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Marsupial uncoupling protein 1 sheds light on the evolution of mammalian nonshivering thermogenesis

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Jastroch M, Withers KW, Taudien S, Frappell PB, Helwig M, Fromme T, Hirschberg V, Heldmaier G, McAllan BM, Firth BT, Burmester T, Platzer M, Klingenspor M. Marsupial uncoupling protein 1 sheds light on the evolution of mammalian nonshivering thermogenesis. *Physiol Genomics* 32: 161–169, 2008. First published October 30, 2007; doi:10.1152/physiolgenomics.00183.2007.— Brown adipose tissue expressing uncoupling protein 1 (UCP1) is responsible for adaptive nonshivering thermogenesis giving eutherian mammals crucial advantage to survive the cold. The emergence of this thermogenic organ during mammalian evolution remained unknown as the identification of UCP1 in marsupials failed so far. Here, we unequivocally identify the marsupial *UCP1* ortholog in a genomic library of *Monodelphis domestica*. In South American and Australian marsupials, *UCP1* is exclusively expressed in distinct adipose tissue sites and appears to be recruited by cold exposure in the smallest species under investigation (*Sminthopsis crassicaudata*). Our data suggest that an archetypal brown adipose tissue was present at least 150 million yr ago allowing early mammals to produce endogenous heat in the cold, without dependence on shivering and locomotor activity.

marsupials; brown adipose tissue; cold acclimation

THE EVOLUTION OF BROWN ADIPOSE TISSUE (BAT) and its thermogenic uncoupling protein 1 (UCP1) is of major interest in the understanding of successful mammalian radiation. Adaptive nonshivering thermogenesis generated in BAT enables small eutherian mammals to maintain high body temperature independent of daily and seasonal temperature fluctuations (7). Although BAT was first described in 1551 (16), its thermogenic role was not recognized until the 1960s (10, 48), and it is now established that BAT contributes significantly to adaptive nonshivering thermogenesis of rodents, hibernators, and newborns (7). During cold exposure, sympathetic norepinephrine release activates BAT by stimulation of lipolysis and futile

UCP1-dependent mitochondrial respiration, and recruitment of oxidative capacity. UCP1, a mitochondrial carrier protein, is located in the inner membrane of BAT mitochondria and provides the molecular basis for nonshivering thermogenesis (36). The protein increases proton conductance and uncouples oxidative phosphorylation from ATP synthesis by dissipating proton motive force as heat. All eutherian species investigated so far possess UCP1, with the exception of pigs where a naturally disrupted *UCP1* gene results in poor thermoregulation and sensitivity to cold exposure (3). The observation that UCP1-knockout mice are unable to defend their body temperature when exposed to the cold (17) confirms that UCP1 is crucial for adaptive nonshivering thermogenesis. In contrast to previous expectations, an ancient UCP1 ortholog was identified in the ectothermic teleost fish, but it is not expressed in adipose tissue and the physiological function might be other than heat production (28).

Marsupials are proficient thermoregulators and are capable of defending a stable body temperature during cold exposure (11, 15, 46). Evidence for nonshivering thermogenesis is a matter of debate in marsupial mammals, which separated from eutherians about 150 million yr ago (4). Since BAT is innervated by the sympathetic nervous system, norepinephrine released endogenously or injected into the animal leads to an increase in metabolic rate that is generally interpreted as a thermogenic response. In macropods the injection of norepinephrine led to an increase in thermogenesis (35, 37), a response attributed to skeletal muscle and not to BAT as found for eutherians (38, 52). It was suggested that adaptive nonshivering thermogenesis may be of major importance in Australian dasyurids as they belong to the smallest marsupials. Indeed, a thermogenic response to norepinephrine has been observed in *Sminthopsis crassicaudata* acclimated at 24°C (9). No thermogenic response to norepinephrine, however, was observed in *Antechinus stuartii* (41), nor in South American marsupials (12, 40). Despite evidence for nonshivering thermogenesis in some marsupials, no study has demonstrated the molecular basis nor the presence of adaptive nonshivering thermogenesis in response to cold.

Nonshivering thermogenesis in eutherians is usually associated with BAT, but the presence of this specialized adipose organ remains controversial in marsupials. Morphological studies revealed BAT characteristics like multilocular fat droplets and vascularization in the interscapular adipose tissue of Bennett's wallaby pouch young (*Macropus rufogriseus rufogriseus*) (35). Another study investigating 38 different marsupial and one monotreme species precluded the presence of BAT in marsupials (18), by pointing out that morphological features of BAT also occurred in white adipose tissue during cold stress (33, 34). Reliance on morphological features only has led to the erroneous conclusion that birds possess BAT (39).

The discovery of UCP1 (19, 42) and the cloning of the cDNA sequence in rodents (2, 5) have stimulated work to identify UCP1 and its genomic presence in marsupials. Weak UCP1-like immunoreactivity has been seen in the interscapular fat deposit of *S. crassicaudata* (22); however, it is generally accepted that UCP1 antibodies cross-react with other mitochondrial carriers (43) or UCP2/3, both of which have been recently identified in marsupials (27). Previous studies suggested that UCP2/3 do not compensate for the lack of nonshivering thermogenesis mediated by UCP1, suggesting physiological roles other than heat production (17, 27).

Unequivocal detection of marsupial *UCP1* requires genomic or gene transcript sequence data. However, several attempts to identify the *UCP1* sequence have failed so far (27, 30, 31, 45). In this study we searched for the presence of BAT and UCP1 in one South American (*Monodelphis domestica*) and two Australian marsupial species (*S. crassicaudata* and *Antechinus flavipes*). Our approach was to search the genomic trace archives for UCP-like sequence fragments of *M. domestica* and characterize their physiological function.

MATERIALS AND METHODS

Isolation of genomic DNA of M. domestica and polymerase chain reactions. DNA was isolated from a tail tip of a female adult *M. domestica* using a standard phenol-chloroform extraction protocol as described previously (27). Following extraction, 50 ng of photometrically quantified DNA was used in subsequent polymerase chain reactions (PCR).

To define specific primers, we initially searched the *M. domestica* whole genome shotgun data provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Traces/trace.fcgi) for UCP-like sequence fragments using a consensus UCP1 coding sequence deduced from available eutherian sequences. The fragments were assembled according to the intron-exon structure of mouse *Ucp1*. Using the obtained trace alignments for primer definition, we generated primers (MWG Biotech, Ebersberg, Germany) to amplify a *UCP1*-like fragment ("forEx3": 5′-AGTGGCACAGCCTA-CAGATGT-3′; "revEx4": 5′-CTTGGAACGTCATC ATGTTTG-3′).

A second primer pair was deduced from *M. domestica* fragments displaying high identity to UCP2 ("forUCP2": 5′-GCCTACAAGAC-TATTGCCCGAGAGGAG-3′; "revUCP2": 5′-AAGCGGAGAAAG-GAAGGCATGAACCC-3′).

Following 40 cycles of denaturation at 94°C for 1 min, annealing at 54°C (or 58°C for UCP2) for 1 min and extension at 72°C for 1 min (2 min for UCP2) were performed. A final extension at 72°C was applied for 10 min followed by rapid cooling to 4°C. The PCR product was gel-purified and ligated into a pGEMT-easy vector (Promega) for sequencing. Nested oligonucleotides were used for the screening of a genomic *M. domestica* bacterial artificial chromosome (BAC) library.

Sequence analysis of opossum BAC clones. High-density arrayed grids of the genomic opossum (*M. domestica*) BAC library VMRC-6 (Virginia Mason Research Center, distributed by BACPAC Resources, Oakland, CA; http://bacpac.chori.org) were screened by hybridization with radioactively end-labeled (T4-Polynucleotidekinase, Roche) oligonucleotides (13):

The *M. domestica* UCP1 specific primers were: md1.F 5′-GG-GACTTTCCATGCCTACAA-3′, md2.R 5′-CAATAGCATTTCTTGC-CACG-3′, md3.F 5′-AATAGCATCCGCAGAAGGAA-3′, md4.R 5′-CGTCCCTGGAAAGAGGAAAT-3′, and the *M. domestica* UCP2 specific primers were: md5.F 5′-CTCTTGCAGGTGGCATCC-3′, md6.R 5′-GACATTGGGCGAAGTTCCT-3′.

The identified BACs were verified by PCR using the probe oligos as primers. BACs VMRC6-66F14 (GenBank acc. no. AC171738, containing md*UCP1*) and VMRC6-60O3 (GenBank acc. no. AC171737, containing md1*UCP2* and md*UCP3*) were sequenced by a combination of shotgun and directed approaches (50). Base calling and assembly were performed by Phred/Phrap. Finishing was performed in accordance to the Human Genome Project standards with the support of external *M. domestica* whole genome shotgun data (http://www.ncbi.nlm.nih.gov/Traces/trace.fcgi).

Phylogenetic inference. The coding and amino acid sequences of *M. domestica* UCP1, UCP2, and UCP3 were deduced from the corresponding genes. A comprehensive search for UCP sequences was performed in public databases (Ensembl genome browser, www.ensembl.org/NCBI, www.ncbi.nlm.nih.gov) by employing the basic local alignment search tool algorithm (1). An alignment of the UCP amino acid sequences was generated using ClustalX 1.81 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and adjusted by eye. Bayesian phylogenetic analyses were performed employing MrBayes 3.1.2 (http://mrbayes.csit.fsu.edu/) (44). The WAG model of amino acid substitution (51) with gamma distribution of rates was applied. Substitution rates were allowed to change across the tree under the covarion model (23). Prior probabilities for all trees were equal; starting trees were random. Two analyses were run in parallel for 1,000,000 generations. Trees were sampled every 100th generation, and posterior probabilities were estimated on the final 3,000 trees (burnin = 7,000). The tree was visualized using Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview). The branch lengths are mean branch lengths of the consensus tree representing substitution rates.

Animal care and experimental protocol. The gray short-tailed opossums (*M. domestica*) were kindly donated by P. Giere and U. Zeller (Museum für Naturkunde, Humboldt-Universität zu Berlin, Germany). The opossums were held individually in the animal facility at the Philipps-Universität Marburg on a 12-h/12-h light/dark cycle (12:12 L:D) at an ambient temperature (T_a) of 24°C ± 2°C given water and fed cat food, curd mixed with fruit, and insects ad libitum. For cold acclimation experiments, two individuals were transferred into a separate chamber maintained at 12°C for 14 days.

Seven yellow-footed Antechinus (*A. flavipes*) were captured with Elliott traps in several subtropical habitats in Southeast Queensland (Australia) between January and March 2005. Fourteen fat-tailed dunnarts (*S. crassicaudata*) were obtained from a breeding colony at La Trobe University, Melbourne. Both species were housed individually in the animal facility of the University of Southern Queensland (12:12 L:D, lights on at 0700 h) at a T_a of 24°C ± 2°C. To investigate the effect of cold acclimation, seven *S. crassicaudata* and four *A. flavipes* were transferred to a climate chamber adjusted to 10°C for 17–22 days, whereas the other individuals remained at 24°C. Animals were given water and fed mealworms and cat food mix including calcium carbonate and vitamins ad libitum.

For cytochrome *c* oxidase (COX) activity assays, 10 *S. crassicaudata* were acclimated to 14 and 28°C at the University of Adelaide animal holding facilities in November 1990.

Experimental protocols for the use of Australian marsupials were approved by the Animal Ethics Committee of the University of

Southern Queensland, Queensland Environmental Protection Agency (permit number WISP02633304) and Environment Australia (export number WT2005-12380). Animal experiments involving *M. domestica* were performed in accordance with the German Animal Welfare Laws.

Tissue dissection. Two 22- and 25-day-old *M. domestica* embryos were euthanized and immediately frozen on dry ice and stored at −70°C prior to cryosectioning. All other individuals of *M. domestica*, *S. crassicaudata*, and *A. flavipes* were euthanized (carbon dioxide), and tissues were dissected. The samples were immediately snap frozen in liquid nitrogen. Frozen tissue samples were stored at −70°C until use. Liver, skeletal muscle, and adipose tissue of *S. crassicaudata* and *A. flavipes* were shipped from Australia in liquid nitrogen to Marburg, Germany.

In situ hybridization of M. domestica embryos. Sagittal body sections (20 μm) were processed using a cryosectioning microtome (Leica CM 3050) and were transferred to precooled object slides. A riboprobe complementary to *M. domestica* UCP1 (225 bp; primers forEx3, revEx4) and UCP2 (350 bp; forUCP2, revUCP2) was generated from a linearized cloned cDNA. Radioactive riboprobes using [^{35}S]UTP (1–2 × 10^7 cpm ml^{-1}), pre- and posthybridization procedures were performed as described previously (26). Controls were performed by hybridizing sections with equal-length sense riboprobes of UCP1 and UCP2.

RNA isolation and reverse transcriptase-PCR. Total RNA was isolated with TRIzol (GIBCO-BRL) according to the manufacturer's protocol. As an additional step, the RNA pellet was redissolved in a solution containing 6.3 mol/l guanidinium thiocyanate, 40 mmol/l sodium citrate pH 7, 0.8% sarcosyl, 8 mmol L^{-1} 2-mercaptoethanol, precipitated with 1 volume isopropanol, washed in 75% ethanol, and finally dissolved in DEPC-treated water. Total RNA was photometrically quantified at 260 nm and stored at −70°C. The isolated RNA was used for first strand cDNA synthesis (SUPERSCRIPT II, GIBCO/BRL) according to the manufacturer's protocol.

M. domestica UCP1 primers 5′-AGGTGAAGCCCAGACCATG-GAT-3′ and 5′-GGCTGACACAAAGTGGCAAGGT-3′, comprising 6.7 kb of the *UCP1* gene and resulting in 550 bp cDNA sequence, were subjected to PCR with cDNAs of selected tissues. We performed 40 cycles of 94°C (1 min), 59°C (1 min), and 72°C (1 min) and terminated them by a 10 min extension at 72°C. The PCR products were gel-purified and ligated into a pJET vector (Fermentas). The full coding sequence of *S. crassicaudata* UCP1 including 5′- and 3′-untranslated region (UTR) was amplified using the smart RACE cDNA amplification kit (Clontech) combined with gene-specific primers deduced from *M. domestica* UCP13′-UTR:5′-CTACAGATGTG-GTGAAAGTCAGAC-3′ and 5′-UTR:5′-GGCTGACACAAAGTG-GCAAGGT-3′.

Subsequent sequencing was used to confirm the identity of the PCR products.

Northern blot analysis. RNA was separated by gel electrophoresis, transferred onto a nylon membrane, and hybridized as described previously (27). After hybridization, the blots were washed with 2× SSC/0.1% SDS for 20 min, 1× SSC/0.1% SDS for 10 min, 0.5× SSC/0.1% SDS for 10 min at room temperature, blots where then transferred to 0.1× SSC/0.1% SDS and washed for 10 min at 60°C. Signal intensities were then monitored by exposure to a PhosphorScreen (Molecular Dynamics). The hybridized probes were then detected by phosphor imaging (Storm 860, Molecular Dynamics), and signal intensities were quantified using ArrayVision 7.0 (Imaging Research). Ethidium bromide staining of total RNA served to normalize gel loading.

COX activity. COX activity of interscapular fat deposits of *S. crassicaudata* was measured polarographically at 25°C with a Hansa Tech oxygen electrode chamber as described previously (21, 32). These experiments were performed in 1990 after tissue transfer to Germany.

Statistical analysis. Values for COX activity and UCP1 mRNA are expressed as means ± SE. The Mann-Whitney *U*-test was applied for two-sample comparisons. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Identification of UCP1 in M. domestica and S. crassicaudata. Following our trace archive search for UCP-like sequence fragments, a 346 bp fragment was amplified from genomic DNA of *M. domestica* containing a putative 121 bp intron. The 225 bp partial coding sequence displayed highest identity to eutherian UCP1 (76%) but lower similarity to eutherian UCP2 and UCP3 (69%). A second fragment was amplified using UCP2 primers exhibiting high identity to UCP2 of *A. flavipes* (92%, summarized in supplement 1).[1] A genomic *M. domestica* BAC library was screened using homologous primers deduced from the cloned UCP fragments. The isolated BAC clones were sequenced, analyzed, and aligned to the human reference sequence (Fig. 1).

The UCP-like gene of BAC VMRC6-66F14 is flanked by highly conserved orthologs of human ELMOD2 and human TBC1D9 and thereby resembling the region syntenic to the human UCP1 locus at chromosome 4. The two UCP genes on BAC VMRC6-60O3 found in juxtaposition as human UCP2 and UCP3 on chromosome 11 and were also enclosed by the orthologs of human DNAJB13 and DKFZP586P0123. The conserved synteny of the loci in vertebrates unequivocally identified the three *M. domestica* genes as UCP1, 2, and 3. Therewith, VMRC6-66F14 (GenBank AC171738) contains the *M. domestica* UCP1 and VMRC6-60O3 (GenBank AC171737) the UCP2 and UCP3 orthologs. Compared with the corresponding human UCP orthologs, the deduced amino acid sequence of *M. domestica* UCP2 exhibited highest identity (91%, 95% similarity), followed by *M. domestica* UCP3 (82%, 90% similarity), and *M. domestica* UCP1 (65%, 77% similarity).

Primers amplifying the *M. domestica* cDNA were also used to amplify a 250 bp UCP1 cDNA fragment of *S. crassicaudata*. Using 5′- and 3′-RACE-PCR, we identified 1,386 bp of UCP1 transcript including the full coding sequence (GenBank acc. no. EF622232). An alignment of the *S. crassicaudata* UCP1 coding sequence showed highest identity with *M. domestica* UCP1 (92%) and lower identity to eutherian and marsupial UCP2 and UCP3 (70–75%) (supplement 2).

Although an ancestral UCP1 ortholog appears in the vertebrate lineage as early as the divergence of ray-finned and lobe-finned fish 420 million yr ago (28), UCP1 disappears during evolution in the bird lineage (e.g., the chicken genome, unpublished observation) and became inactivated in pigs among eutheria (3). Biochemical studies suggest that fish UCP1 is an uncoupling protein with broadly the same activatory and inhibitory characteristics as mammalian UCP1 (26). The physiological relevance of ancient UCP1 in fish liver, despite of protein activity similar to the mammalian ortholog, may be other than heat production. Significant thermogenic uncoupling activity not only requires the presence of UCP1-mediated proton translocation but also a high mitochondrial oxidative capacity to achieve sufficient uncoupled respiration

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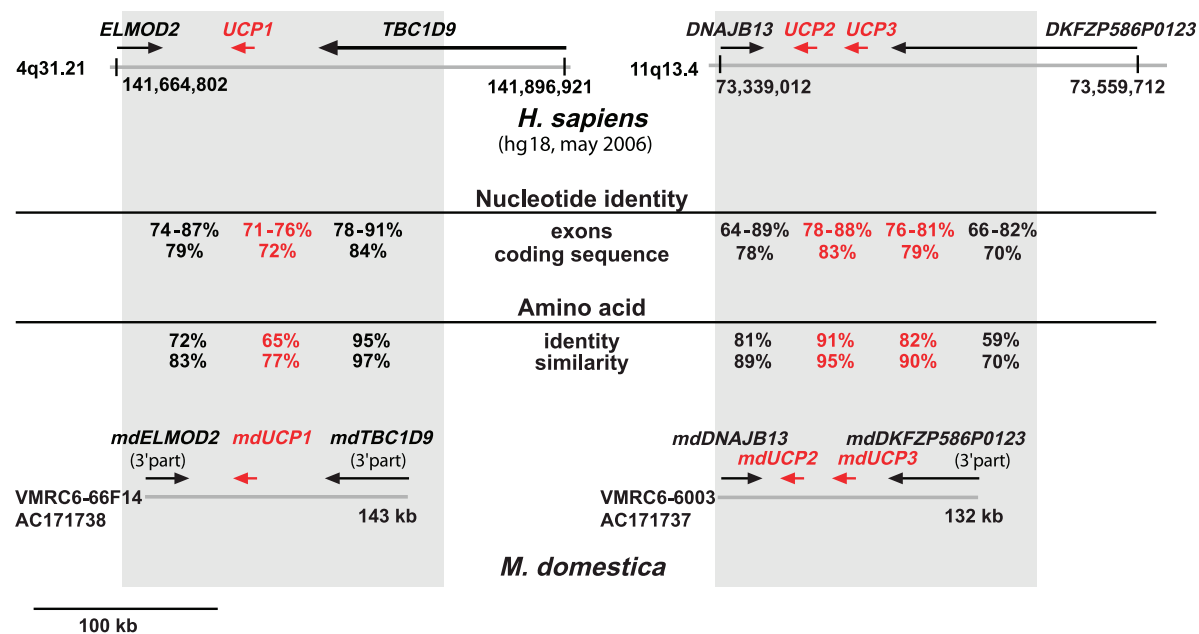[1] The online version of this article contains supplemental material.

Fig. 1. Conservation of uncoupling protein (*UCP*) *1*, *UCP2*, *UCP3* and their flanking genes between human and opossum (*Monodelphis domestica*). The *top* scheme illustrates the genomic organization of the human *UCP1, 2,* and *3* locus; the *bottom* scheme the opossum orthologs. Nucleotide and amino acid identities between the orthologs are compared in the *middle* panel.

leading to relevant heat dissipation. The low oxidative capacity in fish hepatocytes can only contribute marginally to heat production compared with thermogenic brown adipocytes. Although fish UCP1 may already catalyze proton translocation, we must assume that during mammalian evolution this function was improved by natural selection. Our demonstration of marsupial *UCP1* in the present study is not only important because previous studies failed to demonstrate *UCP1* in marsupials, but distinct differences in the coding sequences may represent mutation events that improved the proton translocation function of marsupial and eutherian UCP1 (see supplement 2). Further experiments directly comparing different UCP1 orthologs in test systems will clarify if the proton transport activity increased during evolution and identify the functional residues.

Phylogenetic inference. For classification of the UCP sequences from *M. domestica* and *S. crassicaudata*, we generated a phylogenetic tree by a Bayesian method (24). Our comprehensive search for UCP sequences in public databases revealed 80 UCPs in the animal kingdom. The addition of further sequences, including UCP1 of *M. domestica* and *S. crassicaudata*, allowed a solid reconstruction of the UCP1, UCP2, and UCP3 clades (Fig. 2*A*, supplement 3). In contrast to previous studies (27, 29), this phylogenetic tree clearly resolves a monophyletic clade of all UCP1 proteins, including the fish UCP1 orthologs (Fig. 2*B*). The overall structure of the UCP1 clade reflects the phylogeny of the major vertebrate groups. A closer inspection of the UCP1 clade revealed that the branch length (substitution rate) between marsupials and eutherians is twice the length (0.4 expected mutations per site) of that between marsupials and amphibians (0.2 expected mutations per site). This is remarkable as marsupials are more distantly related to amphibians compared with eutherians. The large distance between marsupial and eutherian UCP1 sequences may indicate an accelerated evolution of UCP1 in eutherians in contrast to steady substitution rates found in the UCP2/3 clades.

Eutherian UCP1 may have developed faster in response to so far unknown selection pressures. This would also explain why direct sequence comparisons result in a closer relationship of the fish and marsupial UCP1 sequences to UCP2/UCP3 than to eutherian UCP1.

Tissue-specific UCP gene expression in the South American marsupial M. domestica. Based on the identification of the *UCP1* gene in *M. domestica*, we investigated *UCP1* gene expression. We sampled cryosections of pouch embryos (22 and 25 days old) and sampled tissues of a juvenile (70 days old, post-nest vacation) and young adults (3 mo old).

Only in the juvenile we found dispersed adipose tissue deposits (brownish appearance) on the ribcage embedded in between pectoral muscle fibers (pectoral fat). Northern blotting analysis with a *UCP1* cDNA probe was insensitive, but using exon-spanning *M. domestica UCP1* primers comprising 6.7 kb of genomic sequence amplified a 550 bp cDNA fragment by PCR only in the pectoral fat (Fig. 3*A*), and subsequent sequencing clearly identified *UCP1* cDNA.

Hybridization techniques in all other individuals using a *M. domestica UCP1* cDNA probe demonstrated the lack of significant *UCP1* mRNA expression whereas *UCP2* mRNA was detectable (Fig. 3, *B* and *C*). In whole body cryosections of the embryos, *UCP2* mRNA was ubiquitously expressed with highest levels in spleen, heart, and liver (Fig. 3*B*). Nonspecific signals, as judged by comparison to the sense-control, occurred in calcified bone tissue. Northern blot analysis of selected tissues from the young adult revealed *UCP2* mRNA in all fat tissues, spleen, and intestine (Fig. 3*C*). Notably, *UCP1* mRNA expression in the interscapular fat, a typical BAT site in eutherians (in particular rodents), was undetectable.

Although the observed expression pattern in *M. domestica* is different from rodents, we have to consider that numerous eutherians do not possess significant amounts of BAT during their whole lifespan. In contrast to rodents and hibernators possessing
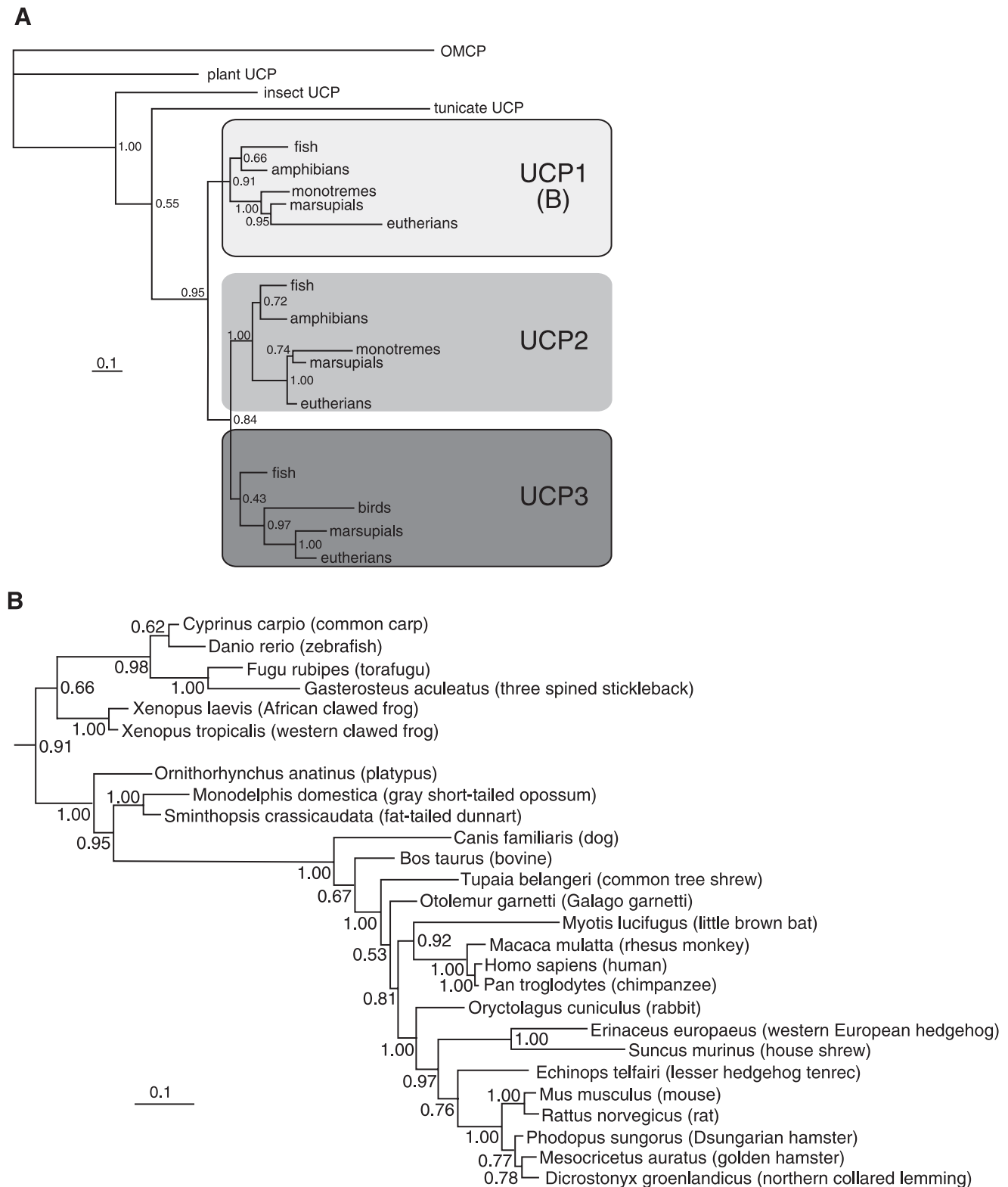
A

B

Fig. 2. Bayesian phylogeny of the core UCP family in vertebrates including marsupial UCP1 (*M. domestica* and *Sminthopsis crassicaudata*). An alignment of all available UCP sequences was analyzed by MrBayes 3.1.2, assuming a Whelan and Goldmann model of evolution. *A*: simplified tree resolving the phylogenetic relations of the core UCP family. The oxalacetate-malate carrier (OMCP) represents the out-group. *B*: detailed illustration of the UCP1 subgroup. Bayesian posterior probabilities are given at the branch nodes, and the scale bar indicates the substitution rate per aligned amino acid position. The complete phylogenetic tree can be found in supplement 3.
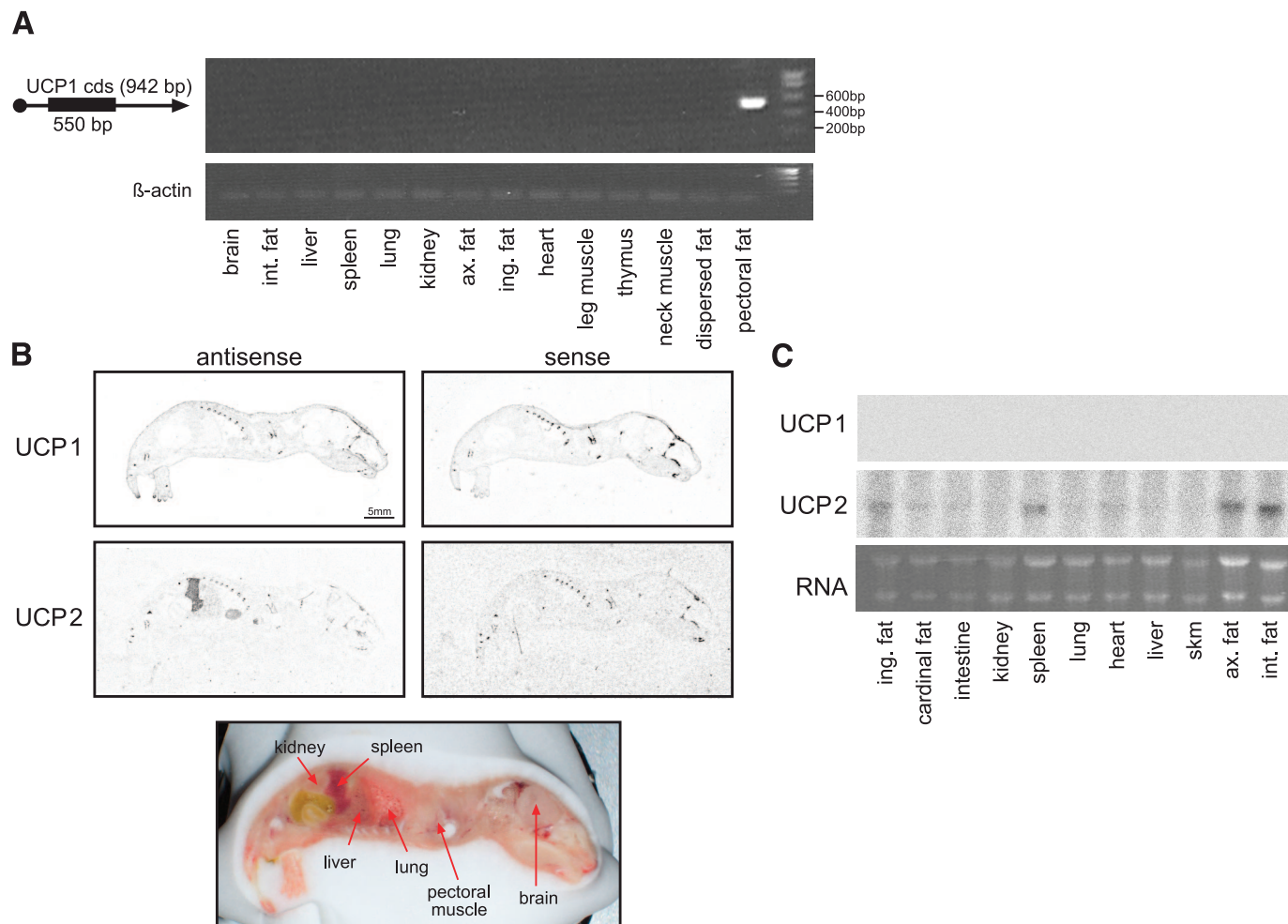
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Fig. 3. Regulation of UCP1 and UCP2 gene expression in multiple tissues of the developing marsupial *M. domestica.* Homologous primers and radioactively labeled cDNA and riboprobes used. *A*: screening for UCP1 cDNA by PCR in multiple tissues of a 70-day-old juvenile. The 550 bp UCP1 fragment was amplified from adipose tissue embedded in the ribcage (pectoral fat). β-Actin mRNA served as a cDNA quality control. *B*: representative sagittal section showing whole body in situ hybridization of 22- to 25-day-old embryos. The UCP2 antisense riboprobe clearly hybridized with UCP2 mRNA in spleen, heart, and liver, while no UCP1 signals could be detected using an *M. domestica* UCP1 antisense riboprobe (*left*). All riboprobes hybridized artefactually with calcified bone as judged by comparison to sense controls (*right*). A photograph of a sagittal transection served to assign radioactive signals to organs. *C*: multiple tissue Northern blot analysis of 3-mo-old *M. domestica* including interscapular fat of warm-acclimated and cold-acclimated individuals (*n* = 4) and mouse BAT controls. Total RNA (10 μg) isolated from selected tissues was hybridized with a 225 bp UCP1 and a 350 bp UCP2 cDNA fragment of *M. domestica*. Total RNA from mouse BAT served as a control. Posthybridization for UCP1 was performed under less stringent conditions, detecting mouse UCP1. The *M. domestica* UCP2 probe detected mRNA in spleen, inguinal, and interscapular fat of *M. domestica*. Skm, skeletal muscle; int. fat, interscapular fat deposit; ing. fat, inguinal fat; ax. fat, axilliar fat.

BAT during their entire life, rabbits lose the ability to express *UCP1* 1 mo after birth (6), while in newborn bovine and lambs *UCP1* expression is of significance only 2 days after physiological birth (8). BAT, or at least *UCP1*, in marsupials may therefore only be of importance to overcome cold-stress around pouch or nest vacation. Increased responsiveness to norepinephrine coincides with pouch vacation in the wallaby and in the Eastern barred bandicoot *Perameles gunnii* (25, 35). Given the identification of marsupial *UCP1* in this study, these observations can be revisited and the contribution of BAT investigated.

Analysis of the UCP1 promoter region in M. domestica. Our experiments show a high specificity of marsupial *UCP1* expression in distinct adipose tissue sites. In rodents and humans, an enhancer box in the upstream promoter region contains condensed elements targeting *UCP1* expression to BAT and allows responsiveness to the cold (for review see Ref. 47). We searched a 10 kb genomic sequence upstream of the *UCP1* transcriptional start site

of *M. domestica* for the presence of the enhancer box. Although we localized the enhancer box in all eutherians, including the ancient Afrotherian species *Echinops telfairii*, *M. domestica* lacks this distinct region, suggesting that the enhancer box first evolved in eutherian mammals (supplement 4). Despite the lack of the enhancer box, marsupial *UCP1* shows a remarkably high tissue-specificity targeting gene expression to distinct adipose tissue sites. The respective response elements may be dispersed across the promoter upstream region, and their presence cannot be categorically excluded.

Tissue-specific UCP gene expression in the Australian marsupials S. crassicaudata and A. flavipes. In *S. crassicaudata*, but not *A. flavipes*, we detected *UCP1* mRNA expression exclusively in the interscapular fat deposit, whereas no signal was detectable in liver and skeletal muscle (Fig. 4*A*). Probing with a *UCP2* fragment cloned from *Sminthopsis macroura* (27) detected highest *UCP2* mRNA levels in the
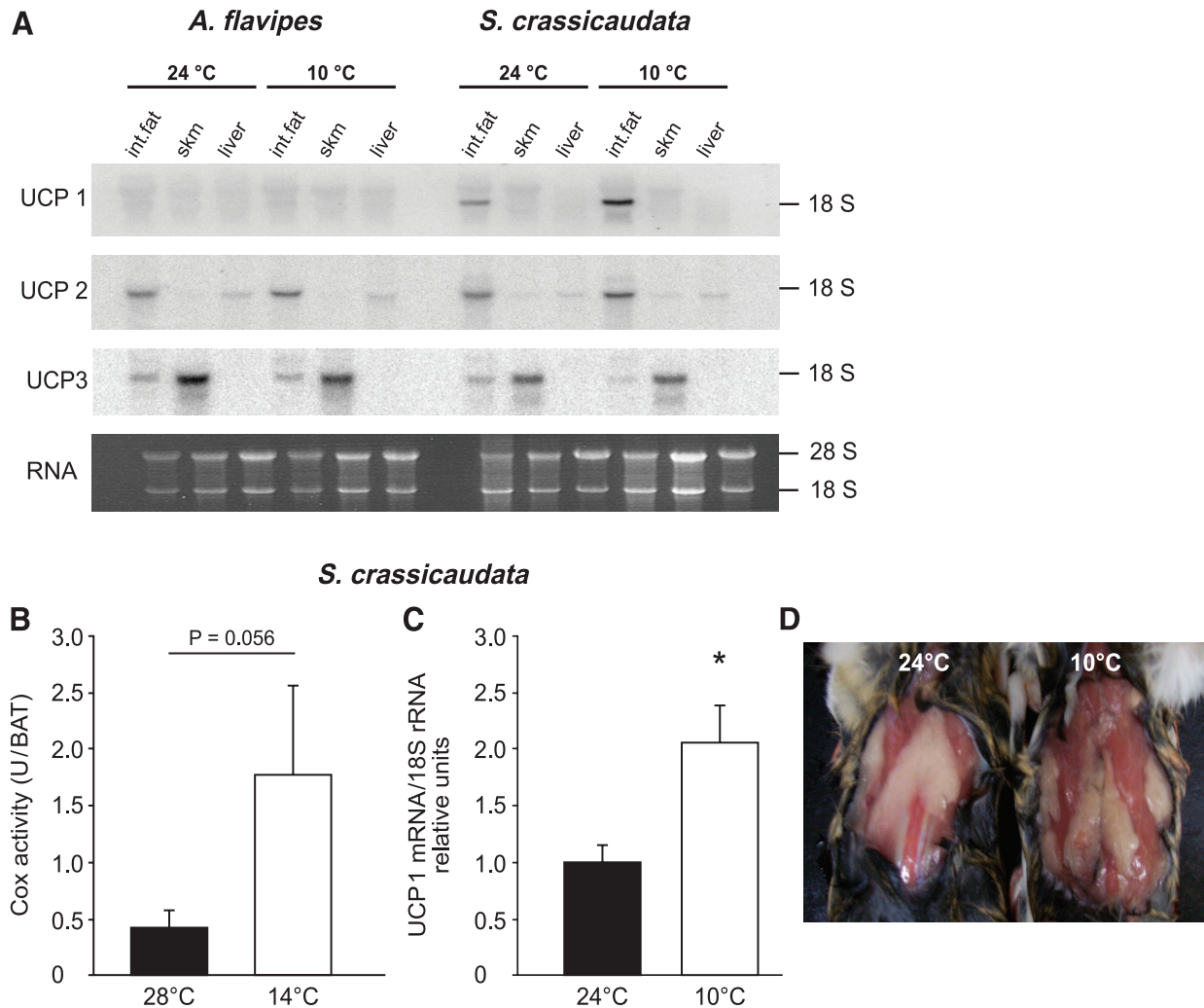
Fig. 4. *A*: UCP expression in selected tissues of the marsupials *S. crassicaudata* and *A. flavipes*. We hybridized 10 μg of total RNA with a *M. domestica* UCP1, *S. macroura* UCP2, and an *A. flavipes* UCP3 cDNA probe. *B*: cytochrome c oxidase (COX) activity in interscapular adipose tissue homogenates of warm-acclimated and cold-acclimated *S. crassicaudata*. *C*: effect of cold exposure (10°C) on *UCP1* gene expression in *S. crassicaudata*. Radioactive intensities of specific signals are shown as relative units corrected by ethidium bromide staining of the 18S rRNA. The effects of cold acclimation was evaluated by Mann Whitney *U*-test; $*P < 0.05$. *D*: appearance of interscapular fat due to cold exposure. The photograph shows a dorsal view on the interscapular fat deposits of a warm-acclimated and a cold-acclimated *S. crassicaudata*.

interscapular fat region of *S. crassicaudata* and *A. flavipes,* whereas mRNA levels in liver and skeletal muscle were rather low (Fig. 4*A*). Cross-reactivity of the *UCP1* probe to *UCP2* could be excluded as the *UCP1* probe exhibited only 61–69% identity to *Sminthopsis* and *Antechinus* UCP2, respectively. Using an *A. flavipes UCP3* probe, we detected *UCP3* mRNA in skeletal muscle of both species confirming a previous study (27).

Here, we demonstrate that UCP1 is constitutively expressed in *S. crassicaudata*, the smallest marsupial under investigation, in contrast to a close dasyurid relative, *A. flavipes*. Previous studies in *Sminthopsis* and *Antechinus* species support the interdependence of nonshivering thermogenesis and marsupial UCP1. As would be likely to occur in the presence of brown adipose tissue, *Sminthopsis ssp.* elevate metabolic rate by 30% in response to 0.25 mg/kg norepinephrine at 24°C (*S. crassicaudata*) (9) or in response to cold exposure (*S. macroura*) (15). In contrast, *Antechinus ssp.* does not show a thermogenic response to norepinephrine (41). It is likely that the differences seen in UCP expression are a functional adaptation to reflect the significant life history differences between these species. *Sminthopsis ssp.* from arid Australia are exposed to pronounced seasonal fluctuations in environmental temperature, while the coastal *Antechinus ssp.* experiences less climatic fluctuations (14).

The lack of UCP1 in adult *A. flavipes* and *M. domestica* is a distinct difference to eutherian species of similar body mass (20). Conventional heating mechanisms like shivering in these marsupials may be adequate to defend body temperature in mild climates. This is, however, not the favored mode of thermogenesis during long-term cold exposure in eutherians, but the major mechanism when nonshivering is insufficient.

Effect of cold exposure on the marsupial interscapular fat deposit. Adaptive nonshivering thermogenesis in rodents requires the recruitment of oxidative capacity and UCP1 to increase heat production. In a preliminary study on *S. crassicaudata* in 1990, there was a strong trend toward increased COX activity in the interscapular fat deposit of cold-acclimated

individuals (Fig. 4*B*), but we were not able to detect *UCP1* mRNA using a rat *UCP1* cDNA probe. By using a marsupial *UCP1* probe in the present study, we demonstrated a significant upregulation of *UCP1* gene expression in response to cold. *UCP1* mRNA levels in cold-acclimated *S. crassicaudata* were twofold higher than in animals exposed to 24°C ($n = 7$, $P = 0.018$, Fig. 4*C*). Furthermore, appearance of the interscapular fat deposit changed from white in animals held at 24°C to brown in cold-acclimated *S. crassicaudata* (Fig. 4*D*), a transition that was absent in *M. domestica*.

Together with the absence of brownish color, *UCP1* mRNA expression was absent in the interscapular fat deposit in young adult *M. domestica* (3 mo old) even after cold exposure (supplement 5). Even posthybridization procedures under less stringent conditions revealed no signal in the interscapular fat of *M. domestica* but visualized cross-reactivity of the *M. domestica UCP1* cDNA probe to mouse UCP1. *M. domestica UCP2* mRNA levels remained unchanged in interscapular fat after cold exposure (supplement 4).

Despite some evidence for nonshivering thermogenesis in marsupials, no studies so far had investigated adaptiveness to the cold. In this study, cold-exposure elevated oxidative capacity and UCP1 expression in the interscapular fat of *S. crassicaudata* resembling adaptive molecular adjustments of eutherian BAT. Response of *S. crassicaudata UCP1* gene expression to cold exposure demonstrates different transcriptional control compared with *M. domestica*. Therefore, genomic *UCP1* promoter data of an Australian marsupial are required to identify *UCP1* response elements that are conserved during mammalian evolution.

Concluding Remarks

The successful radiation of eutherian mammals to cold environments was most likely facilitated by classical adaptive nonshivering thermogenesis depending on BAT and its crucial protein UCP1 (7). However, the origin and evolution of this thermogenic organ are unknown. Textbooks illustrate BAT as monophyletic trait of eutherians (49), and both UCP1 and BAT have been regarded as absent in marsupials (12, 18, 27, 30, 31, 38, 46); however, our study represents the first unequivocal demonstration of *UCP1* gene expression in adipose tissue of South American and Australian marsupials. In some marsupials like *M. domestica* or *A. flavipes*, UCP1 may be recruited transiently during early stages of development and is lost during adulthood, whereas other marsupials like *S. crassicaudata* retain UCP1 expression during the entire lifespan. These findings provide the molecular basis to investigate adaptive nonshivering thermogenesis and lead to interesting insights into the evolution of UCP1-mediated heat production. Our results suggest the presence of an archetypal BAT before the divergence of marsupials and eutherians more than 150 million yr ago allowing early mammals to pursue life in the cold.

REFERENCES

1. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** Basic local alignment search tool. *J Mol Biol* 215: 403–410, 1990.
2. **Aquila H, Link TA, Klingenberg M.** The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequence homologies and of folding of the protein in the membrane. *EMBO J* 4: 2369–2376, 1985.
3. **Berg F, Gustafson U, Andersson L.** The uncoupling protein 1 gene (UCP1) is disrupted in the pig lineage: a genetic explanation for poor thermoregulation in piglets. *PLoS Genet* 2: e129, 2006.
4. **Bininda-Emonds OR, Cardillo M, Jones KE, MacPhee RD, Beck RM, Grenyer R, Price SA, Vos RA, Gittleman JL, Purvis A.** The delayed rise of present-day mammals. *Nature* 446: 507–512, 2007.
5. **Bouillaud F, Ricquier D, Thibault J, Weissenbach J.** Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein. *Proc Natl Acad Sci USA* 82: 445–448, 1985.
6. **Cambon B, Reyne Y, Nougues J.** In vitro induction of UCP1 mRNA in preadipocytes from rabbit considered as a model of large mammals brown adipose tissue development: importance of PPARgamma agonists for cells isolated in the postnatal period. *Mol Cell Endocrinol* 146: 49–58, 1998.
7. **Cannon B, Nedergaard J.** Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277–359, 2004.
8. **Casteilla L, Champigny O, Bouillaud F, Robelin J, Ricquier D.** Sequential changes in the expression of mitochondrial protein mRNA during the development of brown adipose tissue in bovine and ovine species. Sudden occurrence of uncoupling protein mRNA during embryogenesis and its disappearance after birth. *Biochem J* 257: 665–671, 1989.
9. **Clements F, Hope PJ, Daniels CB, Chapman I, Wittert G.** Thermogenesis in the marsupial *Sminthopsis crassicaudata*: effect of catecholamines and diet. *Aust J Zool* 46: 381–390, 1998.
10. **Dawkins MJR, Hull D.** Brown adipose tissue and the response of new-born rabbits to cold. *J Physiol* 172: 216–238, 1964.
11. **Dawson TJ, Dawson WR.** Metabolic scope and conductance in response to cold of some dasyurid marsupials and Australian rodents. *Comp Biochem Physiol* 71A: 59–64, 1982.
12. **Dawson TJ, Olson JM.** Thermogenic capabilities of the opossum Monodelphis domestica when warm and cold acclimated: similarities between American and Australian marsupials. *Comp Biochem Physiol A* 89: 85–91, 1988.
13. **Galgoczy P, Rosenthal A, Platzer M.** Human-mouse comparative sequence analysis of the NEMO gene reveals an alternative promoter within the neighboring G6PD gene. *Gene* 271: 93–98, 2001.
14. **Geiser F.** Daily torpor and thermoregulation in Antechinus (Marsupialia). Influence of body mass, season, development, reproduction, and sex. *Oecologia (Berl)* 77: 395–399, 1988.
15. **Geiser F, Drury RL, McAllan BM, Wang DH.** Effects of temperature acclimation on maximum heat production, thermal tolerance, and torpor in a marsupial. *J Comp Physiol [B]* 173: 437–442, 2003.
16. **Gesner C.** *Medici Tigurini Historiae Animalium Liber I de Quadrupedibusuiuiparis.* Froschauer 1551, 840–844.
17. **Golozoubova V, Hohtola E, Matthias A, Jacobsson A, Cannon B, Nedergaard J.** Only UCP1 can mediate adaptive nonshivering thermogenesis in the cold. *FASEB J* 15: 2048–2050, 2001.
18. **Hayward JS, Lisson PA.** Evolution of brown fat: its absence in marsupials and monotremes. *Can J Zool* 70: 171–179, 1992.
19. **Heaton GM, Wagenvoord RJ, Kemp A, Nicholls DG.** Brown-adipose-tissue mitochondria: photoaffinity labelling of the regulatory site of energy dissipation. *Eur J Biochem* 82: 515–521, 1978.
20. **Heldmaier G.** Zitterfreie Wärmebildung und Körpergröβe bei Säugetieren. *Z vergl Physiol* 73: 222–248, 1971.
21. **Heldmaier G, Buchberger A.** Sources of heat during nonshivering thermogenesis in Djungarian hamsters: a dominant role of brown adipose tissue during cold adaptation. *J Comp Physiol [B]* 156: 237–245, 1985.
22. **Hope PJ, Pyle D, Daniels CB, Chapman I, Horowitz M, Morley JE, Trayhurn P, Kumaratilake J, Wittert G.** Identification of brown fat and mechanisms for energy balance in the marsupial, Sminthopsis crassicauda. *Am J Physiol Regul Integr Comp Physiol* 273: R161–R167, 1997.
23. **Huelsenbeck JP.** Testing a covariotide model of DNA substitution. *Molecular Biology Evolution* 19: 698–707, 2002.

24. **Huelsenbeck JP, Ronquist F.** MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755, 2001.
25. **Ikonomopoulou MP, Rose RW.** The development of endothermy during pouch life in the eastern barred bandicoot (Perameles gunnii), a marsupial. *Physiol Biochem Zool* 79: 468–473, 2006.
26. **Jastroch M, Buckingham JA, Helwig M, Klingenspor M, Brand MD.** Functional characterisation of UCP1 in the common carp: uncoupling activity in liver mitochondria and cold-induced expression in the brain. *J Comp Physiol [B]* 177: 743–752, 2007.
27. **Jastroch M, Withers K, Klingenspor M.** Uncoupling protein 2 and 3 in marsupials: identification, phylogeny, and gene expression in response to cold and fasting in *Antechinus flavipes*. *Physiol Genomics* 17: 130–139, 2004.
28. **Jastroch M, Wuertz S, Kloas W, Klingenspor M.** Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis. *Physiol Genomics* 22: 150–156, 2005.
29. **Jimenez-Jimenez J, Zardoya R, Ledesma A, Garcia dL, Zaragoza P, Mar Gonzalez-Barroso M, Rial E.** Evolutionarily distinct residues in the uncoupling protein UCP1 are essential for its characteristic basal proton conductance. *J Mol Biol* 359: 1010–1022, 2006.
30. **Kabat AP, Rose RW, Harris J, West AK.** Molecular identification of uncoupling proteins (UCP2 and UCP3) and absence of UCP1 in the marsupial Tasmanian bettong, Bettongia gaimardi. *Comp Biochem Physiol B Biochem Mol Biol* 134: 71–77, 2003.
31. **Kabat AP, Rose RW, West AK.** Non-shivering thermogenesis in a carnivorous marsupial Sarcophilus harrisii, in the absence of UCP1. *J Therm Biol* 28: 413–420, 2003.
32. **Klaus S, Heldmaier G, Ricquier D.** Seasonal acclimation of bank voles and wood mice: nonshivering thermogenesis and thermogenic properties of brown adipose tissue mitochondria. *J Comp Physiol [B]* 158: 157–164, 1988.
33. **Loncar D, Afzelius BA, Cannon B.** Epididymal white adipose tissue after cold stress in rats. I. Nonmitochondrial changes. *J Ultrastruct Mol Struct Res* 101: 109–122, 1988.
34. **Loncar D, Afzelius BA, Cannon B.** Epididymal white adipose tissue after cold stress in rats. II. Mitochondrial changes. *J Ultrastruct Mol Struct Res* 101: 199–209, 1988.
35. **Loudon ASI, Rothwell NJ, Stock MJ.** Brown fat, thermogenesis and physiological birth in a marsupial. *Comp Biochem Physiol* 81A: 815–819, 1985.
36. **Nicholls DG, Locke RM.** Thermogenic mechanisms in brown fat. *Physiol Rev* 64: 1–64, 1984.
37. **Nicol SC.** Non-shivering thermogenesis in the potoroo, Potorous tridactylus (Kerr). *Comp Biochem Physiol* 59: 33–37, 1978.
38. **Nicol SC, Pavlides D, Andersen NA.** Nonshivering thermogenesis in marsupials: absence of thermogenic response to beta 3-adrenergic agonists. *Comp Biochem Physiol A* 117: 399–405, 1997.
39. **Oliphant LW.** First observations of brown fat in birds. *Condor* 85: 350–354, 1983.
40. **Opazo JC, Nespolo RF, Bozinovic F.** Arousal from torpor in the Chilean mouse-opossum (Thylamys elegans): does non-shivering thermogenesis play a role? *Comp Biochem Physiol A Mol Integr Physiol* 123: 393–397, 1999.
41. **Reynolds W, Hulbert AJ.** Cold acclimation in a small dasyurid marsupial: Antechinus stuartii. In *Carnivorous Marsupials*, edited by Archer M. Mosman, N. S. W. Australia: Royal Zoological Society of NSW, 1982, p. 278–283.
42. **Ricquier D, Kader JC.** Mitochondrial protein alteration in active brown fat: a sodium dodecyl sulfate-polyacrylamide gel electrophoretic study. *Biochem Biophys Res Commun* 73: 577–583, 1976.
43. **Ricquier D, Raimbault S, Champigny O, Miroux B, Bouillaud F.** Comment to Shinohara et al. (1991) *FEBS Letters* 293, 173–174. The uncoupling protein is not expressed in rat liver. *FEBS Lett* 303: 103–106, 1992.
44. **Ronquist F, Huelsenbeck JP.** MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574, 2003.
45. **Rose RW, West AK, Ye JM, McCormick GH, Colquhoun EQ.** Non-shivering thermogenesis in a marsupial (the Tasmanian bettong Bettongia gaimardi) is not attributable to brown adipose tissue. *Physiol Biochem Zool* 72: 699–704, 1999.
46. **Schaeffer PJ, Villarin JJ, Lindstedt SL.** Chronic cold exposure increases skeletal muscle oxidative structure and function in Monodelphis domestica, a marsupial lacking brown adipose tissue. *Physiol Biochem Zool* 76: 877–887, 2003.
47. **Silva JE, Rabelo R.** Regulation of the uncoupling protein gene expression. *Eur J Endocrinol* 136: 251–264, 1997.
48. **Smith RE.** Thermoregulation by brown adipose tissue in cold. *Fed Proc* 21: 221, 1962.
49. **Vaughan TA, Ryan JM, Czaplewski NJ.** *Mammalogy* (4th ed.). Saunders College Publishing, 2000.
50. **Wen G, Ramser J, Taudien S, Gausmann U, Blechschmidt K, Frankish A, Ashurst J, Meindl A, Platzer M.** Validation of mRNA/EST-based gene predictions in human Xp11.4 revealed differences to the organization of the orthologous mouse locus. *Mamm Genome* 16: 934–941, 2005.
51. **Whelan S, Goldman N.** A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol Biol Evol* 18: 691–699, 2001.
52. **Ye JM, Edwards SJ, Rose RW, Steen JT, Clark MG, Colquhoun EQ.** Alpha-adrenergic stimulation of thermogenesis in a rat kangaroo (Marsupialia, Bettongia gaimardi). *Am J Physiol Regul Integr Comp Physiol* 271: R586–R592, 1996.