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The role of skeletal-muscle-based thermogenic mechanisms in vertebrate endothermy

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Abstract

Thermogenesis is one of the most important homeostatic mechanisms that evolved during vertebrate evolution. Despite its importance for the survival of the organism, the mechanistic details behind various thermogenic processes remain incompletely understood. Although heat production from muscle has long been recognized as a thermogenic mechanism, whether muscle can produce heat independently of contraction remains controversial. Studies in birds and mammals suggest that skeletal muscle can be an important site of non-shivering thermogenesis (NST) and can be recruited during cold adaptation, although unequivocal evidence is lacking. Much research on thermogenesis during the last two decades has been focused on brown adipose tissue (BAT). These studies clearly implicate BAT as an important site of NST in mammals, in particular in newborns and rodents. However, BAT is either absent, as in birds and pigs, or is only a minor component, as in adult large mammals including humans, bringing into question the BAT-centric view of thermogenesis. This review focuses on the evolution and emergence of various thermogenic mechanisms in vertebrates from fish to man. A careful analysis of the existing data reveals that muscle was the earliest facultative thermogenic organ to emerge in vertebrates, long before the appearance of BAT in eutherian mammals. Additionally, these studies suggest that muscle-based thermogenesis is the dominant mechanism of heat production in many species including birds, marsupials, and certain mammals where BAT-mediated thermogenesis is absent or limited. We discuss the relevance of our recent findings showing that uncoupling of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) by sarcolipin (SLN), resulting in futile cycling and increased heat production, could be the basis for NST in skeletal muscle. The overall goal of this review is to highlight the role of skeletal muscle as a thermogenic organ and provide a balanced view of thermogenesis in vertebrates.

Keywords

endothermy; skeletal muscle; thermogenesis; SR calcium transport; brown adipose tissue

I. INTRODUCTION

The maintenance of a constant core body temperature T_c (Mitchell *et al*., 1992) is one of the most exquisite homeostatic mechanisms of modern-day birds and mammals. Present-day

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endotherms have both the ability to produce heat (thermogenesis) as well as dissipate heat, which enables them to maintain T_c in hot and/or cold environments. Importantly, the evolution of thermogenic mechanisms in vertebrates has enabled species not only to survive in fluctuating environments but also to invade new territories from the Arctic to the Antarctic. However, the origins and evolution of thermogenic mechanisms in vertebrates remain poorly understood. Current knowledge of thermogenesis is largely derived from studies performed in present-day mammals (mostly rodents) and to a lesser extent in birds. These studies suggest that during cold exposure both shivering and non-shivering thermogenic mechanisms are activated in mammals and birds (Chaffee & Roberts, 1971; Block, 1994; Silva, 2011). It is well known that shivering, a repetitive process of muscle contraction, is an important mechanism of heat production and is a first line of defence during acute cold exposure. Studies on various genetically modified mouse models show that shivering alone is insufficient for the maintenance of T_c and non-shivering mechanisms must be activated to sustain heat production (Thomas & Palmiter, 1997; Guerra *et al.*, 1998; Bachman *et al.*, 2002; Ukropec *et al.*, 2006; Arruda *et al.*, 2008; Aydin *et al.*, 2008; Anunciado-Koza *et al.*, 2011; Bal *et al.*, 2012).

Pioneering studies performed in newborn mammals and adult rodents have identified brown adipose tissue (BAT) as an important site of heat production *via* non-shivering thermogenesis (NST) (Dawkins & Scopes, 1965; Heim & Hull, 1966; Heim, Kellermayer & Dani, 1968; Knight & Myant, 1970; Alexander, Bennett & Gemmell, 1975; Lean & James, 1983; Lean *et al.*, 1986; Casteilla *et al.*, 1989; Giralt *et al.*, 1990; Houstek *et al.*, 1993; Zancanaro *et al.*, 1995; Cambon, Reyne & Nougues, 1998; Rose *et al.*, 1999; Cannon & Nedergaard, 2004). Rodents (the most common animal model used in research), like neonates, rely heavily on BAT for thermogenesis; as a result, much of the research on NST during the last two decades has been focused on BAT. There is no doubt that these studies have contributed to a greater understanding of BAT-dependent thermogenesis in mammals, but at the same time, these studies have overlooked the existence of other equally important NST mechanisms, especially the role of skeletal muscle. While BAT function is important in many mammals, it is most often restricted to neonatal stages and is downregulated in adult large non-hibernating mammals, including humans. In addition, there are several endotherms such as birds, marsupials, and wild boars that can maintain constant T_c and thrive in cold climates without BAT. Several studies have suggested that skeletal muscle could be an important site of NST (Davis, 1967; Barre *et al.*, 1989; Bourhim *et al.*, 1990; Duchamp *et al.*, 1991; Duchamp & Barre, 1993; Eldershaw *et al.*, 1997); however, the mechanistic details of muscle-based NST are not completely understood.

In addition to cold-induced thermogenesis, NST mechanisms have been shown to be recruited in diet-induced thermogenesis (Duchamp *et al.*, 1993) as a way to increase energy expenditure during diet overload (Lowell & Spiegelman, 2000; Feldmann *et al.*, 2009; Tseng, Cypess & Kahn, 2010). This area of research has received considerable attention because it can be targeted to increase energy expenditure, thereby providing a therapeutic strategy against obesity (Lowell & Spiegelman, 2000; Tseng *et al.*, 2010). Indeed, in recent years, there has been renewed interest in finding ways of increasing BAT activity in adult humans as a strategy to increase energy expenditure. However, to tackle the recent obesity

epidemic, a detailed understanding of other NST mechanisms in humans is of increasing significance.

The purpose of this review is (*i*) to trace the origins of various thermogenic processes throughout vertebrate evolution, (*ii*) to highlight the role of skeletal muscle as the original thermogenic organ, and lastly, (*iii*) to show that skeletal muscle is an important site of NST. In addition, we will discuss the relative contributions of muscle *versus* BAT-dependent thermogenic mechanisms in extant endothermic homeotherms. A major emphasis is placed on illustrating how muscle-based thermogenesis might be critical in animals where BAT is absent or limited in adult life. In addition, we discuss recent data from our laboratory that implicates uncoupling of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump by sarcolipin (SLN) as a mechanism for skeletal-muscle-based NST. This finding suggests that skeletal muscle has the capacity to activate futile Ca^{2+} cycling as a mechanism to generate heat. Thus, the overall goal of this review is to highlight the importance of skeletal muscle to thermogenesis throughout vertebrate evolution.

In addition to thermogenesis, many other adaptive features contributed to the evolution of endothermic homeothermy among vertebrates. These include: behavioural adaptations such as parental care and nesting/herding, heat conservation (by fur, feather, integument modifications), heterothermic adaptations (that reduce the energy cost of rigid homeothermy) such as torpor and hibernation, and predation and predator evasion, as these adaptations include locomotion thereby energy expenditure. Moreover, neurohormonal control of the activation and maintenance of thermogenesis will also have played a key role in the evolution of homeothermy. Such adaptations are beyond the scope of this review.

II. EVOLUTION OF SKELETAL MUSCLE AS A THERMOGENIC ORGAN

(1) Muscle is intrinsically capable of producing heat

Muscle contractions from physical activity generate considerable amounts of heat, and contraction-based heat production is exploited by shivering during cold exposure (Kosaka, Simon & Thauer,1967*a*; Kosaka, Takagi & Koyama, 1967*b*; Kosaka & Simon, 1968; Minaire & Chatonnet, 1968; Tkachenko, 1968; Nikki, 1969; Tanche & Therminarias, 1969; Chaffee & Roberts, 1971; Vybiral & Jansky, 1974; Lefaucheur *et al.*, 2001; van Marken Lichtenbelt & Daanen, 2003; Silva, 2011). Indeed, when maximally recruited, as during exercise or an intense bout of shivering, muscle can account for up to 90% of whole-body oxygen uptake, an indirect measure of heat production (Stainsby & Lambert, 1979; Zurlo *et al.*, 1990). During muscle contraction, heat is generated by the hydrolysis of ATP from three different ATPases: myosin ATPase (Stewart *et al.*, 2010; Cooke, 2011; Little & Seebacher, 2013), which performs the contractile work, and SERCA (Block, 1994; Dumonteil, Barre & Meissner, 1995; Simonides *et al.*, 2001; Morrissette, Franck & Block, 2003; de Meis, Arruda & Carvalho, 2005; Arruda *et al.*, 2007; Kjelstrup *et al.*, 2008; Bal *et al.*, 2012; Inesi & Tadini-Buoninsegni, 2013; Little & Seebacher, 2013; Sahoo *et al.*, 2013) and Na^+/K^+ ATPase (Guernsey & Morishige, 1979; Muller & Seitz, 1984; Herpin, McBride & Bayley, 1987; Kelly & McBride, 1990; Rolfe & Brown, 1997; Karbowski, 2009), which reset resting ion gradients and membrane potential. To sustain these processes, ATP generation must be

increased to match demand. These obligatory metabolic processes also generate heat, adding to total heat production.

(2) Muscle as the first organ to be recruited in heat production

Striated muscle can be considered the most primitive facultative thermogenic organ. Fish were among the earliest vertebrate species to evolve, and evidence for muscle-based heat production can be observed in certain fish species that show characteristics of endothermy. While the vast majority of fish are ectotherms, species from the families Lamnidae (sharks) and Scombridae (tunas) can achieve some level of whole-body homeothermy through a modified myotomal muscle (Dickson & Graham, 2004; Runcie *et al.*, 2009). In these species, this slow-twitch, oxidative muscle is located in a medial location, in contrast to the myotomal muscle of ectothermic fish, permitting heat conservation (Carey & Teal, 1966; Dickson & Graham, 2004). The muscle is also perfused by counter-current heat exchangers, which minimize heat loss (Dickson & Graham, 2004). A known characteristic of these species is their continuous swimming behaviour; these sustained contractions combined with the heat-conservation mechanisms described above result in net heat production and represent the source of endothermy in these fishes (Dizon *et al.*, 1978; Guppy, Hulbert & Hochachka, 1979; Hochachka & Mommsen, 1991; Dickson & Graham, 2004; Runcie *et al.*, 2009).

Another fish species known to exhibit endothermy is the opah (*Lampris guttatus*). Rather than achieving whole-body homeothermy, opahs have developed regional endothermy, which utilizes localized heat production specifically to warm the cranial region (Runcie *et al.*, 2009). Cranial endothermy is an important ecological adaptation that provides a selective advantage as it protects the central nervous system from rapid changes in ambient temperature and enhances vision and detection of prey. To achieve cranial endothermy, opahs employ contractions of the extraocular muscles in order to elevate cranial temperatures when water temperatures are low (Runcie *et al.*, 2009).

Further evidence for heat production as a result of muscular work is found in the brooding python (*Python molurus* and *Python spilotes spilotes*), a reptile that exhibits “facultative endothermy.” Brooding pythons use shivering thermogenesis, i.e. repetitive muscle contractions, to keep their eggs warm during embryonic development but revert to heterothermy after brooding (Hutchiso, Dowling & Vinegar, 1966; Harlow & Grigg, 1984; Slip & Shine, 1988; Grigg, Beard & Augee, 2004). Collectively, the evidence thus shows that heat can be generated by muscle, and that muscle can be recruited, in non-homeothermic species; perhaps this represents the most primitive form of regulated heat production. In fact, it has been postulated that behavioural thermoregulation and facultative endothermy were factors involved in the attainment of homeothermy (Hochachka & Mommsen, 1991). Modifications of these thermogenic mechanisms, along with a sophisticated thermoregulatory system, likely contributed to the development of obligate endothermy (Hochachka & Mommsen, 1991; Grigg *et al.*, 2004). Acquiring insulation, such as fur, feathers, and subcutaneous fat would additionally have contributed to this process.

(3) The role of skeletal muscle in the transition from ectothermy to endothermy

Out of all groups of vertebrates only birds and mammals achieved whole-body homeothermy. For centuries the specific mechanism(s) that led to the evolution of endothermy among vertebrates have remained elusive despite intense research effort. Comparative analyses of ectotherms and endotherms have provided clues to the heat-producing mechanisms that must have defined the path to the evolution of endothermy. When comparing similar-sized reptiles (ectotherms) to mammals (endotherms), skeletal muscle is found to be much more massive (30% greater) in mammals (Ruben, 1995). Based on such comparative analyses it has been proposed that the expansion of skeletal muscle mass was a key adaptation during the evolutionary transition of vertebrates from ectothermy to homeothermy (Newman, 2011). Studies also indicate that the surface area of the inner mitochondrial membranes in muscle is 220% greater in mammals than in reptiles and isolated mammalian mitochondrial membranes have twice the enzymatic activity (Else & Hulbert, 1985; Hulbert & Else, 1989; Ruben, 1995). Because the energetic requirements for thermogenesis are high, an increase in mitochondrial respiratory capacity suggests that muscle must have played a key role as a site for thermogenesis during the adaptive transition towards homeothermy. Therefore, increased skeletal muscle mass, along with the means to sustain higher metabolic activity are likely to have been critical in the acquisition of endothermy among vertebrates. However, due to the different genetic backgrounds of ectotherms and endotherms (i.e. reptiles *versus* mammals), direct comparisons cannot clearly define the mechanism(s) that mediated the transition from ectothermy to endothermy.

To circumvent this problem, researchers have utilized chicken embryos as an ideal model system to study the mechanisms underlying the transition from ectothermy to endothermy (Walter & Seebacher, 2007, 2009; Ijiri *et al.*, 2009). Before hatching, chick embryos switch from an ectothermic state to an endothermic state; therefore, one can perform controlled experiments without potentially confounding species-dependent variations. Observations from chick embryos show that muscle acquires a more oxidative phenotype during the transition, and respiration in skeletal muscle increases after hatching, whereas respiration in the liver remains unaltered (Walter & Seebacher, 2007, 2009; Ijiri *et al.*, 2009). Collectively, these data suggest that muscle is one of the earliest sites of thermogenesis and plays a key role in the transition from ectothermy to endothermy.

III. NON-SHIVERING THERMOGENIC ORIGINS IN SKELETAL MUSCLE

(1) Modified skeletal muscle as a site of NST

The above sections emphasized that muscle can be recruited for thermogenesis, even in non-homeothermic species, but in these cases, the heat-producing capability of muscle is dependent upon contraction. However, muscle can be modified to produce heat in a non-shivering manner without being coupled to contraction. Evidence for muscle-based NST is found in certain fishes with the capacity for regional endothermy. These species, including billfishes, tuna, and mackerel, utilize modified muscle tissue as a means of achieving endothermy (Carey, 1982; Block, 1986, 1994; Dickson & Graham, 2004). This specialized muscle tissue, termed the “heater organ”, is derived from eye muscle and is located in the cranial region. The heater organ is composed of cells that lack the typical myofibrillar

structure and have very low expression of actin and myosin (Tullis, Block & Sidell, 1991). Heater organ cells are densely packed with mitochondria and sarcoplasmic reticulum (SR) networks (Hochachka & Mommsen, 1991; Block, 1994). The extensive SR of heater organs is organized into tightly packed stacks, which optimizes surface area for SERCA. Studies have indicated that, in addition to SERCA, the heater organ expresses a Ca^{2+} release channel (CRC) and calsequestrin (CASQ), which are distributed rather homogeneously throughout the SR (Block & Kim, 1992; Block, Obrien & Meissner, 1994). This is in contrast to skeletal muscle, where the CRC and CASQ are commonly located at specific sites in the SR, called triad junctions. Although the precise mechanism of heat production in heater organ cells has not been proven, it has been linked to Ca^{2+} transport across the SR membrane. The mechanism proposed is as follows: neuronal stimulation depolarizes the heater cell, which leads to Ca^{2+} release from the SR. The resulting increase in cytoplasmic Ca^{2+} stimulates SERCA to actively remove the Ca^{2+} by utilizing energy derived from ATP hydrolysis. Simultaneously, the free cytosolic Ca^{2+} can also enter through the mitochondrial uniporter and stimulate mitochondrial respiration (and heat generation) directly (Block, 1994). Thus, in the heater organ, heat is produced by SERCA-catalysed ATP hydrolysis and the stimulation of mitochondrial metabolism required to replenish ATP. Hence, by coupling SR Ca^{2+}-transport with ATP production by mitochondria, and with the loss of myofilaments, a new cell type evolved to produce heat (Fig. 1). Interestingly, the heater organ originated independently in two lineages of fish [billfishes and butterfly mackerel (*Gasterochisma melampus*)], a fact that highlights the relative ease with which muscle can be adapted to fulfill a thermogenic role (Block, 1994).

(2) Muscle-based NST in birds

Perhaps the most convincing evidence for NST in skeletal muscle comes from avian species (Barre *et al.*, 1985, 1989; Barre, Bailly & Rouanet, 1987; Duchamp *et al.*, 1989; Dumonteil, Barre & Meissner, 1993, 1994; O'Brien, Meissner & Block, 1993; Dumonteil *et al.*, 1995; Bicudo, Vianna & Chaui-Berlinck, 2001; Raimbault *et al.*, 2001; Bicudo, Bianco & Vianna, 2002; Toyomizu *et al.*, 2002; Ijiri *et al.*, 2009; Walter & Seebacher, 2009). Birds occupy some of the most diverse climates yet maintain the highest body temperatures (38–42°C) among homeotherms (Butler & Woakes, 2001). Some birds including pigeons, geese, and starlings are able to maintain T_c at ~44°C during flight even at ambient temperatures below 0°C (Hart & Roy, 1967; Aulie, 1971; Miller, 1974; Torre-Bueno, 1976). A common feature of birds is that their skeletal muscle is more massive than in comparably sized reptiles and mammals (Else & Hulbert, 1981, 1985; Butler, 2000; Hutchinson, 2004; Newman, 2011; Newman, Mezentseva & Badyaev, 2013): the Japanese quail (*Coturnix coturnix japonica*) has a skeletal muscle mass that constitutes more than 70% of its body mass (Kawahara & Saito, 1976). Therefore it is speculated that the expansion of avian skeletal muscle, particularly the breast and thigh muscles, provided a unique survival advantage that permitted more efficient heat generation. These muscles are highly specialized for aerobic respiration with high levels of myoglobin and mitochondria, making them well suited for flight (Butler, 1991). Cold-adaptation experiments on birds have shown that, while shivering is the first line of defence used to maintain body temperature as in mammals, birds are also known to use NST mechanisms (Barre *et al.*, 1989; Duchamp *et al.*, 1989, 1991; Duchamp &

Barre, 1993; Eldershaw *et al.*, 1997). Hence, the same characteristics that enable flight very likely provide the proper physiological milieu for NST in muscle.

Although others had previously recognized cold-induced NST in birds, C. Duchamp and H. Barre were the first to identify skeletal muscle as the major site of NST (el-Halawani, Wilson & Burger, 1970; Duchamp *et al.*, 1989; Duchamp & Barre, 1993). Using blood-flow measurements from thermoneutral (TN, 25°C) and cold-acclimated (CA, 4°C for 5 weeks) ducklings (*Cairina moschata*) exposed to 8°C, they were able to show that total muscle blood flow increased equally in the TN and CA ducklings, although the CA ducklings did not shiver. Both groups were also able to maintain T_c in the optimal range (Duchamp & Barre, 1993). Thus, skeletal muscles from CA ducklings were able to produce the same amount of heat as muscles from shivering TN ducklings, demonstrating the existence of NST in skeletal muscle. Similar results have also been found for sparrows (*Passer domesticus*), king penguin chicks (*Aptenodytes patagonicus*), and chickens (*Gallus domesticus*) (Barnett, 1970; el-Halawani *et al.*, 1970; Duchamp *et al.*, 1989; Eldershaw *et al.*, 1997). Although this work defined muscle as the site of NST, it did not provide a detailed mechanism for the source of heat production. In a follow-up study, Dumonteil *et al.* (1995) performed timed cold exposures to determine how changes in gene expression patterns coincided with the activation of NST. In their experiment, the onset of NST correlated with the timing of increases in SERCA and CRC expression, whereas CASQ levels were unaffected (Dumonteil *et al.*, 1995). Thus, these studies indicated that Ca^{2+} cycling could be responsible for muscle-based NST. Earlier studies substantiated corroborated these findings by showing increases in both SERCA and the CRC after 6 weeks of cold acclimation in ducklings (Dumonteil *et al.*, 1993). Further support for Ca^{2+} cycling in NST is the finding that long-chain fatty acylcarnitines and related metabolites induce Ca^{2+} release from the avian CRC. During cold acclimation, long-chain fatty acids and their metabolites have been shown to be increased in muscle (Dumonteil *et al.*, 1994). Therefore, fatty acyl-CoA/carnitine-induced Ca^{2+} release could be a mechanism by which Ca^{2+}-cycling is activated in response to cold. Further research is required to define the exact mechanism of NST in avian skeletal muscle.

(3) NST in mammals: evolution of BAT and its role in mammalian thermogenesis

It appears that birds followed an evolutionary pathway that led to muscle hyperplasia along with enrichment in mitochondrial content (Ruben, 1995; Newman, 2011) but did not evolve BAT (Walter & Seebacher, 2007, 2009; Mezentseva, Kumaratilake & Newman, 2008; Teulier *et al.*, 2010). Mammals did not follow the same route. The more recent mammals, the eutherians, invented a whole new tissue type, BAT, which prototherian mammals lack. This evolutionary bifurcation in NST mechanisms could have been due to the ecological and ecogeological constraints prevailing during the times of diversification of these groups from their respective ancestors. During this period, birds adopted an aerial mode of life that permitted muscle hyperplasia and the evolution of muscle-based NST. Mammals, however, remained predominantly terrestrial, and maintenance of T_c from basal metabolism alone became insufficient once cooler land masses began to appear. The appearance of BAT as a specialized thermogenic organ among mammals allowed mammalian habitation of colder climatic zones.

Among vertebrates, only mammals and birds developed whole-body homeothermy. The mammals and birds originated from different amniote lineages at very different time points in vertebrate evolutionary history and thus, must have undergone the transition to endothermic homeothermy independently (Mcnab, 1978; Ruben, 1995; Hillenius & Ruben, 2004; Geiser, 2008; Clarke & Portner, 2010). Synapsids (the lineage including modern mammals) diverged at the very beginning of the amniote radiation during the Late Carboniferous (Luo & Crompton, 1994; Sidor & Hopson, 1998; Luo, 2007; Geiser, 2008), while birds diversified from their sauropsid ancestors at least 80 million years later during the mid-to-late Jurassic (Ruben, 1995; Hillenius & Ruben, 2004; Luo, 2007; Nespolo *et al.*, 2011; Ruta *et al.*, 2013), as depicted in Fig. 2. By the Devonian Period of the Palaeozoic Era, the land plants had established and new ecological niches were available on land for animals to invade. In the Carboniferous, amphibians gave rise to the first amniotes, which made life on land possible. By the mid-Permian, when fauna on land became abundant, positive selection would have existed towards organisms that could remain active (i.e. better predators or escapers) even at low ambient temperatures. This evolutionarily advantageous feature was only possible *via* mechanisms of endothermic heat production (Clarke & Portner, 2010). Maintenance of elevated metabolic rate and muscular activity (including shivering) might have been the first two mechanisms of endothermic heat production; however, they are very costly and would be limited by nutritional availability in the environment.

The ectothermy to endothermy transition has a close association with the transition from brady- to tachy-metabolism (Bennett & Ruben, 1979; Clarke & Portner, 2010; Hayes, 2010). It seems likely that the ancestral amniotes, like their anamniote tetrapod ancestors, were brady-metabolic with very low basal metabolic rates and an inability to support maximal metabolic rate for long periods. As ancestral amniotes underwent adaptations for terrestrial life (early tetrapods were probably like modern amphibians, needing water to complete their life-cycle), the transition to an erect posture (elevated pelvic joint) must have aided in overcoming the physiological constraints imposed by a sprawling posture, including slow locomotion (friction with ground) and heat loss (ventral surface in close proximity to ground) (Kielan-Jaworowska & Hurum, 2006). Moreover, efficient terrestrial locomotion required animals to overcome “Carrier’s constraint”, the observation that postural and anatomical features precluded the ability to run and breathe at the same time, by adopting an elevated posture along with other skeletal modifications (Carrier, 1987; Brainerd & Owerkowicz, 2006). This sprawling-to-erect postural transition appeared among synapsid and sauropsid lineages independently in the Mid-Permian. In order to sustain the higher energetic demands of the newly evolved postural muscles and running ability, the development of skeletal muscles with both fast contractile properties and a more robust metabolic capacity (characterized by a high number of mitochondria), termed fast-oxidative muscles, must have been necessary. Thus, during the late Permian, it seems possible that skeletal-muscle-based NST must have been used for the first time for a transition to tachy-metabolic status with a rudimentary level of homoeothermy among both synapsid and sauropsid lineages. In order to recruit muscle-based thermogenesis more effectively, increased muscle mass (i.e. muscle hyperplasia) and increased mitochondrial number and activity were required. Palaeontological work on various dinosaur lineages during the last

three decades, suggests that many of these lineages had achieved a basal level of homeothermy (Schweitzer & Marshall, 2001; Pontzer, Allen & Hutchinson, 2009; Clarke & Portner, 2010; Kohler *et al.*, 2012; Clarke, 2013; Seymour, 2013), concomitant with muscle hyperplasia, ultimately giving rise to ancestral birds in the Jurassic. Muscle-based NST was further utilized by birds as the sole mechanism for their true endothermic homeothermy [as the uncoupling protein 1 (UCP1) gene was lost and no BAT tissue evolved], supplemented by the anatomical and physiological modifications required for an aerial mode of life, as shown in Fig. 3.

Mammalian evolution remained shadowed during almost the entirety of the Mesozoic Era by the successful radiation of the dinosaurs. During the Triassic and Jurassic, mammals remained primarily nocturnal (most dinosaurs were diurnal) and small in size (less than 1 kg). Therefore, given these ecological constraints, the evolution of muscle hyperplasia as a means to achieve homeothermy during this period seems unlikely. Moreover, most of the land masses were warm during the Triassic and Jurassic, remaining so until the Early Cretaceous, when some land masses began to become cooler. In this scenario, the evolution of endothermic mechanisms would not have been under positive selection and could occur only by 'neutral drift' until conditions changed in the Early Cretaceous. Among mammals, BAT is not found in any prototherian, but recent data shows a primitive form of cold-activated BAT in a metatherian mammal (*Sminthopsis crassicaudata*; family Dasyuridae) (Jastroch *et al.*, 2008). This prototype BAT could have provided UCP1 with the correct molecular milieu to perform a thermogenic function [elaborately discussed by Saito, Saito & Shingai (2008), Oelkrug *et al.* (2013) and Hughes *et al.* (2009)]. Based on these data it seems likely that BAT made its first appearance at or before the diversification of Metatheria and Eutheria, which occurred in the Late Jurassic, but subsequent to the split of Theria and Prototheria. Once colder climatic niches were available starting in the Cretaceous, natural selection would have positively selected animals with better endothermic capability. Further, with the elimination of ~85% of the large reptilian species from the earth during the late Cretaceous mass extinction, many ecological niches became available, allowing the expansion of mammalian species. Eutherians further evolved a sophisticated BAT-based mechanism and show a variety of NST strategies depending on their ecological and physiological requirements.

(4) NST in small *versus* large mammals: the complementary roles of BAT and skeletal muscle

BAT-dependent thermogenesis is highly efficient in newborn humans and rodents and a great deal of research has focused on this tissue leading to a clear understanding of its mechanism of heat production (Dawkins & Scopes, 1965; Heim & Hull, 1966; Heim *et al.*, 1968; Jenkinson, Noble & Thompson, 1968; Knight & Myant, 1970; Alexander *et al.*, 1975; Lean & James, 1983; Lean *et al.*, 1986; Schubring, 1986; Slebodzinski, 1988; Houstek *et al.*, 1993; Zancanaro *et al.*, 1995; Enerback *et al.*, 1997; Blumberg, Deaver & Kirby, 1999). Support for the existence of BAT-independent NST is provided by the following observations: (*i*) the transition from ectothermy to endothermy did not depend on the evolution of BAT, and (*ii*) secondly, BAT is not a characteristic trait of all endotherms (see above and Figs 2 and 3) (Grigg *et al.*, 2004). Comparisons of large and small mammals and

birds yield striking differences in apparent means of thermogenesis (Table 1). Studies in large mammals, including adult rabbits, dogs, and marsupials, where BAT is less prevalent, underscore the importance of skeletal muscle-based NST in facultative thermogenesis (Davis, 1967; Rose *et al.*, 1999; Arruda *et al.*, 2008); whereas, studies on small mammals, namely rodents, focus on BAT as the principal contributor to NST. These species-specific recruitment strategies are discussed in more detail below.

Rodents rely on the heat-generating capacity of BAT to maintain body temperature in response to temperatures below thermoneutrality (Enerback *et al.*, 1997; Cannon & Nedergaard, 2004). BAT is located primarily in the subcutaneous interscapular region (comprising ~60% total BAT volume) of the mouse and rat, in addition to small depots dispersed throughout the body (Frontini & Cinti, 2010). BAT is defined by the presence of multilocular adipocytes, large numbers of mitochondria, and the expression of UCP1 (Heaton, 1972; Matthias *et al.*, 2000). UCP1 is located in the inner mitochondrial membrane and generates heat by dissipating the proton gradient generated by the electron transport chain (Fedorenko, Lishko & Kirichok, 2012). Numerous studies have shown UCP1/BAT to be recruited and activated upon cold exposure. Furthermore, UCP1, and thus BAT, is required for the survival of mice challenged to an acute cold exposure (Enerback *et al.*, 1997; Ukropec *et al.*, 2006). However, UCP1-null mice are able to survive when gradually adapted to cold, indicating the presence of other adaptive thermogenic mechanisms (Golozoubova *et al.*, 2001; Ukropec *et al.*, 2006; Meyer *et al.*, 2010; Shabalina *et al.*, 2010). Unlike rodents, studies on thermogenesis in large mammals point to a much higher reliance on skeletal muscle. In large mammals such as rabbits, ruminants, and humans, BAT activity is downregulated after the neonatal period and is minimally present or not detectable in adulthood, suggesting that skeletal muscle is the primary site of heat production (Lean *et al.*, 1986; Casteilla *et al.*, 1989; Trayhurn, Thomas & Keith, 1993; Cambon *et al.*, 1998; Rose *et al.*, 1999; Lomax *et al.*, 2007). Interestingly, some species even lack a functional *Ucp1* gene; pigs lost a functional UCP1 protein ~20 million years ago due to a nonsense mutation (Berg, Gustafson & Andersson, 2006). Pigs maintain T_c at ~37°C, and interestingly, are very susceptible to a deadly condition called malignant hyperthermia, where sustained Ca^{2+} release by ryanodine receptor 1 in skeletal muscle results in excessive heat production (Fill *et al.*, 1990; MacLennan & Phillips, 1992). This may suggest that pigs employ muscle-based thermogenesis and that its dysregulation can lead to hyperthermia.

Moreover, cold-exposure studies performed on large mammals provide evidence for skeletal-muscle-based NST. Cold-acclimation experiments performed on dogs in which the hindlimbs were denervated to prevent shivering showed that cold-acclimated dogs were able to significantly increase oxygen consumption of the hindlimbs, similar to levels before denervation. Thus, the authors concluded that the denervated skeletal muscle contributed to NST (Davis, 1967). Similar results were obtained for cold exposures on the Tasmanian bettong *Bettongia gaimardi*, a marsupial in which thermogenic BAT has not been identified (Rose *et al.*, 1999). In addition, in bettongs adapted to cold, heat production and responsiveness to catecholamines are increased. While direct muscle respiration was not measured in this study, earlier experiments had shown that catecholamines stimulated muscle oxygen consumption (Ye *et al.*, 1996; Rose, Kuswanti & Colquhoun, 1998); therefore, it was concluded that muscle contributes to NST in bettongs (Rose *et al.*, 1999).

(5) The recruitment of BAT *versus* muscle-based NST mechanisms: a physioecological perspective

A clear explanation for the reduced volume of BAT in adult large mammals is not available. Using the concept of "thermal inertia (Hochachka & Mommsen, 1991)," one could hypothesize that the larger surface area to volume ratio of small mammals necessitates a more powerful and efficient NST organ to compensate for the greater heat loss. Larger mammals, with smaller surface area to volume ratios, do not suffer from similar rates of heat loss, and therefore, muscle-based thermogenic mechanisms would be sufficient to maintain T_c.

Rodents (i.e. BAT-enriched mammals) possess an abundance of fast-type skeletal muscles, particularly Type IIB fibres (Hesse, Fischer & Schilling, 2010; Schiaffino & Reggiani, 2011). Because of their small size, rodent muscle must be able to contract quickly and more frequently than large mammals (Gasc, 2001; Hesse *et al.*, 2010); in mammals, contraction velocity and stride frequency correlate inversely with body size (Strang & Steudel, 1990; Seow & Ford, 1991). In addition, the effects of gravity on small, lightweight mammals is less than for large mammals, reducing the need for postural muscles, which are typically composed of slow or intermediate oxidative fibres (Hesse *et al.*, 2010). Fast-type muscles, especially Type IIB, rely on glycolytic pathways for ATP production, compared to slow or intermediate fibre types which rely predominantly on oxidative metabolism. Accordingly, the amount of oxidative fibres correlates positively with body mass; therefore, skeletal muscles with more oxidative features, where muscle-based NST would occur, are favoured in large mammals (Emmett & Hochachka, 1981; Hesse *et al.*, 2010).

Muscle composition is also indicative of the ecological coverage and behavioural traits of an animal. Rodents, for instance, have small ecological (spatial) coverage so muscle activity is restricted mostly to small bursts of activity, such as sprinting, as when escaping from a predator. By contrast, most large mammals cover a greater ecological area, even for foraging and other routine activities, necessitating a muscle profile more closely resembling that of a marathon runner (Table 1). Based on these facts, the following explanation seems feasible: because skeletal muscle is used primarily for locomotion, the types and duration of movements a species is adapted for can define the thermogenic capability of the muscle. In simpler terms, animals adapted for extended muscle usage, including locomotion and posture maintenance, will have a greater likelihood of employing muscle-based thermogenesis than animals that require muscle activity for only short periods of time.

IV. SKELETAL MUSCLE AND BAT SHARE A COMMON ORIGIN

(1) BAT and skeletal muscle: divergence from a common cellular precursor

BAT and skeletal muscle are distinct organs with disparate thermogenic mechanisms. However, recent research has established that skeletal muscle and BAT develop from a common progenitor: myogenic factor 5 (Myf5)-expressing precursors, originally believed to give rise specifically to skeletal muscle (Seale *et al.*, 2008). The cell fate switch from skeletal muscle to BAT is controlled by the PR domain-containing 16 (PRDM16) transcriptional complex (Seale *et al.*, 2008; Kajimura *et al.*, 2009; Ohno *et al.*, 2013; Harms

et al., 2014). Recent work has shown that the related protein, PRDM3, can also induce this switch in the absence of PRDM16 (Harms *et al.*, 2014). Interestingly, expression of PRDM16 drives brown fat development in Myf5-expressing skeletal myoblasts, whereas loss of PRDM16 from primary brown fat preadipocytes is sufficient to induce differentiation into skeletal muscle. Intriguingly, white adipocytes do not develop from these predecessor cells, thus BAT and white adipose tissue (WAT) have distinct developmental origins (Seale *et al.*, 2008). Moreover, gene expression profiles of BAT, WAT, and skeletal muscle indicate disparities between BAT and WAT, and similarities between muscle and BAT (Timmons *et al.*, 2007). Further, comparisons of mitochondrial proteomic signatures show more similarities between BAT and skeletal muscle than BAT and WAT (Forner *et al.*, 2009). Therefore, BAT, in spite of having some adipocytic features, shares a closer lineage with skeletal muscle (Fig. 4).

These new findings might provide some clue to why the two organs share a common function of NST. Both are endowed with characteristics that could support NST, including a high capacity for lipid oxidation, dense mitochondria, neural innervation, and the ability to respond to circulating adrenergic factors through beta-adrenoreceptors (Farmer, 2008; Forner *et al.*, 2009). It can be speculated that skeletal muscle became the first thermogenic organ in ancestral endotherms, but evolutionary pressure resulted in the development of a more efficient organ devoted entirely to heat production, so as not to interfere with muscle function, when the thermogenic demand became greater. This new organ evolved from one already possessing the capacity to meet the demand. All organisms with skeletal muscle have an inherent ability to generate heat. However, small mammals, especially rodents with prevalent BAT, may be less reliant on muscle NST, while others, including birds and large mammals, actively utilize muscle thermogenesis. Barbara Block, a pioneer in muscle-based thermogenesis, similarly hypothesized that “a source of heat is readily available in all skeletal muscle fibers that cycle Ca^{2+} for continuous contraction for sustained periods of time” (Block, 1994).

(2) Beige or brite fat: an analog of classical BAT

More recent work in the field of thermogenesis has been focused particularly on the inducible brown fat within the WAT depot, termed “beige” or “brite” fat. Rodent beige fat possesses the thermogenic properties of classical BAT; i.e. expression of UCP1 and increased mitochondrial enzymes, while retaining many of the properties of WAT (Wu *et al.*, 2012; Harms & Seale, 2013; Wu, Cohen & Spiegelman, 2013). At thermoneutrality beige fat is minimally present; however, its differentiation/proliferation can be induced by the same stimuli that induce BAT activity and proliferation, i.e. cold exposure or beta 3-adrenoreceptor agonism (Young, Arch & Ashwell, 1984; Loncar, Afzelius & Cannon, 1988*a*,*1988b*; Cousin *et al.*, 1992). In fact, “beiging” can compensate for decreased brown fat activity or in other conditions where thermogenic needs are not met (Xue *et al.*, 2007; Schulz *et al.*, 2013). Excitement has been generated in this field, not only because of its recruitable nature, but because human brown fat has a molecular signature more closely resembling rodent beige fat than classical brown fat, making it an attractive therapeutic target (Wu *et al.*, 2012).

Compared to brown fat, the developmental origin(s) of beige fat are less well defined. Though brown fat and beige fat are functionally similar, they do not originate from the same lineage; beige fat arises from a Myf5-negative lineage (Fig. 4A) (Sanchez-Gurmaches *et al.*, 2012). Interestingly, Long *et al.* (2014) recently showed that beige fat also develops from a muscle origin, that is, from smooth-muscle-(like) precursors. The precise cellular lineage(s) and developmental factors regulating beige fat specification, however, are yet to be elucidated.

V. THE RECRUITMENT OF CA^{2+}-CYCLING IN MUSCLE AS A NST MECHANISM

For muscle to be an effective site of NST, one or more of the processes present in muscle must be recruitable and adaptable to heat generation in the absence of a muscle contraction. However, it would be disadvantageous to muscle function and energetics to evolve an additional machinery devoted entirely to NST. There are published accounts that indicate a role for all three muscle ATPases as well as metabolic/mitochondrial heat in muscle-based NST (Else & Hulbert, 1987; Hulbert & Else, 1989, 1999; Kelly & McBride, 1990; Couture & Hulbert, 1995; Cadenas *et al.*, 2000; Toyomizu *et al.*, 2002; Fernstrom, Tonkonogi & Sahlin, 2004; Shabalina *et al.*, 2006; Cooke, 2011). However, the evidence for SERCA in NST is much more convincing than that for the myosin ATPase, Na^+/K^+ ATPase, or mitochondria alone. In fact, SERCA has three unique properties that would permit NST without affecting muscle function: (*i*) SERCA is abundant in muscle (Vangheluwe *et al.*, 2005; de Jonge *et al.*, 2006; Babu *et al.*, 2007; Periasamy & Kalyanasundaram, 2007; Kinnunen & Manttari, 2012; Fajardo *et al.*, 2013; Lamboley *et al.*, 2013), therefore, a portion of the SERCA population can be recruited without affecting muscle function, (*ii*) the energy liberated as heat from SERCA can be increased or decreased depending on the cellular conditions (de Meis, 2001, 2002; de Meis *et al.*, 2005; Arruda *et al.*, 2008,2007; Kjelstrup *et al.*, 2008; Mahmmoud, 2008; Mahmmoud & Gaster, 2012), and (*iii*) the Ca^{2+}-uptake function of SERCA can be uncoupled from ATP hydrolysis by its regulatory partner, sarcolipin (SLN) (Smith *et al.*, 2002; Mall *et al.*, 2006; Bal *et al.*, 2012; Bombardier *et al.*, 2013*b*; Gorski *et al.*, 2013; Inesi & Tadini-Buoninsegni, 2013; Sahoo *et al.*, 2013; Toyoshima & Cornelius, 2013; Toyoshima *et al.*, 2013; Winther *et al.*, 2013).

(1) SERCA function and heat production

A considerable amount of data has been published concerning the molecular and biochemical basis of NST in rabbits. Most work hinges on the unifying theme that Ca^{2+} cycling in muscle is the major thermogenic mechanism in rabbits, and by extrapolation, other large mammals where BAT is not a major thermogenic organ. In particular, ATP hydrolysis by SERCA is considered to be the dominant heat producer (de Meis *et al.*, 2005; Arruda *et al.*, 2007; Kjelstrup *et al.*, 2008). When activated by high cytosolic Ca^{2+} levels, as during excitation–contraction coupling, SERCA pumps Ca^{2+} into the SR, using the energy derived from ATP hydrolysis to restore the SR load. In the optimal “coupled” state, two Ca^{2+} ions are transported per ATP hydrolysed (Lytton *et al.*, 1992; Lee & East, 2001; Lee, 2002; Periasamy & Kalyanasundaram, 2007; Periasamy, Bhupathy & Babu, 2008). However, SERCA has a unique ability to become “uncoupled,” a state in which fewer (than

two) Ca^{2+} ions are transported per ATP and the remaining energy from ATP hydrolysis is transformed into heat (Yu & Inesi, 1995; de Meis, 2001). Interestingly, when rabbits are acclimatized to cold, SERCA1 expression is increased in red muscle (SERCA2 levels are unaffected) but not in white muscle (Arruda *et al.*, 2008). Furthermore, *in vitro* preparations of these cold-acclimatized muscles show that cold exposure increased the heat released during ATP hydrolysis twofold in red muscle, where oxidative (mitochondrial) capacity is at least twofold greater than white muscle. Thus, cold exposure increased the heat-generating capacity of rabbit red muscle (Arruda *et al.*, 2008). These results are intriguingly in agreement with those obtained on cold-exposed birds.

(2) Sarcolipin (SLN)-mediated thermogenesis is important for cold- and diet-induced thermogenesis

Despite suggestions that skeletal muscle could be recruited in NST, the mechanistic basis for muscle-based NST has been poorly understood. Recent studies have identified SLN, a regulator of SERCA, as an important player in muscle-based thermogenesis (Tupling *et al.*, 2011; Bal *et al.*, 2012; Yuan *et al.*, 2012; Bombardier *et al.*, 2013*a*,*b*; Gorski *et al.*, 2013; Sahoo *et al.*, 2013). SLN is a 31 amino acid single transmembrane protein that co-localizes with the SERCA pump in the SR of skeletal and cardiac muscles. When bound to SERCA, SLN can uncouple SERCA from Ca^{2+} transport, and as a result can promote futile cycling of SERCA, increasing ATP hydrolysis and heat production (Smith *et al.*, 2002; Mall *et al.*, 2006). This concept was examined using genetically altered mouse models lacking SLN (Bal *et al.*, 2012). This study demonstrated that loss of SLN renders mice cold-sensitive (when interscapular BAT is ablated), but this cold sensitivity can be rescued when SLN is re-expressed. Interestingly, when fed on a high-fat diet, SLN-null mice developed obesity, while control wild-type mice were less obese but upregulated SLN expression in muscle, which suggested that SLN-based thermogenesis is also important for diet-induced thermogenesis (Bal *et al.*, 2012). In addition, recent structure/function studies suggest that SLN interacts with SERCA in a Ca^{2+}-dependent manner. The binding of SLN to SERCA is unique in that SLN remains bound to SERCA throughout the Ca^{2+} transport cycle and thus can result in uncoupling of SERCA (Sahoo *et al.*, 2013). These data collectively suggest that the SLN/SERCA interaction could be the basis for increased heat production in muscle.

VI. SARCOLIPIN: THE MISSING LINK FOR SKELETAL-MUSCLE-BASED NST

In rodents, SLN is expressed in a developmental and tissue-specific manner. Protein expression is high in skeletal muscles during late gestation and at birth, then becomes gradually restricted to oxidative muscle fibres in adulthood (Babu *et al.*, 2007). UCP1 expression follows the opposite pattern, with expression beginning at birth and remaining prominent in adulthood (Giralt *et al.*, 1990). In adult rodents, SLN expression is predominant in slow-twitch, oxidative muscles, including the soleus and diaphragm, and in small amounts in the tibialis anterior, extensor digitorum longus, and red portion of the gastrocnemius and quadriceps (Vangheluwe *et al.*, 2005; Babu *et al.*, 2007). Given its putative role in thermogenesis, it is not surprising that SLN is restricted to slow, oxidative muscles in rodents where thermogenesis would most likely occur. In striking contrast is the expression profile of SLN in adult large mammals, where oxidative muscle predominates,

coinciding with the reduction in BAT content in adulthood. In rabbits, SLN expression is relatively high in all skeletal muscles thus far examined (Fig. 5B); similar results were found in dog and human skeletal muscle (Fig. 5C). These data suggest that SLN plays a more dominant role in large mammals compared to rodents. In support of this, recent studies showed increased uncoupling of SERCA activity in rabbit red muscle after cold adaptation, which was associated with the induction of SERCA1 (de Meis, 2001; Arruda *et al.*, 2003, 2008). In rabbits, SLN expression correlates highly with SERCA1a expression (Fig. 5B). Therefore, it is very likely that SLN was also upregulated after cold exposure and is responsible for this increased uncoupling. However, because this study was performed before SLN was implicated in NST, SLN expression or function was not determined.

Evidence for SLN-mediated thermogenesis in muscle throughout vertebrates can be found by analysing SLN gene/protein structure; SLN sequences are highly conserved across species from fish to mammals (Fig. 5A). Such high conservation among species indicates that SLN may have played a critical role in the evolution of thermogenesis and remains conserved in animals that rely heavily on skeletal muscle thermogenesis. However, to support the energetic requirements of SLN-based thermogenesis, substantial skeletal muscle oxidative capacity is necessary. Therefore, SLN-based thermogenesis must have evolved in concert with the evolution of fast muscles with high oxidative capacity, a trait that probably evolved in the ancestral lineages that gave rise to homeothermic vertebrates (birds and mammals, see Fig. 2).

Based on these new observations and careful re-analysis of the existing data, we now propose a new model for the major thermogenic components of endotherms and their relative contributions (Fig. 5D). In adult non-hibernating mammals, including humans, where UCP1 and BAT are limited, SLN is the dominant source of thermogenesis. By contrast, in species where UCP1 and BAT are abundant, the contribution of SLN to thermogenesis is secondary to that of BAT.

VII. CONCLUSIONS

1. We have attempted to provide a critical analysis of the origin and evolution of thermogenic mechanisms in vertebrates, in particular, the contributions of BAT and muscle to NST. Our analyses suggest that skeletal-muscle-based thermogenesis preceded BAT during evolution, with BAT not evolving until the appearance of placental mammals. Since the divergence of placental mammals occurred after the appearance of endothermy in vertebrate evolution, this suggests that another thermogenic process must have been present prior to this diversification. Here, we provide a thorough analysis of the literature to show that skeletal muscle was likely this thermogenic mechanism. We provide data to show that even when BAT is present muscle is still a dominant thermogenic organ.
2. However, there remain important gaps in our knowledge with regard to the role of each of these mechanisms in different mammalian species. Although existing data suggest that both these mechanisms are recruited, the unique roles of each of these mechanisms in acute cold exposure *versus* gradual cold adaptation remain unclear.

Why certain species evolved a preferential utilization of BAT *versus* muscle or *vice versa*, why BAT evolved in some species but not others (birds, marsupials, etc.) and whether either mechanism can compensate for the loss of the other remains unknown.

3. Our sequence comparison reveals that SLN is highly conserved from fish to man, suggesting that SLN could potentially be involved in thermogenesis in non-mammalian endotherms. However, it remains to be determined if SLN has the same function in all vertebrates. In addition, relatively little information is available concerning thermogenic mechanisms in avian species, especially regarding the role of SLN in avian skeletal muscle. Although putative sequences have been identified in many bird and fish species, expression of SLN mRNA or protein has not been investigated. The most promising evidence, so far, has been the identification of SLN-like mRNA sequences in a chicken expressed sequence tag (EST) (BX935884.1) and catfish EST (AF227818.1), both from muscle, although experimental confirmation is lacking.

4. Unfortunately, the answers to many of these questions regarding the contribution of muscle to the evolution of endothermy lie within extinct species, particularly those that were present at different diversification events. The challenge now relies on the ability of researchers to integrate studies from diverse extant species and fossil records to begin to understand some of these answers. An ongoing challenge for researchers studying skeletal muscle thermogenesis has been the delineation of heat production originating from contraction *versus* NST processes. Therefore, new methodologies to localize and capture thermogenesis more precisely *in vivo*, particularly skeletal muscle-based NST, must be developed.

5. With an increase in obesity worldwide, there is a significant interest in thermogenic mechanisms as a strategy to increase energy expenditure in humans. With skeletal muscle being a major consumer of metabolites, activation of NST could be an effective strategy to increase energy expenditure. However, the mechanism(s) by which muscle-based NST is activated and recruited is not known, including whether this is under the regulation of hormonal and/or neuronal circuits. Because skeletal muscle contractile activity is primarily activated through nervous stimulation, whether muscle-based NST could be recruited through this mechanism remains to be understood. Some studies suggest a potential role for hormonal regulation of NST in muscle: observations that dogs with denervated skeletal muscle can maintain NST suggests that hormonal factors are involved in the recruitment of NST (Davis, 1967). Other studies have also pointed to various neurohormonal factors in the regulation of NST, including catecholamines, thyroid hormones, glucagon, etc. (Barre, Cohen-Adad & Rouanet, 1987; Eldershaw *et al.*, 1997; Marmonier *et al.*, 1997; Thomas & Palmiter, 1997; Rose *et al.*, 1999; Arruda *et al.*, 2003, 2008; Silva, 2006).

6. The detailed molecular mechanisms of the SLN–SERCA interaction, including uncoupling of SERCA leading to heat production is not well understood, nor are the signalling mechanisms, including selective activation of Ca^{2+} cycling, that

promote SLN-based heat production. We believe that understanding the detailed molecular basis of thermogenesis has important clinical implications in regulating whole-body metabolism and obesity.

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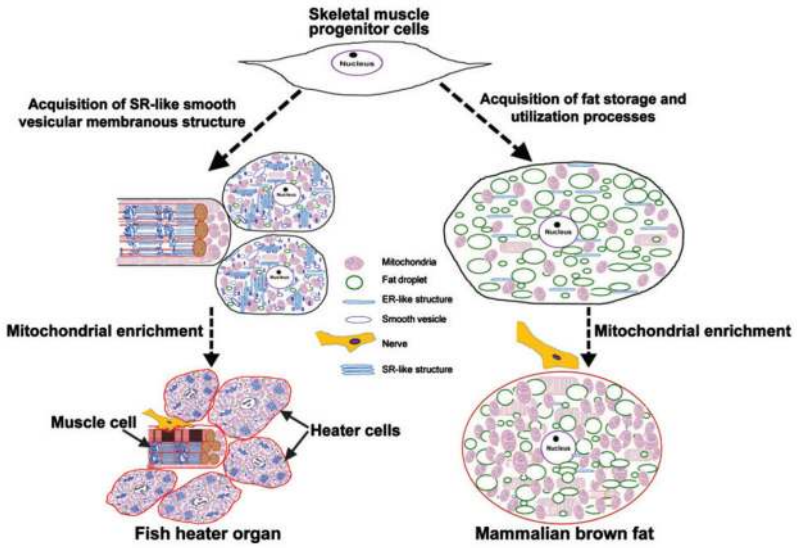

Fig. 1.

Modification of the skeletal muscle lineage during evolution to acquire a non-shivering thermogenic function. Electron microscopy of the heater organ from various fishes has shown that the sarcoplasmic reticulum (SR) network [with SR Ca^{2+}-ATPase (SERCA), Ca^{2+} release channel (CRC) and Ca^{2+}-buffering proteins] and mitochondria act in partnership to produce heat (Block & Franzini-Armstrong, 1988; O'Brien & Block, 1996; Londraville *et al.*, 2000; Morrissette *et al.*, 2003). This suggests that during evolution, skeletal muscle progenitor cells must have adapted by decreasing the expression of myofibrillar proteins to give rise to a new cell type that ultimately became heater cells of the fish heater organ. It can be speculated that among mammals, brown adipose tissue (BAT), which is functionally analogous to the fish heater organ, followed a similar evolutionary path. By acquiring more prominent fatty acid uptake, storage, and utilization processes and mitochondrial enrichment, a skeletal-muscle-like progenitor cell could have evolved into a BAT-progenitor cell type that would ultimately become modern (eutherian) BAT cells. Recent findings that skeletal muscle and BAT share a common cellular origin (see Fig. 4) provide support for this scenario. ER, endoplasmic reticulum.

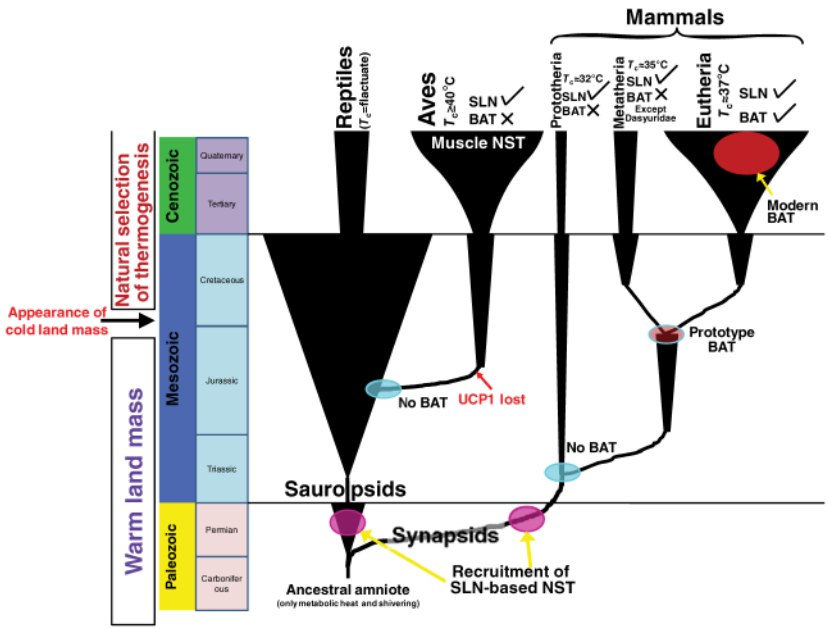

Fig. 2.

Proposed scheme illustrating the evolution of brown adipose tissue (BAT) and muscle-based thermogenesis that contributed to vertebrate endothermic homeothermy. The common ancestor of amniotes must have been bradymetabolic with metabolic heat (with or without the ability to shiver) as the only source of endothermic heat production. With the appearance of an erect posture (which appeared independently in many sauropsid and synapsid lineages in the mid-to-late Permian) among early amniotes, continuously recruited postural muscles would have provided a feasible molecular environment for sarcolipin (SLN)-based muscle non-shivering thermogenesis (NST) to be recruited. Many of the sauropsid lineages relied on muscle hyperplasia (birds and some dinosaurs) (Newman, 2011), which would have had a twofold benefit: bigger muscles that equated to faster locomotion and better chase/escape ability as well as greater cumulative heat production. An aerial mode of locomotion (i.e. flying) is accompanied by muscle hyperplasia, eliminating the need for additional thermogenic mechanisms. The synapsids took a different route to homeothermy that did not rely primarily on muscle hyperplasia. BAT-based NST has largely been attributed to a eutherian innovation that enabled utilization of uncoupling protein 1 (UCP1) for heat production. However, recent findings have identified the presence of a primitive BAT in a single metatherian species. According to palaeontological evidence, the divergence between Metatheria and Eutheria took place in the Late Jurassic, suggesting that UCP1 presumably was not recruited for thermogenesis until just before metatherian/eutherian divergence. Hence, SLN and UCP1 must have been recruited for thermogenesis at very different time frames in the evolutionary history of vertebrates. The evolution of BAT among mammals also overlaps the evolution of viviparity, which occurred between the prototherian–therian and metatherian–eutherian divergence events. It is interesting to note that birds and monotremes (that lack BAT) are completely oviparous, while only therian mammals (that have BAT) are viviparous. Therefore, BAT may have aided the survival of viviparous newborns, where skeletal muscle is often not fully developed at birth. However, the role of

BAT (which can influence the energy trade-off between the neonate and parent) in the evolution of viviparity has not been studied.

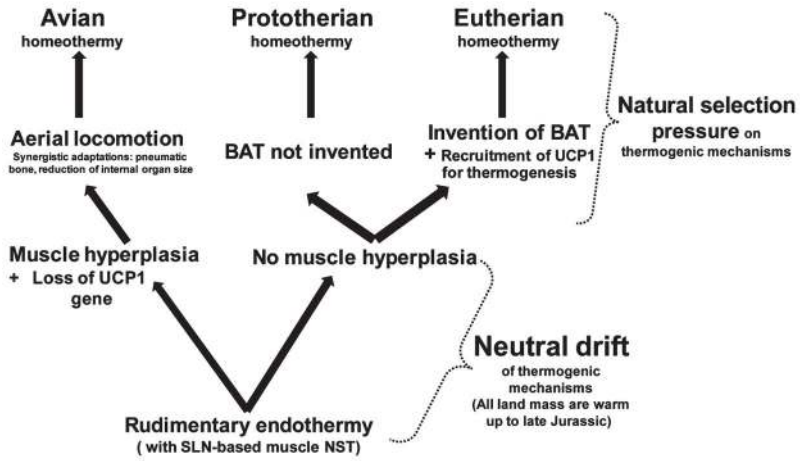

Fig. 3.

The evolution of two routes of vertebrate homeothermy in mammals. The common ancestor of synapsids and sauropsids must have recruited sarcolipin (SLN)-based muscle non-shivering thermogenesis (NST) achieving a rudimentary level of endothermy. However, for muscle-based NST to be used to achieve homeothermy, the muscle mass to body mass ratio would have to be increased, which only birds achieved. Evolution of thermogenic mechanisms might not have had a selective evolutionary advantage until the appearance of colder climatic niches in the Cretaceous; under this scenario most endothermic mechanisms would have been evolving more or less under neutral drift (because a minimal thermogenic need can be achieved by sustaining an elevated metabolic rate). Ancestral mammals (the common ancestor of Metatheria and Eutheria) evolved brown adipose tissue (BAT) possibly in the Jurassic; the Prototheria do not have this tissue. Thereafter, mammals possessing two adaptive thermogenic processes will have utilized and fine-tuned them according to the demands of their particular environmental/ecological niche.

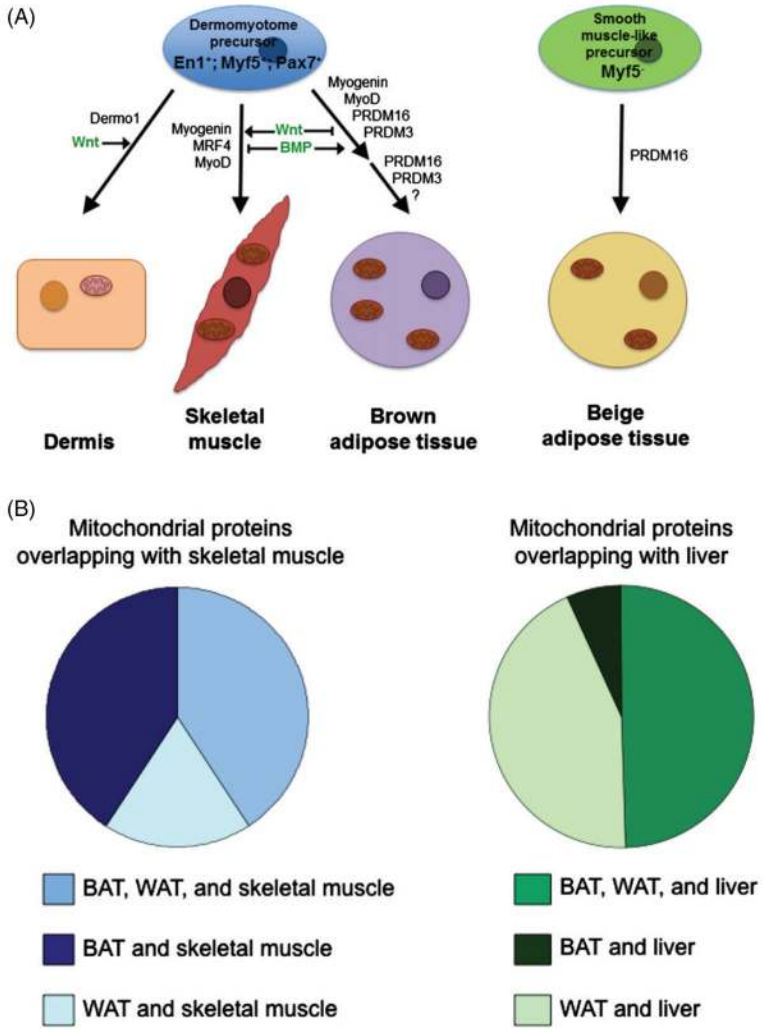

Fig. 4.

Skeletal muscle and brown adipose tissue share a common cellular lineage. (A) Lineage tracing analyses show that En1, Pax7, and Myf5-expressing cells of the dermomyotome of the paraxial mesoderm are tripotent, giving rise to dermis, skeletal muscle, and brown adipose tissue. Dermis develops from the dermomyotome near the surface ectoderm, and its differentiation is dependent on the expression of Dermo1 and Wnt signalling from the ectoderm (Atit *et al.*, 2006; Gensch *et al.*, 2008; Lepper & Fan, 2010). Skeletal muscle and brown adipose tissue develop from the medial dermomyotome. Skeletal muscle differentiation is initiated by the sequential expression of myogenin, myogenic regulatory factor (MRF4), and myogenic determination factor (MyoD) and by spatiotemporal Wnt signalling from the ectoderm and neural tube (Francetic & Li, 2011; von Maltzahn *et al.*, 2012). Early brown adipose tissue precursors express the myogenic transcription factors myogenin and MyoD but downregulate them upon terminal differentiation (Timmons *et al.*, 2007). Brown fat specification is regulated by expression of PR domain-containing 16 (PRDM16), or the functionally redundant PRDM3 (Harms *et al.*, 2014), and BMP (4 and 7) signalling further directs brown fat differentiation by inhibiting MyoD and Myf5 expression

and promoting adipogenesis (Kajimura, Seale & Spiegelman, 2010; Francetic & Li, 2011). Furthermore, Wnt signalling inhibits adipogenesis, promoting skeletal muscle specification (von Maltzahn *et al.*, 2012). Due to common transcription factors (myogenin and MyoD) found in early brown fat and skeletal muscle precursors, it seems possible that the dermomyotome initially gives rise to two cell types rather than three: dermal precursors and a common skeletal muscle/brown fat precursor, which further differentiates into skeletal muscle and brown fat. Details of this process have not been elucidated. The development of beige adipose and white adipose tissue is much less clear. Beige adipose tissue develops from a separate lineage to skeletal muscle and classical brown adipose tissue. Beige fat arises from Myf5-negative precursors of the smooth muscle lineage, and its differentiation is directed by PRDM16 (Cohen *et al.*, 2014; Long *et al.*, 2014). Because smooth muscle develops from heterogeneous cellular lineages (i.e. neural crest, proepicardium, mesothelium, etc.), beige fat may have multiple developmental origins that are depot-specific (Majesky, 2007; Long *et al.*, 2014). White adipose tissue also has diverse cellular origins, developing from ectoderm and mesoderm, including neural crest (ectoderm), mesenchyme (lateral mesoderm), dermis, etc. (Billon & Dani, 2012; Wojciechowicz *et al.*, 2013). (B) Mitochondria are the energetic workhorses of cells; therefore, their properties match the metabolic requirements and/or demand of the cell type. The mitochondrial proteomes of brown adipose tissue (BAT) and white adipose tissue (WAT) were compared to the mitochondrial proteomes of skeletal muscle and liver. A large portion of the proteomes of WAT and BAT overlapped, which mostly included proteins integral to basic mitochondrial structure and function. The mitochondrial proteome of BAT closely resembles that of skeletal muscle. Both are enriched in proteins involved in catabolic processes such as lipolysis, fatty acid metabolism, citric acid cycle, etc. On the other hand, WAT mitochondria share a similar mitochondrial protein profile with liver. WAT mitochondria, in contrast to BAT mitochondria, are specialized for anabolic functions including lipogenesis and detoxification processes. Adapted from Forner *et al.* (2009)

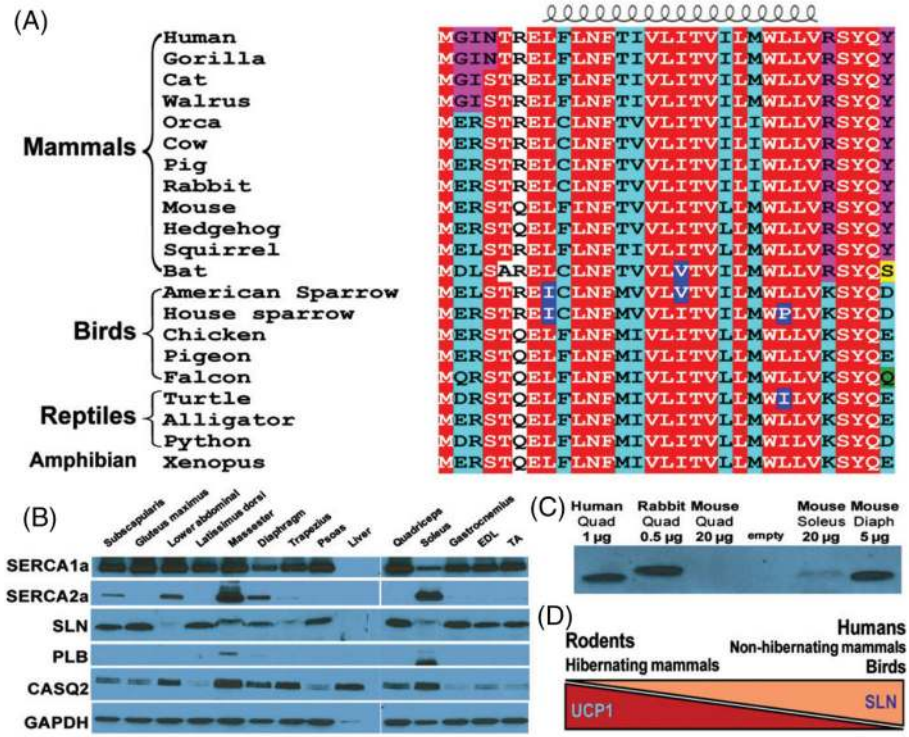

Fig. 5.

Sarcolipin (SLN) is evolutionarily conserved and is abundant in large mammals. (A) Alignment of SLN protein sequences from various vertebrates. SLN is highly conserved among vertebrate species. Importantly, the transmembrane and C-terminal domains, responsible for inhibition of sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA), are remarkably conserved from amphibians to humans. The N-terminal region has unique conservation among different vertebrate groups; however, the physiological relevance of these unique residues has yet to be studied. All sequences were obtained from GenBank. The house sparrow (*Passer domesticus*) sequence was obtained, by our laboratory (L.A. Rowland and N.C. Bal), by DNA cloning from the pectoralis muscle and is highly homologous to the published SLN sequence of the American sparrow (*Zonotrichia albicollis*). The function of SLN in avian species is yet to be investigated. (B) Analyses of SLN and SERCA expression in rabbit skeletal muscle. SLN is abundantly expressed in most skeletal muscle tissues, whereas the homologous protein, phospholamban (PLB), is only found in slow muscles. CASQ2, calsequestrin; EDL, extensor digitorum longus; TA, tibialis anterior. (C) Expression of SLN in large mammals, including humans and dogs (Vangheluwe *et al.*, 2005; Babu *et al.*, 2007), is significantly greater than in rodents. SLN protein is detectable in 0.5 and 1.0 µg of rabbit and human quadriceps muscles, respectively, but is not detectable in 20 µg mouse quadriceps. In mouse muscles where SLN protein is detectable (soleus and diaphragm), total SLN content is still significantly lower than in rabbit and human muscle. Quad, quadriceps; Diaph, diaphragm. (D) Proposed model showing relative contributions of uncoupling protein 1 (UCP1) and SLN to thermogenesis in birds and mammals. We propose that in adult humans, birds, and other non-hibernating mammals, SLN-based muscle thermogenesis constitutes the largest component of thermogenesis,

whereas in rodents and hibernating mammals, UCP1 (BAT) is the dominant heat producer. Tissues used to generate (B) and (C) were approved by the institutional animal care committee and institutional review board.

Table 1

Characteristics and compositions of tissues contributing to thermogenesis in extant endotherms

	Eutherian mammals		
Characteristic	**Small**	**Large**	**Birds**
BAT (volume)	High (~16% body mass) *	Low (scant to <1%) *	None
SLN expression	Low, restricted to slow-twitch muscles	High, present in most/all muscles	Unknown
Muscle composition (fibre type and mitochondrial abundance)	Partitioned fibre types; restricted muscles with abundant mitochondria; preponderance of Type IIB fibres; lesser reliance on postural muscles	Muscles are mostly mixed with high mitochondrial number; considerable reliance on postural muscles; Type IIB fibres minimal or absent	Most muscles rich in mitochondria
Body temperature	~37°C	~37–38°C	38–42°C
Surface area to volume ratio	High	Low	High
Physical activity	Usually bursts of activity (occupy small ecological space)	High range of activity (occupy large ecological space)	Long range of activity (occupy large ecological space)

BAT, brown adipose tissue; SLN, sarcolipin.

* Adapted from Frontini & Cinti (2010) and Virtanen & Nuutila (2011).