Functional Attenuation of UCP1 as the Potential Mechanism for a Thickened Blubber Layer in Cetaceans

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Abstract

Uncoupling protein 1 (UCP1) is an essential protein in the mitochondrial inner membrane that mediates nonshivering thermogenesis (NST) and plays an important role in thermoregulation and fat deposition. However, the relationship between the evolution of UCP1 and fat deposition in the blubber layer in cetaceans remains unclear. Here, frameshift mutations, premature termination, and relaxed selection pressure (ω = 0.9557, P < 0.05) were detected in UCP1 in cetaceans, suggesting that UCP1 was inactivated during cetacean evolution. By time estimation, it was found that the inactivation of UCP1 in cetaceans occurred between 53.1 and 50.2 Ma. However, combined with findings from immunohistochemical analysis of the blubber layer of the Yangtze finless porpoise and in vitro functional assays, a premature termination of cetacean UCP1 resulted in a reduction of UCP1-mediated NST capacity (about 50%) and lipolytic capacity (about 40%), both of which were beneficial to maintain blubber layer and body temperature without excessive fat consumption. This study provides new insights into the molecular mechanisms of the blubber thickening in cetaceans and highlights the importance of UCP1 attenuation in cetaceans for secondary aquatic adaptation.

Key words: cetaceans, UCP1, blubber, thermoregulation, pseudogenization.

Introduction

Cetaceans have evolved adaptively in morphology and physiology since their return from land to sea. The thermal conductivity of water is about 25 times that of air, and maintaining the central body temperature of cetaceans at 37 °C is a tremendous challenge (Parry 1949; Scholander et al. 1950; Wright et al. 2021). To cope with this challenge, the blubber layer is about 20–30 cm thick in cetaceans, more than ten times thicker than in other even-toed ungulates (Pond 1978; Struntz et al. 2004; Ellis 2009). The blubber is a reservoir of abundant fat and energy, playing an important role in ecology, reproduction, and survival, as well as being a major source of energy and freshwater balance during the fasting season (Ellis 2009). More critically, it effectively reduces heat conduction and heat loss, which are central to the entire process of thermoregulation in cetaceans, and acts as an insulator (Ellis 2009; Hashimoto et al. 2015). Therefore, it is particularly important for cetaceans to maintain adequate blubber thickness in order to adapt to the aquatic environment. However, the evolutionary mechanisms by which cetaceans effectively regulate and maintain their thickened blubber have not been well studied.

In mammals, brown adipose tissue (BAT) is a specialized nonshivering thermogenesis (NST) tissue that consumes white adipocytes to increase heat production and regulate body temperature during cold stress (Golozoubova et al. 2001; Cannon and Nedergaard 2004). As a core protein mediating NST in BAT, UCP1 (uncoupling protein 1) plays an essential role in NST and is mainly expressed in the mitochondrial inner membrane (Nicholls and Locke 1984); when activated, UCP1 promotes proton leakage into the mitochondrial matrix (Heaton et al. 1978), but without a concomitant production of adenosine triphosphate (ATP), it would lead to a futile cycle of enhanced substrate oxidation and thermogenesis (Ledesma et al. 2002; Cedikova et al. 2016; Nowack et al. 2017). In addition to thermoregulation, increased BAT activity could be effective against diet-induced metabolic diseases such as obesity, type 2 diabetes, and hyperlipidemia (Hamann et al. 1995; Poher et al. 2015; Kim and Plutzky 2016). For example, mice with increased UCP1 mRNA stability or BAT function were more resistant to high-fat diet-induced obesity (Qiang et al. 2012; Dzempmier et al. 2015; Takahashi et al. 2015). Alternatively, after ectopic expression of UCP1 in pigs, UCP1-KI pigs showed increased thermoregulation and significantly reduced body fat and backfat thickness (Zheng et al. 2017). On the other hand, it has been reported that mice lacking UCP1 or BAT-deficient mice show significantly increased fat deposition and obesity susceptibility under high-fat diet conditions (Lowell et al. 1993; Kopecky et al. 2007).
In humans, increased obesity was significantly associated with decreased BAT activity (Vijgen et al. 2011, 2012). Previous studies revealed that mammals adapted to extremely cold environments, such as cetaceans, sirenians (Trichechus manatus), and woolly mammoth (Mammuthus primigenius), tend to show inactivation of UCP1 (Gaudry et al. 2017). Notably, these species exhibit a remarkable convergence in significant thickening of subcutaneous fat, especially in cetaceans. Therefore, it is interesting to explore the evolution of the UCP1 gene and its potential role in promoting the thickening of the subcutaneous fat layer associated with secondary aquatic adaptation.

In this study, we identified inactivating mutations in the cetacean UCP1 gene through a comparative analysis of the UCP1 gene in 27 cetacean species. Furthermore, immunohistochemical (IHC) and in vitro functional analyses revealed reduced expression, stability, and uncoupling activity of the cetacean UCP1 protein. These findings may provide new insights into the molecular mechanisms of blubber thickening in secondary aquatic adaptation.

Results
Pseudogenization of Cetacean UCP1
To investigate, the role of the UCP1 gene in cetacean fat consumption and metabolic thermogenesis, we identified UCP1 sequences in 42 cetartiodactylan species with high-quality genome assemblies, including 27 cetacean species. Numerous variations in the exons of UCP1 in cetaceans were widely observed, whereas large fragment deletions were found in the toothed whales, such as Delphinidae and Hyperoodontidae (complete deletion of UCP1). Phocoenidae and Monodontidae (deletion of exons 1–4), fin whales (Balaenoptera physalus) and boutou (Inia geoffrensis) (deletion of exons 3 and 4), and franciscana (Pontoporia blainvillei) (deletion of exon 6) (fig. 1A). Although the complete coding sequence (CDS) of UCP1 was conserved in most baleen whales (except fin whale), many frameshift mutations were identified. Five shared mutations were found in all cetaceans, including splice site mutations in exon 1 (AG→AT) and exon 3 (GT→GG), a 4-bp deletion in exon 4, and a 2-bp deletion and premature termination (CGA→TGA) in exon 6 (fig. 1A). All of these mutations were verified in the Sequence Read Archive (SRA) database (fig. 1B). Mutations in the Yangtze finless porpoise, baiji, minke whale, and sperm whale were verified and corrected by polymerase chain reaction (PCR), and the 1 bp deletion of exon 2 in baiji, the deletion of exon 2 in Yangtze finless porpoise, and the 2 bp deletion of exon 2 in sperm whale were found to be due to sequence errors (supplementary fig. S1, Supplementary Material online).

In addition, UCP1 is associated with ADP/ATP carriers, and is a member of the mitochondrial carrier family (MCF) consisting of three ~100-amino acid homologous domains. The MCF domains function to interact to form a pseudo-symmetric salt bridge network to maintain stability, which is essential for UCP1 protein function. A comparison of UCP1 protein domains revealed that sperm whale conserved three MCF domains and maintained the UCP1-specific motifs after pseudogenization, whereas other cetaceans had only one or two MCF domains (fig. 2). Since these domains are essential for the UCP1 protein to perform its function, we speculated that pseudogenized UCP1 may still have some function in cetaceans.

Relaxation of Selection Pressure and Dating of UCP1 Inactivation in Cetaceans
Pseudogenes are generally under a relaxed selective pressure. To explore the evolutionary pattern of the UCP1 gene in cetaceans, we firstly used one-ratio CODEML model to detect an overall selection pressure for all species. In this model, mammalian UCP1 exhibited an overall selection constrained dN/dS ratio (ω = 0.57822, P < 0.05; table 1). Notably, in the branching model, the cetacean UCP1 gene showed a nearly neutral evolutionary rate (ω = 0.9557, P < 0.05), whereas the intact UCP1 branch showed purifying selection (ω = 0.14593, P < 0.05, table 1). Furthermore, the relaxed selection pressure on cetacean lineages was supported by the RELAX results using cetaceans with inactivating mutations as test branches (K = 0, P < 0.0001, supplementary fig. S2, Supplementary Material online).

Based on the measured dN/dS ratios and the divergence time of cetaceans, the UCP1 gene in cetaceans was estimated to have been inactivated at 50.2–53.1 Ma, shortly after the ancestral cetaceans diverged from the hippopotamuses. On the other hand, complete deletion of UCP1 occurred in the middle Oligocene in Hyperoodontidae, and in the early Miocene in Delphinidae, Phocoenidae, and Monodontidae (fig. 3).

Expression of UCP1 in the Blubber Layer of Cetaceans
The increased number of mitochondria and miniaturization of lipid droplets in adipocytes are important markers of enhanced UCP1-mediated NST in adipocytes. The inner, middle, and outer layers were defined by the location of the blubber layer relative to skeletal muscle (fig. 4A), and hematoxylin–eosin (HE) staining revealed that adipocytes in the inner layer were small unilocular fat droplets (90–170 μm), significantly smaller than those in the middle and outer layers (190–290 μm; fig. 4B). This suggested that adipocytes in the inner layer of blubber had the potential to undergo fatty acid β-oxidation.

Western blotting (WB) analysis of extracts from the blubber layer showed that UCP1 protein expressed in the inner layer was significantly increased compared with the outer and middle layers of the blubber (fig. 4C). Furthermore, IHC analysis using mouse anti-UCP1 antibody showed that UCP1 protein was highly expressed in the inner layer of the blubber. Additionally, voltage-dependent anion channel protein 1 (VDAC1) was the most abundant protein in the outer mitochondrial membrane and played an important role in regulating mitochondrial energy metabolism, and the amount of VDAC1 protein can be a marker of mitochondrial energy metabolism and
quantity. WB and IHC staining results revealed that VDAC1 protein was significantly enriched in the inner layer of the blubber (fig. 4D and E), suggesting that adipocytes in the inner layer of the blubber contained more mitochondria. Taken together, these results suggested that the pseudogene UCP1 was highly expressed in the inner layer of blubber, and its inner layer adipocytes were similar to brown adipocytes with UCP1-mediated NST function.

Decreased Gene Function and Protein Stability of Cetacean UCP1

Pseudogenization of a gene could affect the expression, translation, and function of the targeted gene and its downstream-related genes. WB analysis showed that, although pseudogenic UCP1 is normally translated into protein, total protein expression and stability were significantly reduced (fig. 5A and B). When the UCP1 protein
performs its uncoupling function, mitochondrial membrane potential (MMP) is reduced, and changes in MMP can be as a characterization of UCP1 protein function. As a result, a significant increase (∼1-fold) in MMP was detected in pseudogenic UCP1 compared with intact gene copy (fig. 6; supplementary fig. S3, Supplementary Material online). Furthermore, pseudogene UCP1 was found to significantly increase triglyceride (TG) content in 3T3-L1 cells (fig. 5C) and significantly decrease expression levels of thermogenic genes (TFAM and CD137) and lipolytic genes (ATGL and CPT1A) in C2C12 cells (fig. 7). These results suggest that pseudogenized UCP1 in cetaceans may significantly attenuate uncoupling and lipolytic functions.

**Discussion**

The blubber is recognized as the primary site of fat and energy storage in cetaceans. It is central to the entire process of thermoregulation and maintenance of body streamliner and is an essential guarantee for cetaceans to adapt to the aquatic environment (Ellis 2009). However, the mechanism of how cetaceans effectively balance blubber thickness and energy metabolism has remained unclear. In this study, we combined bioinformatics analysis and in vitro functional assays to explore the role of the UCP1 gene in cetacean aquatic adaptation, and provided new insights into blubber thickness and body temperature regulation in cetaceans.
Comparative analysis of 27 cetacean UCP1 genes identified frameshifting insertions and deletions, splice site disruption mutations, and in-frame stop codon mutations as signals of gene inactivation mutations in the cetacean UCP1 gene. This is similar to the results of Gaudry et al. (2017), but we corrected some of these putative pseudogene signals using PCR (supplementary fig. S1, Supplementary Material online). This suggested that the UCP1 gene in cetaceans may have been pseudogenized. However, further analysis of the sequence alignment implied that the pseudogenized cetacean UCP1 gene seemed to still retain its function. For example, these mutations appeared in nonfunctional domains or splice sites. Other studies have shown that such a kind of mutations do not necessarily lead to functional changes or inactivation of the protein (Gaudry and Campbell 2017; Crichton et al. 2017). Although two premature stop codons were identified in cetacean UCP1, both were located in the C-terminal region, indicating that the MCF domains were not significantly disrupted, and that retention of the MCF domain is important for UCP1 protein to perform its function (Nelson et al. 1998; Pebay et al. 2003; Ruprecht et al. 2014; Crichton et al. 2017). However, the number of MCF domains varied among cetaceans, and this variation might influence the stability of the pseudo-symmetric salt bridge network, the formation of the central cavity, and the rate of substrate exchange (Robinson et al. 2008; Divakaruni et al. 2012; Crichton et al. 2015). Furthermore, considering that the TGA stop codon showed significantly lower termination efficiency than other stop codons such as TAG and TAA (Cridge et al. 2018; Huang et al. 2020), the two shared TGA stop codons from the frameshift mutation of the cetacean UCP1 gene may have had a lower effect on transcriptional termination of this gene. In summary, the cetacean UCP1 gene may still retain some function, although it shows some signals of pseudogenization.

The retention of a functional UCP1 gene with a premature stop codon in cetaceans was supported by further functional assays. For example, the UCP1 gene in cetaceans can be expressed and translated into UCP1 protein (fig. 5A and B) and showed uncoupling activity (supplementary fig. S3, Supplementary Material online), indicating that the pseudogene UCP1 is functional. This was further confirmed by IHC analysis, which showed that UCP1 protein is highly expressed in the inner blubber layer of Yangtze finless porpoise (fig. 4D). Indeed, several previous studies have demonstrated that parts of pseudogenes are transcribed or expressed and fully or partially functional as intact genes in humans and mice (Zheng et al. 2007; Tarn et al. 2008; Poliseno et al. 2010; Han et al. 2011; Muro et al. 2011; Cheetham et al. 2020). For example, the pseudogene CX43 could not only be transcribed normally,
but also encode the same cell growth inhibitory protein as the parental functional CX43 gene (Kandouz et al. 2004).

UCP1, as a core protein of BAT thermogenesis, can activate the sensitive lipase (HSL) and lipid TG lipase (ATGL) to promote lipolysis in adipocytes (Michurina et al. 2021).

**Fig. 4.** Histology and IHC analysis of cetacean blubber tissue. (A) Schematic diagram of the blubber layer. (B) Western blot analysis of blubber layer of the Yangtze finless porpoise. Mouse BAT extracts were loaded as a positive control (Mus BAT). (C) Immunohistochemical analysis of Yangtze finless porpoise blubber using UCP1 antibody. (D) Immunohistochemical analysis of Yangtze finless porpoise blubber using VDAC1 antibody. Scale bar = 100 μm. (E) HE staining of Yangtze finless porpoise blubber. Unedited Western blot images are in the supplementary figure S4, Supplementary Material online. Data are shown as mean ± SEM, **P < 0.01.

**Fig. 5.** Inactivation of the UCP1 gene weakens its function. (A) Western blot and time course of Mus-UCP1, Mus-UCP1-4del, and Mus-UCP1-Stop abundance after CHX was supplied to transfected 293T cells. (B) Western blot of Mus-UCP1, Mus-UCP1-4del, and Mus-UCP1-Stop abundance to transfected 293T cells. (C) UCP1 overexpression on intracellular triglyceride content in 3T3-L1 cells (n = 3). Unedited Western blot images are in supplementary figure S4, Supplementary Material online. Data are shown as mean ± SEM, ns, P > 0.05, *P < 0.05, **P < 0.01.
Surprisingly, when the *UCP1* gene was pseudogenized, expression levels of two lipolysis rate-limiting enzymes (*ATGL* and *CPT1A*) were detected to be significantly reduced (fig. 7), suggesting a mechanism to avoid excessive lipolysis. *ATGL* is a rate-limiting enzyme that catalyzes TG hydrolysis. It has been reported that TG hydrolysis activity is reduced and fat deposition is accelerated in *ATGL* knockout mice (Haemmerle et al. 2006; Yamaguchi 2010; Wong et al. 2011). Similarly, *CPT1A*, the rate-limiting enzyme for fatty acid β-oxidation, can regulate TG accumulation in the body by mediating fatty acid β-oxidation (Gagnon et al. 2014). Suppression of *CPT1A* gene expression may promote the expression of fatty acid synthase and acetyl-core carboxylase (*ACCα*) genes, resulting in TG accumulation (Bhuiyan et al. 1994). For these reasons, the pseudogenized *UCP1* gene may decrease lipid degradation in adipocytes and promote TG accumulation in cetaceans. This was further evidenced by experiments at the cellular level. The pseudogenized *UCP1* gene significantly increased (40% increase) TG accumulation in 3T3-L1 cells (fig. SC). On the other hand, it was found specific convergent amino acid substitutions in several genes (i.e., *NFIA*, *SEMA3E*, and *MFN2*) in full aquatic marine mammals, and these genetic changes probably contribute to the development of blubber and the formation of a counter-current heat exchange system, that can effectively limit heat loss rather than increasing *UCP1*-mediated NST to maintain body temperature (Yuan et al. 2021). Notably and interestingly, inactivating mutations were also detected in *UCP1* of sirenians and woolly mammoths with thick subcutaneous fat, but the mutations occurred at different amino acid sites. Although all these mutations occurred independently and no mutations were shared with cetaceans, the pseudogenized *UCP1* gene in these species suggesting that these species may have evolved a functionally convergent mechanism to reduce heat dissipation in cold environments to maintain subcutaneous fat thickness. The present study suggested that the pseudogenized *UCP1* gene identified in cetaceans has weakened the uncoupling function of this gene (fig. 6; supplementary fig. S3, Supplementary Material online) and may have significantly reduced *UCP1*-mediated NST. This may be another indirect mechanism by which cetaceans may no longer need to increase lipolysis for heat production, to prevent unrestricted lipolysis and maintaining the blubber layer for aquatic adaptation.

In conclusion, it was found that pseudogenization of the *UCP1* gene in cetaceans may effectively reduce lipolysis in adipocytes and promote fat deposition. Therefore, pseudogenization and reduced function of the *UCP1* gene in cetaceans may be an evolutionary mechanism that aids secondary aquatic adaptation experienced by cetaceans during the transition from land to sea. This is supported to some extent by the fact that the period of inactivation of the *UCP1* gene in cetaceans (∼53.1–50.2 Ma) coincided with the period when they returned to the sea (∼56–53 Ma).

**Conclusion**

In this study, we combined evidence from bioinformatics, immunohistochemistry, and functional analysis to comprehensively examine the adaptive evolution of the *UCP1* gene in cetaceans for the first time. Bioinformatics analysis showed that the cetacean *UCP1* gene exhibited pseudogenization signals, but functional evidence suggested that cetaceans still retain partial functions that may help them effectively prevent excessive lipolysis and maintain adequate blubber thickness. This may provide new insights into the evolutionary mechanisms of secondary aquatic adaptation in cetaceans.

**Materials and Methods**

**Sample Collection, DNA Extraction, and PCR Amplification**

Muscle samples of sperm whale (*Physeter catodon*), minke whale (*Balaenoptera acutorostrata*), Yangtze finless porpoise (*Neophocaena asiaeorientalis*), baiji (*Lipotes vexillifer*), melon whale (*Peponocephala electra*), and Blainville’s beaked whale (*Mesoplodon densirostris*) were stored in a −20 °C freezer of the Jiangsu Key Laboratory for Biodiversity and Biotechnology, Nanjing Normal University. Whole-genomic DNA was extracted according to the conventional phenol-chloroform protocol (Sambrook and Russel 2001) and transferred to −20 °C for long-term storage. To obtain CDS of the *UCP1* gene, six specific primer pairs for PCR amplification were designed (supplementary table S1, Supplementary Material online). PCR profiles were 95 °C/3 min, 95 °C-15 s,
Collection of Blubber Layers from Yangtze Finless Porpoise

All samples examined in this study were taken from individuals estimated to die within 48 h prior to sampling. Full-depth samples, including skin, blubber, and a portion of the muscle, were taken from the mid-thoracic aspect of each individual, and the blubber layer was separated into three regions (inner, middle, and outer layers). All samples from the different sections were immediately fixed in 4% paraformaldehyde (PFA) for histological analysis or frozen at −80 °C until use. At least three tissue blocks were taken from each sampling region.

Hematoxylin–Eosin Staining and Immunohistochemistry Staining

HE staining was performed according to standard methods (Hashimoto et al. 2015). Briefly, blubber was fixed in 4% PFA at 4 °C for 24 h. After dehydration, blubber tissue was embedded in paraffin and sectioned to 5 μm. Sections were stained with HE. Within each layer, the cytoplasmic size of adipose droplets and adipocytes was measured in three randomly selected fields using Image-ProPlus 6.0.

For IHC staining, deparaffinized sections were incubated with H2O2 and reacted with mouse anti-UCP1 antibody (Proteintech, 23673-1-AP, 1:100) or mouse anti-VDAC1 antibody (Proteintech, 55259-1-AP, 1:100) overnight at 4 °C. The sections were incubated with 1X phosphatidylinositol (1:100), washed three times with 1X phosphate-buffered saline, and then incubated with secondary antibodies (goat anti-mouse IgG, SA00001-1, 1:10,000; goat anti-rabbit IgG, SA00001-2, 1:10,000) for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole.

Cell Culture and Differentiation

Sequences encoding mouse UCP1 and its mutants were amplified from mouse cDNA sequences using specific primers (supplementary table S1, Supplementary Material online) and cloned into pEGFP-C1 or pcDNA 3.1(+) plasmids. All sequences were confirmed by Sanger sequencing. C2C12 cells and 293T cells were purchased from the American Type Culture Cell Collection. 3T3-L1 adipocytes were donated by Zhu Li of the Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, Sichuan, China. Mouse C2C12, 293T, and 3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S) in a culture vessel maintained at 37 °C and 5% CO2 for 48 h. Then, LipoD3000 DNA in vitro transfection reagent (Ver. II; SignaGen Laboratories), plasmid pEGFP-C1 or pcDNA 3.1(+) (negative control) and recombinant plasmids were separately transfected into C2C12, 3T3-L1, or 293T cells. After 12 h, the medium was replaced with DMEM supplemented with 10% FBS and 1% P/S.

For adipocyte differentiation, 3T3-L1 and C2C12 cells were allowed to reach 100% confluency and treated for 48 h with an induction medium containing DMEM, 10% FBS, 1% P/S, 5 μg/ml insulin (Solarbio), 0.5 mM isobutylmethylxanthine (Sigma), 1 μM dexamethasone (Solarbio), 1 μM triiodothyronine (T3, Solarbio), and 1 μM rosiglitazone in induction medium for 48 h. The cells were then maintained in a differentiation medium supplemented with 5 μg/ml insulin, 1 μM T3, and 1 μM rosiglitazone for another 6 days. During differentiation, the medium was changed every 2 days. To induce the expression of thermogenes, C2C12 cells were incubated with 10 μM forskolin for 4 h.

Quantitative Analysis of Triglycerides

Triglyceride concentration in mature adipocytes was detected using a TG assay kit (Solarbio Science &
Technology, Beijing, China) according to the manufacturer’s instructions. Briefly, mature adipocytes were collected in six-well plates, reacted with reagents supplied with the TG content detection kit, and the OD value was measured by a microplate reader at a wavelength of 420 nm and the TG concentration was calculated based on a standard curve.

**Mitochondrial Membrane Potential Assay**

Mitochondrial membrane potential was detected using the JC-10 Assay Kit (Solarbio Science & Technology) according to the manufacturer’s protocol. Briefly, mature adipocytes were seeded in six-well plates, stained with JC-10 dye for 20 min in an incubator maintained at 37 °C, washed twice with JC-10 staining buffer, and 2 ml of cell culture medium was added. Fluorescence was detected in a microplate reader at wavelengths of 490 nm (excitation), 530 nm (emission), 525 nm (excitation), and 590 nm (emission), respectively. All experiments were performed at least three times.

**qPCR and Western Blotting**

Total RNA was isolated using Trizol Extraction Reagent (Vazyme, Nanjing, China) according to the manufacturer’s instructions. Extracted total RNA (2 μg) was converted back to cDNA using Hifair III First Strand cDNA Synthesis SuperMix qPCR (YEASEN, Shanghai, China) and analyzed with Roche LightCycler 480 II instrument (Roche Applied Science, Mannheim, Germany) for real-time PCR system and mRNA expression was quantitatively measured using SYBR Green (YEASEN) with β-tublin as an internal normalization control. Primer sequences are shown in supplementary table S1, Supplementary Material online.

For WB, cells and blubber tissue were lysed at 4 °C in radioimmunoprecipitation assay buffer (Solarbio) mixed with protease and phosphatase inhibitors. Protein samples were incubated with primary antibodies against UCP1 (Proteintech, 23673-1-AP, 1:800), 3× FLAG-tag (Proteintech, 20543-1-AP, 1/3,000), or β-tublin (Proteintech, 10094-1-AP, 1/3,000) at 4 °C overnight. Proteins were detected by incubation with horseradish peroxidase-conjugated secondary antibodies and visualization was detected with a chemiluminescence system (Fusion Solo S; Vilber Lourmat, France). Quantitative analysis of WB bands was performed using Image-ProPlus 6.0 software.

For protein stability assays, cycloheximide (CHX; 100 μg/ml; Santa Cruz Biotechnology) was added to cultures for the desired treatment time (0, 1, 3, and 5 h). All cells were collected 36 h after transfection, and lysates were assayed by WB. Quantitative analysis of WB bands was performed using Image-ProPlus 6.0 software.

**Identification of the Loss of Cetacean UCP1**

To detect the adaptive evolution of the UCP1 gene in cetaceans, a total of 42 cetartiodactylan species (including 27 cetaceans) with high-quality genomes were used in this study (supplementary table S2, Supplementary Material online). First, CDSs of the UCP1 genes of cattle (Bos taurus), common bottlenose dolphin (Tursiops truncatus), and minke whale were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/). The UCP1 sequence for the bowhead whale (Balaena mysticetus) was downloaded from http://www.bowhead-whale.org/. BLAST alignments of other mammals were performed using ~100 bp upstream and downstream of each exon of these species as reference sequences. Sequence alignment was performed in MUSCLE in MEGA6 (Edgar 2004; Tamura et al. 2013). Frameshift mutations and overlapping regions were also aligned manually.

We followed the method described by Sharma et al. (2020) to detect the inactivation of the UCP1 gene in 42 mammals. To verify the presence of inactivating mutations in the ancestors of cetaceans, the ancestral sequences were reconstructed by adding the “-showanc” parameter to PRANK (Löytynoja and Goldman 2010). In addition, validation of putative inactivating mutations was performed using raw sequence reads in the SRA data in the NCBI online database (https://www.ncbi.nlm.nih.gov/sra/term). In addition, the UCP1 sequences of Yangtze finless porpoise, baiji, sperm whale, and minke whale were amplified using PCR with the primer sequences shown in supplementary table S2 (Supplementary Material online).

**Detection of Selection Pressure**

To assess whether the UCP1 CDS was subjected to different evolutionary selection pressures across species, the CODEML program in PAML 4 (Yang 2007) was used to calculate the nonsynonymous substitution rate (dN)/synonymous substitution rate (dS) ratio (ω = dN/dS). All frameshift insertions/deletions and nonsense mutations in the alignments were removed prior to analysis; the phylogenetic tree at PAML run-time was downloaded from the TimeTree Database (http://www.timetree.org/).

Five models were used to determine the selection pressure in different branches of the UCP1 gene. Briefly, model A assumes that all branches have the same ω; model B is the same as model A, but ω is fixed at 1 (ω = 1); model C assumes that pseudogenized branches (cetacean branches) have the same ω2 and intact branches (noncetacean branches) have the same ω1; model D is the same as model C, but ω2 is fixed at 1 (ω2 = 1); and model E assumes that each branch has ω independently. The five models are then compared pairwise (A vs. B, C vs. A, D vs. C, and E vs. C). A likelihood ratio test with a χ2 test was performed on the nested likelihood models. The mean ω for each rank or super rank was calculated according to the results of the free likelihood model.

In addition, RELAX is a hypothesis testing framework that asks whether the intensity of natural selection has relaxed or strengthened along a given set of test branches. It is the most common tool for identifying trends or shifts in the severity of natural selection for a given gene (Wertheim et al. 2014). In this study, the program
RELAX, available on the Datamonkey website (http://www.datamonkey.org/), was used to further examine relaxed selection.

**Time Estimate for the Pseudogenization of the Cetacean UCP1 Gene**

To assess when UCP1 was inactivated in cetaceans, we applied the methods described by Chou et al. (2002) and Mu et al. (2021). Genes evolve under the same selection pressures \( K_n \) as other species until they are inactivated. Once inactivated, genes are assumed to evolve neutrally \( (K_n = 1) \) and accumulate nonsynonymous and synonymous mutations at a neutral rate. The \( dN/dS \) values \( (K) \) estimated for the entire branch are the average \( dN/dS \) values of the branch under selection \( (K_s) \) and the \( dN/dS \) values of the branch under neutral evolution \( (K_n = 1) \). The genes were then weighted by the proportion of time they were evolving under selection \( (T_s/T) \) and neutrally \( (T_n/T) \) (Chou et al. 2002; Mu et al. 2021).

\[
K = K_s \times T_s / T + K_n \times T_n / T \\
T_s = T - T_n
\]

where \( T \) is the time since divergence from the last common ancestor; upper and lower confidence intervals for species divergence time \( T \) were obtained from the TimeTree Database (http://www.timetree.org/); \( T_s \) represents the time for a gene to evolve under selection pressure; \( T_n \) is the time for a gene in neutral evolution.

**Statistical Analyses**

All data are presented as mean ± SEM. Significance was analyzed by using Student’s t-test or one-way analysis of variance; \( P < 0.05 \) was considered statistically significant. Data analysis was completed by applying SPSS 20.0 (SPSS Inc, Chicago, IL, USA).

**Supplementary Material**

Supplementary data are available at Molecular Biology and Evolution online.

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**Author Contributions**

G.Y. and S.X. conceptualized and supervised the study. M.Z. and T.W. collected the data and conducted the bioinformatics analyses. M.Z. performed all cell culture, cloning, expression assays, western blotting, protein stability assays, and histochemical analysis. Y.C. performed DNA extraction and PCR amplifications. M.Z. prepared the original draft, and G.Y. revised the manuscript. All authors read and approved the final manuscript.

**Data Availability**

There are no data to be archived. All data were included in supplementary materials for readers to validate.

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