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REVIEW

Insights into brown adipose tissue evolution and function from non-model organisms

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ABSTRACT

Brown adipose tissue (BAT) enables adaptive thermoregulation through heat production that is catalyzed by mitochondrial uncoupling protein 1 (UCP1). BAT is frequently studied in rodent model organisms, and recently in adult humans to treat metabolic diseases. However, complementary studies of many non-model species, which have diversified to many more ecological niches, may significantly broaden our understanding of BAT regulation and its physiological roles. This Review highlights the research on non-model organisms, which was instrumental to the discovery of BAT function, and the unique evolutionary history of BAT/UCP1 in mammalian thermogenesis. The comparative biology of BAT provides a powerful integrative approach that could identify conserved and specialized functional changes in BAT and UCP1 by considering species diversity, ecology and evolution, and by fusing multiple scientific disciplines such as physiology and biochemistry. Thus, resolving the complete picture of BAT biology may fail if comparative studies of non-model organisms are neglected.

KEY WORDS: Beige adipose tissue, Uncoupling protein, Thermogenesis, Marsupials, Endothermy, Metabolic disease

Introduction: heat production and brown adipose tissue

Endogenous heat production is one of the most fascinating evolutionary achievements of nature and permits elevated rates of metabolism, activity, reproduction, brain function and many other processes, that are independent of environmental temperatures. Endothermy has independently evolved in different animal groups, even within predominantly ectothermic groups such as insects and fish, and is at the roots of avian and mammalian evolution.

Only mammals possess brown adipose tissue (BAT), a specialized organ that enables them to produce heat to maintain a high body temperature below the thermoneutral zone of many mammals (Cannon and Nedergaard, 2004). In contrast to typical white adipose tissue (WAT), which mainly stores nutrients in the form of large unilocular fat droplets, the multiple cellular lipid droplets of BAT provide an enhanced lipid surface to facilitate lipolysis for rapid breakdown and lipid oxidation (Keipert and Jastroch, 2014). The brown color originates from the high mitochondrial density and the dispersed vascularization network within BAT. The vascularity collects the warmed blood and the circulatory system distributes the heat throughout the body (reviewed by Oelkrug et al., 2015). The high mitochondrial mass in brown adipocytes, which comprises up to 8% of total protein, provides the engine to harvest oxidation energy from lipids and glucose (Rousset et al., 2004); mitochondrial uncoupling protein 1 (UCP1) acts as a valve to rapidly release this energy as heat instead of synthesizing ATP. Intracellular levels of purine nucleoside diphosphates and triphosphates constitutively inhibit UCP1, whereas rising intracellular free fatty acid levels upon cold stimulation acutely activate UCP1 (Heaton et al., 1978). How fatty acids activate UCP1 mechanistically, whether other activators exist and how the protein catalyzes the net transport of protons, is still a matter of debate (reviewed by Crichton et al., 2017). The crystal structure of UCP1 has yet to be determined, therefore, it is unclear whether mutational analysis solely identifies specific structure–function relationships of UCP1, or whether the loss of functional features is only a result of unspecific protein disintegration. Mammalian UCP1 appears to be mainly expressed in distinct BAT sites, and is induced within WAT during various physiological stress conditions, including cold exposure (reviewed by Bonet et al., 2013; Wu et al., 2013). These UCP1-positive cells with brown-like functional features (Shabalina et al., 2013) have been termed either beige or brite (brown-in-white) adipose tissue (Ishibashi and Seale, 2010; Petrovic et al., 2010). Interestingly, brown-like morphological and molecular characteristics are also enhanced in the WAT of UCP1-ablated mice (*Mus musculus*) (Granneman et al., 2003; Keipert et al., 2015; Ukropec et al., 2006), calling for a relaxation of the 'browning' definition, with enhanced metabolism possibly being independent of UCP1. Although UCP1 is required to maintain body temperature during acute cold exposure (Golozoubova et al., 2001), UCP1 knockout mice survive slow acclimation to the cold without any visible metabolic defects (Golozoubova et al., 2001; Keipert et al., 2017; Meyer et al., 2010; Ukropec et al., 2006). The lifespan of UCP1 knockout mice is unaffected at room temperature (Kontani et al., 2005); however, at 4°C, lifespan is reduced by unknown causes (Golozoubova et al., 2001). Some suggestions include impaired muscle calcium handling owing to damage incurred by chronic shivering (Aydin et al., 2008) and systemic elevation of oxidative stress (Stier et al., 2014).

BAT activity and mass increase in response to environmental cold exposure. To respond to cold environmental temperatures, a centrally processed cold-sensation induces the release of noradrenaline from efferent sympathetic postganglionic nerve fibers in BAT to activate a collection of α- and β-adrenergic receptors residing in the brown adipose plasma membrane (Bartness et al., 2010). In particular the β3-adrenergic receptor is highly abundant and coordinates an array of cellular processes to control heat production, including lipolysis and gene transcription (Boeuf et al., 2001; Klingenspor, 2003). At thermoneutral temperatures, BAT is involved in the combustion of surplus nutrient energy in mice, evoked by high-fat diets (Feldmann et al., 2009). However, at room temperature, UCP1 knockout mice are protected from high-fat diet-induced obesity, a phenomenon that is not understood (Liu et al., 2003). In

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seasonal mammals, the recruitment of BAT is stimulated by the photoperiod, independent of ambient temperature changes. The underlying mechanisms are not fully understood; however, melatonin may play a role (Heldmaier and Hoffmann, 1974).

Historical context of BAT research

Conrad Gessner provided the first rough anatomical description of BAT in the marmot almost 500 years ago (Gesner, 1551). This was followed by morphological and physiological studies in the following centuries (reviewed by Rasmussen, 1923), before the thermogenic role of BAT was clarified in the past century using integrative physiology and biochemistry (reviewed by Smith and Horwitz, 1969). Hibernators have been the focus of much investigation because they possess the highest BAT mass, requiring the 'hibernation gland' (=BAT) to rapidly rewarm from hypothermic conditions. Early research on BAT in non-hibernating rodents played an important part in removing the label 'hibernation gland' (Smith, 1961). Characterizations in non-hibernating species, including human infants, demonstrated that thermogenic BAT played a broader role in the regulation of thermogenesis and energy metabolism of mammals. The molecular era of BAT research was boosted by the identification of its unique protein, UCP1. The 32-kDa mitochondrial protein in BAT was first noticed in stained protein gels without functional characterization (Ricquier and Kader, 1976). Heaton and colleagues attributed fatty-acid inducible, purine nucleotide-sensitive proton conductance of BAT mitochondria to this 32-kDa protein (Heaton et al., 1978). The term 'uncoupling protein' is derived from its molecular function to increase proton leak respiration rates that are not 'coupled' to the generation of ATP. UCP1 was cloned and sequenced (Aquila et al., 1985; Bouillaud et al., 1986), paving the way for a new spectrum of molecular experimentation on this fascinating protein, including structure–function relationships, and the identification of orthologous and paralogous proteins such as UCP2 and UCP3 (Boss et al., 1997; Fleury et al., 1997; Gimeno et al., 1997). The generation of the UCP1 knockout mouse confirmed the pivotal role of UCP1 in thermogenesis (Enerback et al., 1997). A series of studies in the UCP1 knockout mouse have contributed to our understanding of how brown fat works (reviewed by Cannon and Nedergaard, 2004) and the mouse is still under investigation to identify other roles of UCP1 (Jastroch, 2017). Studies on structure–function relationships were important, although not fully conclusive, for the understanding of UCP1 action (Klingenberg et al., 1999 and reviewed by Crichton et al., 2017) To date, crystallization of UCP1 has failed and, therefore, the frequently published structural illustrations are based on threading the UCP1 sequence into the crystal structure of the adenine nucleotide translocase (Pebay-Peyroula et al., 2003).

Owing to the rediscovery of BAT in adult humans (Nedergaard et al., 2007), BAT has now become a focus of human medical research, which is facing a worldwide human epidemic of metabolic syndrome. Researchers hope that UCP1 and BAT activity can be used to treat obesity and its comorbidities, such as diabetes, and this is now the main focus of BAT research (Bartelt and Heeren, 2014). Given these major efforts, several fundamental aspects of brown adipocyte biology have been discovered in recent years, e.g. that brown adipocytes and myocytes derive from common progenitors (Seale et al., 2008).

Comparative physiology of BAT

Nowadays, experimentation on BAT is performed almost exclusively using laboratory rodent models, fostered by established genetic tools, convenient husbandry and their high reproductive capacity. BAT function can be conveniently assessed and manipulated in mice under controlled laboratory conditions. By contrast, assessing BAT function in exotic animals requires capturing exotic mammals from the wild that have been exposed to non-hygienic conditions and are uncontrolled for age and life history; furthermore, trapping these animals in remote locations may even put the researcher's health at risk. Despite these drawbacks, the comparative physiology research community aims to increase our knowledge of BAT biology using the diversity of ∼5000 mammalian species that have adapted to all extremes of environmental and climatic conditions. Profound differences can be expected considering that profound differences in the expression of UCP1 and BAT even exist between some mouse strains (Guerra et al., 1998; Xue et al., 2007). The differences between mouse strains are surprising given that these animals have been inbred at room temperature for the past 100 years (hoping that these unchallenging conditions do not eliminate details of BAT biology that would be required in the real seasonal world). Although *M. musculus* is undoubtedly important as a model to consolidate functional components of BAT with plenty of genetic tools, relying solely on mouse models may limit our complete understanding of the significance of BAT in nature. In the following, we review recent advances in BAT biology, highlighting new insights regarding the evolution of BAT that were put forward by researchers using non-model organisms. Furthermore, we suggest new avenues of research opened up by comparative approaches for understanding molecular mechanisms in BAT, involving transcriptomics and proteomics in evolutionary distant species (as discussed later). The strategy of integrating omics data was recently applied to hibernators, and has assisted in increasing our understanding of BAT function in the extreme physiological condition of hibernation (Ballinger et al., 2016; Grabek et al., 2015; Hampton et al., 2013).

Evolution of UCP1 and BAT

BAT is referred to as characteristic of mammals (Vaughan et al., 2000), and its 'development, i.e. the acquisition of brown adipose tissue with its new protein, uncoupling protein-1 (UCP1, thermogenin), may have been the one development that gave us as mammals our evolutionary advantage' to survive cold stress (Cannon and Nedergaard, 2004). However, until recently, there has been a lack of conclusive evidence regarding whether all three major mammalian clades, comprising monotremes, marsupials and placentals, possess BAT and UCP1, or whether BAT exists in all placental mammals.

Tracing the evolution of UCP1 and BAT using comparative approaches has enabled researchers to determine when BAT and UCP1 evolved. Furthermore, the various environmental and physiological challenges of different ecological niches impact thermogenesis and metabolic homeostasis, thus shaping the systemic integration of BAT and UCP1 and stretching the flexibility of BAT metabolism to the maximum. Thermogenic BAT may have contributed to key selection processes in the evolution of mammalian endothermy. Pinpointing the proximate and ultimate causes for the evolution of thermogenic BAT helps to understand genetic and metabolic programming, elucidating which molecular networks were implemented during the course of evolution to allow thermogenesis. These investigations are not only of academic interest but also may assist with accessing thermogenic adipose tissue for translational medicine. One of the major aims of metabolic medicine is the generation of thermogenic BAT in unhealthy humans, which would surely benefit from

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nature's manual on how to construct BAT, which was perfected by selection forces over millions of years.

Pioneering morphological and physiological comparative studies summarized the presence or absence of BAT in neonates of 285 mammalian species, suggesting the absence of BAT in monotremes, marsupials and some groups of placentals (Rowlatt et al., 1971). More than 40 years later, it transpires that these studies, although missing molecular data, were well performed and provided accurate predictions for most placental mammals. The authors themselves made the humble final statement that 'our contribution to the study of brown fat is one of description rather than interpretation. We feel that much more data needs to be collected in wild animals and that speculation on the part played by this substance in meeting the physiological demands of mammals should take into account its non-comprehensive, patchy distribution in that class.' (Rowlatt et al., 1971). In particular, the identification of BAT in marsupials remained controversial: some reports refuted its existence (Hayward and Lisson, 1992) whereas others identified BAT structures in the Bennett's wallaby and other marsupials (Loudon et al., 1985 and summarized in Jastroch et al., 2008). Given that BAT sometimes occurs transiently during juvenile life, e.g. in lambs and calves, the presence of BAT might have been easily missed owing to its short-term occurrence. The confident identification of BAT and UCP1 orthologs in diverse species was possible using the UCP1 gene sequence as an accepted marker for BAT. Thus, genomic sequencing of model and non-model organisms promoted comparative and evolutionary research of BAT by unequivocally providing evidence for the presence or absence of the UCP1 gene.

Comparative genomics investigations in a variety of placental mammals revealed conserved synteny of the UCP1, UCP2 and UCP3 genomic loci among placental mammals (Jastroch et al., 2005). In a variety of other vertebrate species, the initial scanning of upcoming genomic trace libraries before genomic assemblies has revealed UCP-like sequences [e.g. gray short-tailed opossum (*Monodelphis domestica*) and zebrafish (*Danio rerio*); M.J., unpublished observations]. The conserved synteny was instrumental in the annotation of the UCP-like sequences as UCP1, UCP2 and UCP3 orthologs. These studies revealed that UCP1 is present in teleost fishes [e.g. pufferfish (*Fugu rubripes*), *Danio rerio* and the common carp (*Cyprinus carpio*) (Fig. 1A)] and, thus, must have been present before the divergence of ray- and lobe-finned vertebrates ~420 million years ago (Jastroch et al., 2005) (Fig. 2). Subsequent studies have reported the expression of the UCP1 ortholog in amphibians such as the cane toad (*Bufo marinus*) (Trzcionka et al., 2008; Fig. 1B) and shown that the UCP1 gene was extinguished in the Sauropsida lineage comprising reptilians and birds (Emre et al., 2007; Schwartz et al., 2008). In the monotreme lineage, the UCP1 gene has been found in the genomic databases of the platypus. Recently, intact UCP1 messenger ribonucleic acid (mRNA) was identified in RNA sequencing projects of the platypus (*Ornithorhynchus anatinus*), demonstrating the presence of UCP1 gene expression in monotremes (Gaudry and Campbell, 2017). In the marsupial lineage, UCP1 is expressed in both representatives of Australian [e.g. fat-tailed dunnart (*Sminthopsis crassicaudata*), Fig. 1C] and South American marsupials (e.g. *M. domestica*, Fig. 1E) (Jastroch et al., 2008). Interestingly, there are differences in the regulation of UCP1 expression, at least within the Australian marsupials. Although cold exposure triggers the expression of UCP1 in *S. crassicaudata*, UCP1 mRNA was undetectable in adult, cold-exposed yellow-footed Antechinus (*Antechinus flavipes*) (Fig. 1D). Further comparative studies are required to resolve the cause of the differences between these species, which are similar in size but display profound differences in habitats (e.g. semi-arid versus subtropical forests) and biology (e.g. reproduction cycles and the expression of torpor behavior). Although cold-induced UCP1 mRNA levels in *S. crassicaudata* suggested some function for adaptive non-shivering thermogenesis, the metabolic response to noradrenaline that reports BAT activity revealed no cold-adaptation (Polymeropoulos et al., 2012). How marsupials generate heat and whether marsupial BAT plays a role is unresolved.

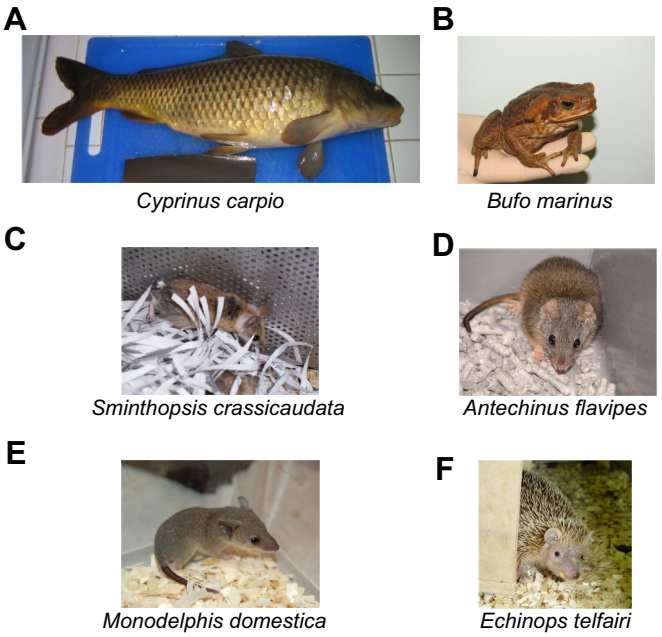

Fig. 1. Photographs of species that were used to elucidate the evolutionary history of UCP1-mediated non-shivering thermogenesis. (A) Common carp (*Cyprinus carpio*) as a representative of teleost fishes; (B) cane toad (*Bufo marinus*) as a representative of amphibians; (C) fat-tailed dunnart (*Sminthopsis crassicaudata*) as a representative of Australian, semi-arid marsupials; (D) yellow-footed Antechinus (*Antechinus flavipes*) as a representative of Australian, subtropical marsupials; (E) gray short-tailed opossum (*Monodelphis domestica*) as a representative of South American marsupials; (F) lesser hedgehog tenrec (*Echinops telfairi*) as a representative of a protoendotherm and the afroinsectiphilian group.

Evolution of UCP1 tissue specificity

UCP1 in rodents and humans is almost exclusively expressed in brown and beige adipose tissue and induced by environmental cold. The molecular basis for this tissue specificity has been studied in mice and humans, showing highly complex recruitment of several transcription factors in proximal and distal genomic regions of the UCP1 gene (reviewed in Cannon and Nedergaard, 2004). In particular, a distal region is found in humans and mice that is dense in recognition sites for transcription factors (del Mar Gonzalez-Barroso et al., 2000). This region is conserved among placental mammals but is missing in *M. domestica* (Jastroch et al., 2008). Detailed genomic studies have shown that although the upstream regulatory region is not universally conserved among placental mammals that express UCP1, it is intact in pigs that possess a pseudogenized UCP1 gene (Shore et al., 2012). Furthermore, some CpG islands close to the UCP1 transcription start are conserved but are not related to differences in UCP1 gene expression levels. Recently, a survey scrutinizing UCP1 transcriptional regulatory elements in 139 mammalian species substantiated that the UCP1 enhancer region is only found in placentals but not in marsupials and monotremes (Gaudry and Campbell, 2017). Other motifs that

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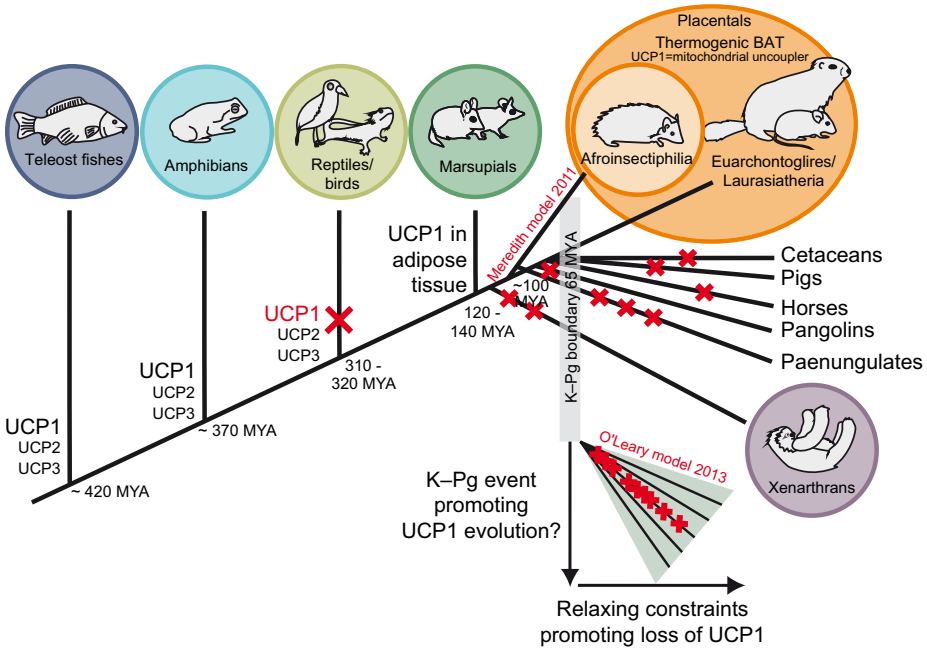

Fig. 2. Timeline showing the evolution of mitochondrial uncoupling protein 1 (UCP1) and brown adipose tissue (BAT). The duplication events leading to distinct UCP1, UCP2 and UCP3 orthologs occurred before the divergence of teleost fishes from the placental lineage (420 MYA). Amphibians investigated to date possess all three UCP genes. In the Sauropsida lineage (reptiles and birds), UCP1 has been lost; in birds, the UCP2/UCP3 loci were condensed to a single UCP, termed avian UCP3. The earliest evidence for targeting UCP1 gene expression to adipose tissue must have occurred in the common ancestor of marsupials and placentals. The timing of placental radiation according to the molecular evolution studies reported by Meredith et al. (2011) suggests thermogenic BAT was present at least 100 MYA, and inactivation events occurred periodically between 75 MYA and 25 MYA (Gaudry et al., 2017). The O'Leary model suggests that placental radiation occurred after the Cretaceous–Paleogene (K–Pg) boundary, and squeeze inactivation events after the K–Pg event. Hence, we speculate that constraints at the K–Pg boundary promoted mutation of UCP1 toward thermogenesis, with relaxed constraints for UCP1 during or after the K–Pg boundary manifesting in the loss of the UCP1 gene. MYA: million years ago.

had previously only been investigated in mice and rats appear not to be fully conserved among other mammalian species. This important work highlights the power of comparative approaches to filter the data for functional elements and to distinguish between specific and general regulation of UCP1 in BAT.

Although mammalian UCP1 expression appears specific for brown and beige adipose tissue, there are some reports of UCP1 expression in the thymus of mice and rats; however, the physiological role is not clarified yet (Adams et al., 2008a,b; Carroll et al., 2004; Carroll et al., 2005). Other claims are less convincing, such as the detection of UCP1 in murine smooth muscle cells, which has been disproven by others (Rousset et al., 2003). The targeting of UCP1 gene expression to adipose tissue must have evolved before the divergence of marsupials and placentals but after the divergence of amphibians. Australian and South American marsupials show adipose-specific expression, whereas UCP1 mRNA has not been found in fat depots of the common carp (teleosts) or in the cane toad (amphibians) (Jastroch et al., 2008; Jastroch et al., 2005; Trzcionka et al., 2008) (Fig. 2). In cyprinids, UCP1 expression is highest in the liver, and substantial levels of expression occur in the kidneys and in the brain. In contrast to mammals, cold water reduces liver UCP1 mRNA whereas brain UCP1 mRNA levels are increased, suggesting major changes in UCP1 transcriptional regulation during the course of evolution from ectothermic vertebrates to mammals. These comparative studies on transcriptional regulation are still patchy and require further experiments using various vertebrates.

Lessons from marsupial BAT

The BAT of the Australian marsupial *S. crassicaudata* shows the typical BAT-like coloration, as well as induction of UCP1 and cytochrome c oxidase activity in response to cold (Jastroch et al., 2008). However, we have no information on the marsupial UCP1 protein levels, and the cytochrome c oxidase activity values are much lower than those found of rodents. In the South American marsupial *M. domestica*, UCP1 is only found during early juvenile development; in three-month-old individuals, UCP1 is not even detectable after cold acclimation.

To date, there is limited evidence that marsupial BAT produces heat (Nicol et al., 1997; Polymeropoulos et al., 2012; Rose et al., 1999; Schwartz et al., 2008), and there is no evidence that marsupial UCP1 uncouples mitochondrial respiration. Indeed, molecular phylogenetic analyses suggest that marsupial UCP1 function is more similar to UCP1 of ectothermic vertebrates than to placental UCP1 (Jastroch et al., 2008). High mutation rates between non-placental versus placental vertebrates are best explained by relaxed constraints (Hughes et al., 2009; Gaudry et al., 2017). In contrast, the paralogs UCP2 and UCP3 show a much higher degree of conservation, reflected in shorter branch lengths, and may be more integral for survival than UCP1 (Hughes et al., 2009; Gaudry et al., 2017; Gaudry and Campbell, 2017). In the scenario of relaxed constraints, the original function of UCP1 may have become obsolete, thus reducing selection pressure on the non-placental UCP1 gene. The increased susceptibility to mutations (seen in the long phylogenetic branch between marsupials and placentals) may have generated a new function and possibly an advantageous physiological role, such as non-shivering thermogenesis in placentals. Once UCP1 gained this new function, the new protein sequence would be under purifying selection to be exactly shaped for placental BAT thermogenesis. What then is the function of marsupial UCP1? To date, no functional studies have been

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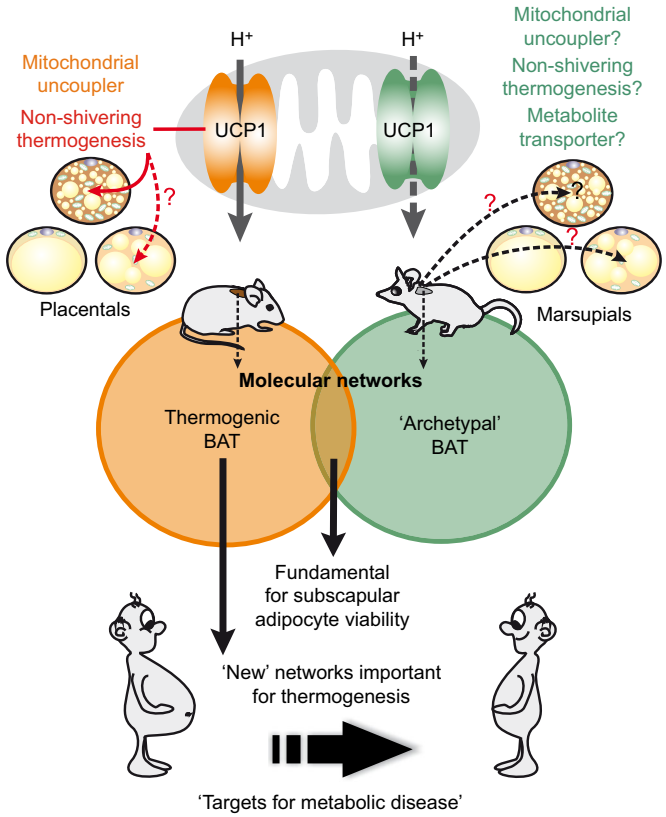

Fig. 3. Significance of comparative biology for brown adipose tissue (BAT) biology in placentals and marsupials. Determining the molecular function of marsupial mitochondrial uncoupling protein 1 (UCP1) may serve to understand metabolic processes that led to the development of thermogenic BAT. Comparing molecular networks of placental versus marsupial interscapular BAT may assist with filtering the data for pathways that are fundamental for subscapular adipocytes but not for thermogenesis. In contrast, specific pathways in thermogenic BAT highlight 'new' networks that are important for thermogenesis and may serve as therapeutic targets for metabolic disease.

performed and the protein may not even relate to net proton translocation and thermogenesis. It would be exciting to determine the function of marsupial UCP1, whether it uncouples or if the archetypal UCP1 transports metabolites, similar to other members of the pleiotropic family of mitochondrial anion transporters. The identification of marsupial UCP1 function is likely key to unlocking the physiological role of archetypal BAT in marsupials. Beyond the molecular function of marsupial UCP1, comparative molecular studies between marsupial and placental BAT may uncover metabolic pathways and networks that are essential to form the potent heat-generating machinery that we know from 'modern' placentals (Fig. 3). For translational medicine, this knowledge is required to recruit molecular networks in non-thermogenic human adipose tissue, to obtain beneficial metabolic effects for the treatment of metabolic disease.

Lessons from afrotherian BAT

Thermogenically competent BAT as well as a UCP1 ortholog that has almost identical proton leak activity to that of mouse UCP1 is found in afroinsectiphilian afrotherians, a group of placentals that is usually associated with the primordial thermoregulatory characteristics of their extant representatives (Mzilikazi et al., 2007; Oelkrug et al., 2013), although extinct ancestors may have possessed superior abilities to thermoregulate. The discovery of BAT in the afrotherian tenrec (Fig. 1F) has significant implications for proximate and ultimate causes of BAT evolution and eutherian endothermy that may be related to offspring incubation and parental care (Oelkrug et al., 2015). With the discovery of BAT in afrotherians, we are able to state that thermogenic BAT did not evolve during mammalian migration to colder environments but was present before, in temperate climates and in the placental ancestor. The molecular evolution of UCP1 suggests that thermogenic UCP1 is possibly restricted to placentals and that thermogenic BAT may have never evolved in marsupials and monotremes.

We previously concluded that thermogenic BAT must have been present at least 100 million years ago when afrotherians diverged from other placentals, based on molecular clock studies (Meredith et al., 2011) (Fig. 2). However, the date of placental speciation is still a matter of debate and phylogenetic calculations based on a combination of phenomic and molecular data suggest that the divergence of placental mammals occurred after the Cretaceous–Paleogene (K–Pg) boundary, ∼65 million years ago (O'Leary et al., 2013) (Fig. 2) – although this progressive field also suggests continuous radiation of placentals over the K–Pg boundary (Liu et al., 2017).

BAT and the radiation of placental mammals

If placentals started radiating after the mass extinction around the K–Pg boundary, thermogenic BAT may have given the placental ancestor, which resided underground during the devastating mass extinction event (Lovegrove et al., 2014), a crucial advantage, enabling it to outcompete other non-placental species. In particular, during the short-term cooled climate after the meteorite impact, when emitted ash and dust blocked the sun, thermogenic BAT would be advantageous, providing accelerated, endogenous rewarming rates from hypometabolic states (Oelkrug et al., 2011). Furthermore, during the short periods of resurfacing for reproduction, BAT would increase fitness by accelerating offspring incubation, as suggested for typical subterranean protoendotherms, the tenrecs (Levesque et al., 2014; Levesque and Lovegrove, 2014; Oelkrug et al., 2013; Oelkrug et al., 2015; Poppitt et al., 1994). Vice versa, did the mass extinction event at the K–Pg boundary promote the evolution of highly thermogenic BAT in placental mammals? For example, we lack a conclusive explanation for why high mutation rates of UCP1 occurred between the ancestors of marsupials and placentals, suggesting a significant environmental selection event promoting placental UCP1 and BAT. Could the selection of the distinct placental UCP1 be promoted by the evolutionary advantages at the K–Pg boundary? The link to this historical event impacting all animals and plants is almost impossible to prove. However, if the placental thermogenic UCP1 gene was under strong constraints during and shortly after this event, we would expect more relaxed constraints on placental UCP1 and thermogenic BAT in the long-term post-catastrophic era. Relaxed constraints could result in the loss of UCP1 and BAT unless a species has entered niches where UCP1 gives a crucial advantage for reproduction and survival, such as migration to the cold. A survey of many placental mammals revealed that the classical non-shivering thermogenesis response is mass-dependent and obsolete above a body mass of 10 kg (Heldmaier, 1971; Oelkrug et al., 2015). Why then would placental mammals maintain BAT if they are larger? Or have they not yet lost BAT, and are large placentals in the process of losing thermogenic BAT?

The loss of UCP1 in pigs

Berg and colleagues were the first to report on the pseudogenization of UCP1 in pigs, providing the molecular rationale for the poor ability

of pigs to thermoregulate that requires an increased reliance on behavioral thermoregulation to maintain body temperature (Berg et al., 2006). The UCP1 exon losses in the pig genome reported by Berg et al. (2006) are unambiguous, contrasting with ambiguous claims by others (discussed in Jastroch and Andersson, 2015). Based on this apparent controversy, the presence of pig UCP1 mRNA and protein was investigated, demonstrating that no protein is translated in pigs (Hou et al., 2017). A recent study comparing cold-sensitive and cold-resistant pig strains concluded that metabolically active adipocytes contribute to enhanced thermogenesis in cold-resistant pigs using UCP3 as an alternative uncoupling protein (Lin et al., 2017). Although the finding of metabolically active adipocytes is interesting and may be referred to as 'beige' adipocytes, even in the absence of UCP1 (Granneman et al., 2003; Keipert et al., 2017; Ukropec et al., 2006), the mechanistic link to UCP3 as an uncoupler appears technically unsubstantiated. Increased proton leak respiration in beige adipocytes of the cold-resistant Tibetan pig versus the cold-sensitive Bama pig has been interpreted as UCP3-dependent proton conductance, ignoring the higher maximal substrate oxidation capacity in adipocytes of the Tibetan pig. The correct interpretation of the proton leak rate is only possible by simultaneous assessment of proton motive force (Affourtit et al., 2012), because increased substrate oxidation capacity will also increase proton leak respiration (Divakaruni and Brand, 2011; Keipert and Jastroch, 2014). Furthermore, knockdown and overexpression of pig UCP3 have a great impact on substrate oxidation and a minor impact on proton leak respiration. If proton leak of the longitudinal plate-based respirometric measurements were properly quantified using the minimum value after inhibiting the ATP synthase (Divakaruni et al., 2014), the genotypic differences in proton leak would disappear. Owing to the lack of proton leak kinetics, there is no experimental evidence that pig UCP3 uncouples mitochondria in a similar manner to UCP1. In rat muscle, however, elevated UCP3 levels do not increase mitochondrial proton conductance (Cadenas et al., 1999) and UCP3 knockout mice have unaltered proton conductance, whereas overexpression causes an uncoupling artefact (Cadenas et al., 2002). Taken together, there is no convincing evidence that UCP3 uncouples mitochondria in rodents, pigs or humans, or even in non-mammalian endothermic birds that possess only the avian UCP (=ortholog of UCP2/3).

The loss of UCP1 in placentals

For more than 10 years, pigs represented a unique placental group with a pseudogenized UCP1 gene. Most recently, a genomic survey of 133 mammalian species surprisingly showed that in eight out of 18 traditional placental orders, the UCP1 gene was pseudogenized (Gaudry et al., 2017). This molecular map of the distribution of BAT within placental mammals represents unequivocal molecular evidence for Rowlatt and colleagues' observations and some of their speculations (Rowlatt et al., 1971), showing that their comparative studies accurately predicted many losses of functional BAT. The molecular clock suggests the earliest UCP1 inactivation events in the ancestors of xenarthrans and pangolins, and the latest in ancestral equids occurred ∼20 million to 25 million years ago. Gaudry and colleagues extensively discuss multiple scenarios for relaxed constraints and driving forces for losing UCP1. Whatever proximate causes pseudogenized the UCP1 gene, any reasoning for ultimate causes must consider that UCP1-dependent non-shivering thermogenesis is mostly important below the lower critical temperature of the animal's thermoneutral zone. Furthermore, alternative thermogenic mechanisms may compensate the requirement for BAT, such as muscle shivering and other unknown non-shivering heat sources, including heat as a byproduct of general metabolism. The presence of powerful mechanisms to reduce heat loss are indicated by the increase of body size, thereby decreasing the surface-to-volume ratio of the animal, and/or counter-current systems to preserve core body temperature. Living in the tropics or poor control of body temperature, or a combination of both, would increase the probability of UCP1 inactivation. Intriguingly, many losses occurred during a period of global cooling and coincided with increases in body mass, suggesting that energy-conserving, rather than energy-dissipating, mechanisms were required for survival and reproduction under environmental conditions that were possibly limited by the food supply. The comprehensive survey of the UCP1 gene locus of 133 mammals (out of more than 5000 species) performed by Gaudry and colleagues spurs exciting new unknowns for further comparative research. Discussing all the possibilities for future research would probably go beyond the scope of this Review; however, one example, the loss of UCP1 in equids that even conquered Polar regions, is puzzling because their close relative the rhinoceros still possesses an intact UCP1 gene despite being the second largest land-mammal that inhabits almost exclusively warm-temperate zones (Gaudry et al., 2017). Does the maintenance of UCP1 represent distinct physiological meaning, or have rhinos coincidentally not lost their UCP1 gene yet? So far, we have avoided discussing UCP1 in beige/brite adipose tissue because we do not understand the physiological role of beige/brite cells. If the role of UCP1 in beige cells goes beyond classical non-shivering thermogenesis, other factors may impact the selection pressure on UCP1, depending on the species. Getting to the bottom of these exciting comparative questions should increase our understanding of the significance of BAT and UCP1 in mammals. To date, 133 species have been genomically examined; what can be expected in the residual 5000 or so species? The current dataset suggests that the last UCP1 inactivation occurred ∼20 million years ago and, thus, is a rare event.

The significance of comparative BAT biology

Modern research investigations of BAT biology appear biased toward well-known model organisms such as mice to explore BAT function. Established genetic manipulation represents a powerful tool for understanding BAT biology; however, there are some drawbacks to solely relying on mouse genetics. The effects of acute gene inactivation are interpreted as thermogenic building blocks in BAT when thermogenesis is compromised. This has been perfectly worked out for UCP1; however, we should not forget that UCP1 tissue specificity and function had been elegantly demonstrated before the gene knockout was performed (Heaton et al., 1978). However, as thermogenesis represents the main function of BAT, any gene that comprises cellular or organ viability will inevitably result in reduced thermogenesis. In these cases, we cannot conclude that the gene/pathway is a thermogenic building block, and it may instead be a fundamental, generic gene/pathway for cell viability. For instance, if we conditionally knockout an apoptosis suppressor gene and the brown adipocyte dies, can we conclude that the gene was crucial for the function of thermogenesis, or was it only crucial for cell viability? Given this example, we may not have fully appreciated the potential of comparative biology and the diversity of non-model organisms that can be used to obtain insights into BAT biology. Nature's selection forces have applied trial and error in innumerable experiments over the course of 500 million years of vertebrate evolution to purify and diversify UCP1 and BAT function. Structure–function relationships of UCP1 provide a stellar example of how nature's experimentation may allow structure–function relationships to be mapped with

confidence given that we are uncertain whether experimental, mutational analysis of UCP1 compromises protein integrity. Evolution provides a diverse collection of UCP1 orthologs with variable activities that can be explored mechanistically. Even within placentals, UCP1 protein sequences evolved rapidly in some lineages, such as canids, some myomorph rodents, vesper bats and members of Eulipotyphla. Thus, it is tempting to speculate that UCP1 in these groups, which include members of seasonal northern regions and some of the smallest placentals on earth, underwent functional adjustments that are crucial to meet their thermoregulatory demands (Gaudry and Campbell, 2017). In particular, these species represent attractive targets that can be used to study important structural, functional and evolutionary traits of UCP1, its tissue and the consequences for non-shivering thermogenesis.

Mouse models are still the gold standard for consolidating experiments because they derive the greatest benefit from established protocols for genetic manipulation and husbandry. However, the shine of the mouse may fade with new molecular methods, such as clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR-Cas) technologies, which allow the genetic manipulation of non-model organisms.

For human metabolic research, improved understanding of BAT evolution may provide clues to translate comparative findings to new therapeutic targets. Nature must have implemented many new pathways and structures into archetypal, non-thermogenic BAT to construct a highly thermogenic organ. Comparative, evolutionary studies, integrating systems biology approaches, enable data to be filtered for important thermogenic pathways (Fig. 3) that translational medical research may be able to utilize to heat up fat in obese humans and treat metabolic disease.

BAT biology started ∼420 million years ago with gene duplication events generating UCP1 next to the UCP2 and UCP3 paralogs (Fig. 2). UCP1 was targeted at later stages to adipose tissue in mammals, before a selection process further purified BAT in placentals. Strikingly, this potent heater organ enables some rodents to maintain body temperatures of 37–38°C even though they are exposed to temperatures of −50 to −70°C at the coldest places on earth. Nature's lessons on BAT have been taught for almost 500 years, starting with the marmot (Gessner, 1551), a non-model mammal. Although mice dominate BAT research nowadays, the fascinating diversity should not be forgotten. New experimental tools pave the way for comparative biology to discover important aspects of BAT in mammalian physiology and evolution.

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Competing interests

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