabetes [50]. However, different behavioral, dietary, and bariatric surgery interventions improve circulating BCAA homeostasis [51]. Studies have recently demonstrated the importance of BCAA metabolism for thermogenic fat activity and its relationship to whole-body energy homeostasis [52,53]. Thermogenic fat, acting as a 'metabolic sink' that takes up circulating macronutrients (e.g., BCAA), is an attractive therapeutic strategy against metabolic diseases. In this regard, quantitative *in vivo* **isotope tracing** studies demonstrate that BAT accounts for 19% of the whole-body oxidation of BCAA in mice under standard housing conditions [54]. Moreover, cold exposure decreases the circulating BCAA levels in mice and humans [52] and defective thermogenic fat BCAA catabolism mitigates the clearance of circulating BCAA in mice [52]. In humans, acute cold stress mainly decreases circulating BCAA compared with other amino acids [52]. However, so far no human studies provide direct evidence that BAT takes up BCAA from circulation under thermoneutral or cold stress.

Interestingly, healthy individuals presenting low versus high BAT activity demonstrate significant differences in systemic BCAA clearance. For example, circulating valine levels are significantly decreased in individuals presenting high BAT but not in low BAT activity under acute cold exposure [52]. More population-based human studies will be needed to further investigate the importance of thermogenic fat activation in clearing systemic BCAA levels. Moreover, it is still unknown

Box 2. Thermogenic futile cycles

Thermogenic futile cycles are ATP-consuming and UCP1-independent thermogenic pathways that are required for brown and beige fat function [66].

Creatine futile cycle: the futile cycling between creatine and phosphocreatine (Pcr) in mitochondria utilizes ATP mitochondrial respiration to phosphorylate creatine under the catalytic activity of the enzyme creatine kinase B (CKB), thereby generating Pcr and releasing ADP in excess, which further stimulates thermogenic respiration [67]. The resulting Pcr is then hydrolyzed by tissue-nonspecific alkaline phosphatase (TNAP), yielding another creatine molecule to re-enter the cycle for a subsequent round of coordinated phosphorylation and hydrolysis steps [68]. According to this model, thermogenic respiration is empowered by the excess of ADP generated as a byproduct of futile creatine cycling.

Calcium futile cycle: Ca^{2+} release from the endoplasmic reticulum (ER) is an acute cellular response to adrenergic stimuli induced by cold stress or pharmacological agonism of β3 and/or α1 adrenoceptors. After its extrusion, Ca^{2+} re-enters the ER through the SERCA2b transporter in a process that demands ATP consumption and consequently leads to energy dissipation. Exceeding cytosolic Ca^{2+} can be imported by mitochondria where it activates pyruvate dehydrogenase, thus boosting glucose oxidation and ATP synthesis [69]. Interestingly, this pathway is only relevant for thermogenesis in beige but not in brown adipocytes.

Lipid futile cycle: hydrolytic breakdown of triglycerides, named lipolysis, results in the release of FFA and glycerol. The resultant free glycerol molecules are utilized as a substrate by glycerol kinase (GK) in ATP-consuming reaction to generate glycerol-3-phosphate (G3P), which in turn is used for triglycerides assembling. This mechanism is present in BAT but absent in WAT due to the lack of GK in white adipocytes. Alternatively, those cells utilize glyceroneogenesis to replenish the G3P pool required for TAG assembly. Glyceroneogenesis yields G3P as an end product under the control of the enzyme phosphoenolpyruvate carboxykinase (PEPCK-C) in an ATP-demanding reaction [70].

Box 3. BCAA catabolism in brown and beige fat thermogenesis

BCAAs are central amino acids related to brown and beige adipocyte thermogenesis [52]. BCAAs are taken up by thermogenic fat through the membrane transporter SLC7A5 [71] and imported into mitochondria through the recently described SLC25A44 [52]. The mitochondrial catabolism of BCAAs involves two main steps. (i) Reversible deamination to produce branched-chain α-keto acids (BCKAs). This reaction is catalyzed by branched-chain aminotransferase (BCAT). (ii) The irreversible oxidative decarboxylation of BCKA by the branched-chain ketoacid dehydrogenase complex (BCKDH). As the final products, BCAA catabolism mostly generates acetyl-CoA and succinyl-CoA, which are then fully oxidized via the TCA in thermogenic fat [72] (see Figure 3 in the main text). Brown and beige adipocytes express high levels of mitochondrial BCAT2 but not cytoplasmic BCAT1 [52]. Transcriptomics and proteomics analyses of human and mouse thermogenic fat revealed the increased expression of most BCAA catabolic enzymes compared with white adipocytes [52]. Interestingly, the fate of BCKA might differ in the BAT of mice in acute versus chronic cold exposure. For instance, acute cold exposure increases the expression of mitochondrial BCAA-oxidation enzymes that provide the intermediates (i.e., acetyl-CoA and succinyl-CoA) that feed the TCA cycle, whereas chronic cold exposure increases the expression of fatty acid synthesis enzymes [52]. The acclimation of BAT during cold exposure suggests a dynamic shift in the fate of BCKA from mitochondrial oxidation to DNL.

if cold-induced BAT activity can improve circulating BCAA levels in populations other than young subjects, considering that BAT mass is reduced (or inactive) in obese and/or elderly subjects.

Thermogenic fat BCAA catabolism and thermogenesis

BCAA catabolism provides the intermediates (acetyl-CoA and succinyl-CoA) that fuel the TCA cycle and enhance brown and beige fat (Box 3). A direct *in vivo* leucine isotope tracing revealed that chronic cold exposure increases the leucine uptake by BAT and inguinal WAT (iWAT) depots; additionally, the use of valine isotope tracer showed higher valine oxidation in both tissues of mice under cold stress. Notably, the loss of function of BCAA transport (**SLC25a44**)- and catabolism [branched-chain alpha-keto acid dehydrogenase (**BCKDHa**)]-related proteins results in decreased brown and beige fat thermogenesis and negatively affects whole-body energy homeostasis [52].

The thermogenic contribution of brown fat BCAA catabolism is also essential in other physiological conditions beyond cold stress. For instance, BAT thermogenesis is a significant heat source of fever induced by intracerebroventricular administration of prostaglandin E2 (**PGE2**) (a well-known pyrogenic mediator) or psychological stress [55]. However, the BAT deletion (Ucp1-Cre-driven) of SLC25a44 (the transporter of BCAA into the mitochondria) reduces PGE2-induced thermogenesis, affecting the febrile response [55].

Another recent study revealed that diet-induced obesity promotes BCAA catabolic defects in BAT [53]. However, the use of a pharmacological BCAA catabolism enhancer [3,6-dichlorobenzo[b] thiophene-2-carboxylic acid (BT2)] increases BAT BCAA oxidation and decreases tissue BCAA and branched-chain α-keto acids (BCKA) levels. Moreover, BT2-treated mice exhibit increased whole-body temperature and prevent HFD-induced obesity. The aforementioned metabolic benefits of BT2 rely exclusively on BAT activity, as the surgical excision of interscapular BAT mitigates the benefits of BT2 on body mass and systemic energy homeostasis. Interestingly, the oral administration of *Bacteroides* probiotics improves BAT BCAA catabolism and attenuates body weight gain, demonstrating a previously unknown link between gut microbiota and BAT BCAA metabolism.

Conversely, the deletion of branched chain amino acid transaminase (Bcat) 2 (Box 3) in adipose tissue depots (adiponectin-Cre lineage) prevents HFD-induced obesity and improves whole-body energy homeostasis in mice due to iWAT browning and enhanced thermogenesis [56]. Mechanistically, the acetyl-CoA derived from BCAA catabolism promotes acetylation at the lysine 915 residue of PR domain containing 16 (PRDM16) and suppresses its interaction with peroxisome proliferator activated receptor gamma (PPARγ) on preadipocytes, which in turn results in a WAT phenotype. However, deletion of Bcat2 mitigates this post-translational modification,

leading to activation of the thermogenic gene program due to enhanced PRDM16 and PPARγ interaction. Caution should be taken regarding the author's proposed mechanism as acetyl-CoA may derive from sources other than BCAA in preadipocytes within iWAT. Moreover, deletion of Bcat2 might affect the browning phenotype by yet-to-be-determined routes.

BCAA are also a carbon source (from leucine and isoleucine) that generate monomethyl branched-chain fatty acids (mmBCFAs) on BAT (Figure 3) [57–59]. The mmBCFAs participate in DNL in differentiated adipocytes [59]. BAT is a source of mmBCFAs in mice under cold exposure [59]. However, the function of these classes of molecules is still unclear during thermogenesis. The

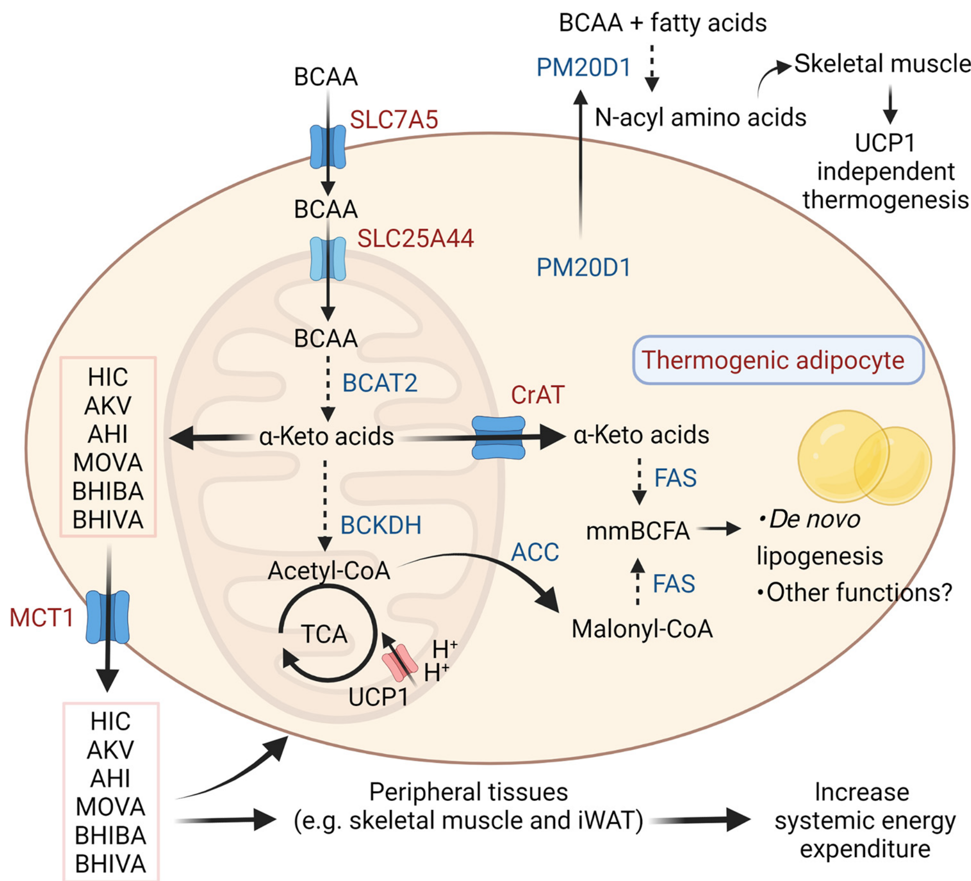

Figure 3. Thermogenic fat BCAA metabolism and systemic energy expenditure. BCAA metabolized into mitochondria provide the intermediates (α-keto acids) that feed the TCA cycle during thermogenesis (detailed description see Box 3 in the main text). α-Keto acids are the carbon source for the synthesis of mmBCFAs, signaling molecules involved in the regulation of DNL. BCAA metabolism also generates signaling molecules (e.g., AHI, AKV, BHIBA, BHIVA, HIC, and MOVA) that increase local UCP1-dependent thermogenesis and act as endocrine molecules, enhancing systemic energy expenditure. Extracellular BCAAs are substrates of the enzymatic activity of PM20D1, which synthesizes *N*-acyl amino acids (lipidated amino acids that act at distal sites, e.g., skeletal muscle), increasing UCP1-independent thermogenesis. Abbreviations: ACC, acetyl-CoA carboxylase; AHI, α-hydroxyisovaleric acid; AKV, α-ketoisovaleric acid; BCAA, branched-chain amino acid; BCAT2, branched-chain aminotransferase 2; BCKDH, branched-chain ketoacid dehydrogenase complex; BHIBA, β-hydroxyisobutyric acid; BHIVA, β-hydroxyisovaleric acid; CrAT, carnitine acetyltransferase; DNL, *de novo lipogenesis*; FAS, fatty acid synthase; HIC, α-hydroxyisocaproic acid; iWAT, inguinal white adipose tissue; MCT1, monocarboxylic acid transporter 1; mmBCFA, monomethyl branched-chain fatty acid; MOVA, 3-methyl-2-oxovaleric acid; PM20D1, peptidase M20 domain containing 1; SLC7A5, solute carrier family 7 member 5; SLC25A44, solute carrier family 25 member 44; TCA, tricarboxylic acid; UCP1, uncoupling protein 1.

abundance of mmBCFAs is relatively low compared with that of other FAs. It is suggested that mmBCFAs might be involved in cellular processes other than the canonical route of lipid synthesis and substrate oxidation.

Interestingly, another class of lipidated amino acids (***N*-acyl amino acids**) that are synthesized by a thermogenic fat-secreted enzyme called peptidase M20 domain containing 1 (PM20D1) act as uncouplers of respiration in a UCP1-independent fashion (Figure 3) [60]. This class of molecules regulates proton flux and heat generation by binding the SLC25 family of inner mitochondrial carriers, particularly ANT1 (Slc25a4) and ANT2 (Slc25a5) [60]. Thus, mmBCFAs might be involved in cellular communication by acting as signaling molecules or serving as components of cell membranes [59]. Future studies are needed to clarify the role of mmBCFAs during thermogenesis in brown and beige adipocytes.

Thermogenic fat BCAA catabolism and intercellular communication

Thermogenic fat BCAA metabolism also generates molecules that are important not only for tissue thermogenesis but also for enhancing whole-body energy expenditure due to interorgan crosstalk. A recent study demonstrated that the BCAA-derived metabolites α-hydroxyisocaproic acid (HIC), α-ketoisovaleric acid (AKV), α-hydroxyisovaleric acid (AHI), 3-methyl-2-oxovaleric acid (MOVA), β-hydroxybutyric acid (BHIBA), and β-hydroxyisovaleric acid (BHIVA) are synthesized in response to thermogenic stimulus and released through MCT1 (Figure 3) [61]. These metabolites increase the expression of brown adipocyte-associated genes in recipient primary adipocytes. The BCAA-derived metabolite (MOVA) increases the expression of mitochondrial and FA oxidation genes and oxygen consumption in murine and human skeletal myocytes. *In vivo*, the administration of MOVA and BHIBA increases mitochondrial oxidation of BAT, iWAT, and skeletal muscle, thereby preventing weight gain, increasing systemic energy expenditure, and improving glucose homeostasis in a mouse model of obesity and diabetes [61]. Interestingly, both BCAA-derived metabolites act through distinct signaling pathways. For example, while MOVA signals through the cAMP–PKA–p38 MAPK axis, BHIBA acts via mTOR to regulate adipocyte and myocyte metabolic function. Finally, the concentrations of BCAA-derived metabolites (e.g., MOVA, BHIVA, and BHIBA) in subcutaneous adipose tissue and plasma are inversely correlated with the human body mass index.

Concluding remarks and future directions

The studies discussed here have revealed details on the mechanisms of uptake and metabolism of glucose, FAs, and BCAA in thermogenic fat. The new evidence highlights that these substrates feed alternative pathways in a time-specific manner to sustain brown and beige fat thermogenesis. Moreover, the metabolism of such energy substrates in thermogenic fat generates signaling molecules that communicate in an autocrine or endocrine manner to enhance whole-body energy expenditure. Although it seems clear that BCAA catabolism yield prothermogenic products, the mechanisms linking glucose and fatty-acid oxidation to heat production remain elusive, even though those steps are necessary for adaptive thermogenesis. It is also of interest to identify druggable targets among those metabolic pathways that are required for thermogenesis, or perhaps identify metabolites that may serve as prototypes for drug development to combat metabolic diseases (see Outstanding questions).

Advances in fluorescence-based metabolic sensors, clustered regularly interspaced short palindromic repeats (CRISPR)-based tools, spatial resolution, chemical biology by proximity-dependent labeling, single-cell omics technologies, and artificial intelligence-based software analysis are all emerging strategies for future research in energy metabolism. The range of applications by combining such technologies is unlimited. Notably, one of the promising strategies for the prospective

Outstanding questions

Could glucose uptake and its catabolism into thermogenic adipocytes directly induce heat production?

What are the mechanisms linking substrate oxidation to heat generation? Are the ATP-dependent futile cycles the mediators of such an effect?

Could we harness the substrate's metabolism in thermogenic fat to develop a novel targeted approach for the treatment of metabolic diseases?

What are the molecular mechanisms determining the partition of metabolic intermediates into different pathways in thermogenic fat upon exposure to cold?

Could thermogenic fat activity be linked to improvements in tissue and/or systemic BCAA levels?

investigation of brown and beige fat energy metabolism will be the use of single-cell metabolomics. Metabolomics defines the chemical information of the cell that is closest to the phenotype [62]. The strategies mentioned earlier will permit scientists to investigate energy metabolism at the cell (or even subcellular) level in a spatiotemporal fashion, interrogate individual or multiple targets, trace metabolites and signaling pathways, and identify molecular mechanisms determining the partition of metabolic intermediates into different pathways in different cellular subtypes. Combining these technologies will help us understand the contribution of brown and beige fat metabolism in unprecedented detail and open new ways to target thermogenic fat.

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Declaration of interests

No interests are declared.

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