RBM3 deficiency impairs body temperature control in response to cold stimulation

Junnosuke Nakamura[1], Takumi Taketomi[3], Fuminori Tsuruta[2,3,4,5*]

[1]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[2]Master's and Doctoral Program in Biology, Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[3]Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[4]Ph.D. Program in Humanics, School of Integrative and Global Majors, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[5]Master's and Doctoral Program in Neuroscience, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan

* Corresponding author
Tel.: +81-29-853-6887
E-mail: tsuruta.fuminori.fn@u.tsukuba.ac.jp

Abstract

Mammals are thermostatic animals and can regulate their body temperature within a precise range, irrespective of ambient temperature conditions. However, the mechanisms of how a body temperature is controlled in cold environments are still unclear. Here, we report that RNA binding motif protein 3 (RBM3) is crucial for maintaining body temperature in response to cold stimulation. RBM3, the RNA binding protein that stabilizes the target mRNA, is one of the significant proteins induced by cold stimulation. The Rbm3 knockout (*Rbm3*$^{-/-}$) juvenile mice are sensitive to cold exposure and cannot maintain their body temperature. Notably, *Rbm3*$^{-/-}$ mice decreased the expression level of UCP1 and UCP3, pivotal for thermogenesis in brown adipose tissue (BAT) and skeletal muscle, respectively. We also found that a defect in RBM3 increases the size of lipid droplets in the BAT. Additionally, UCP1 expression is canceled by cold stimulation in *Rbm3*$^{-/-}$ BAT. On the other hand, RBM3 deficiency has little effect on cold-induced UCP3 expression in skeletal muscle. These data suggest that RBM3 prevents body temperature reduction induced by cold stimulation via expressing UCP1 in BAT. Our findings provide a possibility that RBM3 is a crucial regulator of thermogenesis in juvenile mice with unstable body temperature regulation.

Introduction

Mammals possess physiological mechanisms that maintain a consistent body temperature range[1,2]. The physiological mechanisms involve heat production and dissipation to adapt to external temperature fluctuations. Maintaining an optimal body temperature is crucial for proper physiological functioning, including metabolic reactions. Conversely, deviations from the appropriate temperature range, such as fever and hypothermia, cause an abnormal physiological state, leading to multiple organ failures. Therefore, regulating body temperature is critical for maintaining a normal state.

Recently, it has been reported that heat production in mammals is mainly regulated by two tissues: brown adipose tissue (BAT) and skeletal muscle[3]. Uncoupling proteins (UCPs) play a crucial role in regulating thermoregulation. Specifically, uncoupling protein 1 (UCP1) is consistently recognized as the key gene responsible for heat production in BAT[4]. UCP1 utilizes the proton gradient produced in the electron transport system to generate heat instead of ATP production. Ucp1 expression is induced by the β-adrenergic receptor (βAR) via noradrenaline released from sympathetic nerves[5]. As a downstream target of βAR, the protein kinase A (PKA) pathway activates p38 mitogen-activated protein kinase (p38 MAPK), followed by phosphorylating peroxisome proliferators-activated receptor-γ co-activator-1α (PGC-1α), a co-activator of peroxisome proliferator-activated receptor γ (PPARγ), and another transcriptional factor, activating transcription factor 2 (ATF2)[6,7]. These transcriptional factors play a pivotal role in expressing the UCP1 gene. In addition, PKA has been known to phosphorylate transcriptional factor, cAMP-responsive elements binding protein (CREB). Indeed, CREB can bind to the promoter region of cAMP-responsive elements in the UCP1 gene[8]. Thus, it is likely that CREB activation is also a crucial element for the βAR-PKA axis. Additionally, Ucp1 expression is triggered by thyroid hormone receptors that accept thyroid hormones[1]. Therefore, Ucp1 expression, crucial for life support, is mediated by multiple pathways, although some regulatory mechanisms remain to be elucidated.

UCP3 is predominantly expressed in skeletal muscle[9,10]. Similar to BAT, the skeletal muscle responds to thyroid hormone stimulation, which induces thermogenesis. Importantly, UCP3 is believed to mediate thyroid hormone-induced heat production[11]. Because UCP3 also creates a proton gradient and antagonizes ATP synthesis in the mitochondrial inner membrane[12], UCP3 enhances heat production using similar machinery to UCP1. Additionally, it has been reported that UCP3 affects the activity of sarcoplasmic reticulum Ca^{2+} ATPase 1 (SERCA1)[13], a Ca^{2+} transporter on the endoplasmic reticulum. As SERCA activity may also be involved in thermogenesis[14], it

is possible that UCP3-dependent SERCA activity enhances heat production in skeletal muscle. As the thermogenic machinery in the skeletal muscle develops during the late-postnatal stage, the mechanism underlying temperature regulation may remain unstable after birth[15]. Due to incomplete skeletal muscle development, thermogenesis by skeletal muscle is relatively low during the neonatal period. In contrast, BAT is well-developed during this stage, and its thermogenic activity plays a critical role in regulating body temperature.

Recent studies have revealed that exposure to cold stress can trigger the expression of cold-responsive proteins, including RNA binding motif protein 3 (RBM3). RBM3 is widely expressed in various tissues and is believed to modulate target mRNA translation by directly binding[16]. In addition, it has been shown that cold stress-induced expression of Rbm3 promotes neurogenesis and proliferation of neural stem cells[17–19]. Importantly, the loss of the Rbm3 gene causes abnormal cortical formation[18], indicating that Rbm3 is crucial in modulating brain development. Moreover, RBM3 has been reported to prevent neuronal cell death under neurodegenerative conditions[20]. Indeed, RBM3 expression induced by cold stimuli attenuates degenerative phenotype in model mice such as Alzheimer's disease (AD) and Creutzfeldt-Jakob disease (CJD)[20,21]. Thus, RBM3 is significantly pivotal for regulating brain development and maintenance. Intriguingly, RBM3 is evolutionarily conserved only in mammals with a few exceptions[22]. RBM3 is also increased in RBM3 during hibernation[23,24]. Given that the body temperature significantly decreases during hibernation, it is likely that RBM3 plays a crucial role in controlling homeostasis, which in turn regulates body temperature. Although the relationship between cold shock stimuli and RBM3 expression has been studied, the detailed mechanism by which RBM3 is involved in body temperature control has not been clarified.

In this study, we report that RBM3 is a novel factor that regulates body temperature. We found that RBM3 is important in maintaining body temperature within the precise range. Also, RBM3 is required to suppress a decrease in body temperature after cold exposure. Moreover, under normal conditions, RBM3 increases the expression level of thermogenic genes, Ucp1 and Ucp3. Whereas cold stimulation upregulated the expression level of Ucp1 in BAT but not Ucp3 in skeletal muscle, correlating with RBM3 expression in each tissue. Our data suggest that RBM3-dependent thermoregulation is pivotal for controlling body temperature in thermostatic animals.

Results

Generation of Rbm3 knockout mice

To investigate the mechanisms by which RBM3 regulates body temperature *in vivo*, we generated Rbm3 knockout (*Rbm3*$^{-/-}$) mice using the improved-genome editing via oviductal nucleic acids delivery (*i*-GONAD) technique[25]. The Rbm3 CRISPR RNAs (crRNAs) targeting the protospacer adjacent motif (PAM) in the first intron and fifth intron were delivered with recombinant Cas9 protein (1.0 μg/ml) and trans-activating crRNA (tracr RNA) into the embryo at day 0.7 of gestation in the oviductal ampulla by electroporation. Consequently, we could obtain four founder mice. Next, to confirm the genomic sequence of the *Rbm3* gene, we performed DNA sequencing and polymerase chain reaction (PCR). One mouse strain lacks 1.8 kbp of the genomic region spanning from the first intron to the fifth intron. (Fig. 1A and 1B). We also verified the RBM3 expression using western blotting analysis. RBM3 was expressed in various tissues, including the brain, BAT, heart, and liver. On the other hand, RBM3 expression disappeared in each *Rbm3*$^{-/-}$ mice tissue (Fig. 1C), demonstrating that the *Rbm3*$^{-/-}$ mice line was established.

RBM3 is necessary for maintaining body temperature in response to cold shock

To examine whether RBM3 is necessary for maintaining body temperature, we measured the rectal temperature at postnatal (P) 21 (juvenile) and two months-aged (adult) mice. The body temperatures in WT and *Rbm3*$^{-/-}$ adult mice were not different (Fig. 2A and 2B), suggesting that RBM3 has little effect on regulating body temperature in adult mice. Next, we analyzed the body temperature in juvenile mice. Interestingly, the average body temperature did not differ between the WT and *Rbm3*$^{-/-}$ mice, whereas the variance in body temperatures was significantly higher in *Rbm3*$^{-/-}$ mice than in the WT mice (Fig. 2C and 2D). These data suggest that RBM3 plays an important role in controlling body temperature within the appropriate range in the normal state.

Because RBM3 is a cold-induced protein, we next analyzed whether RBM3 controls the body temperature in response to cold shock exposure. As we expected, the body temperature in adult mice was not largely reduced by cold shock stimulation. However, the body temperature in juvenile mice was fragile against cold shock stimulation (Fig. 2E and 2F). Thus, these data suggest that RBM3 is a crucial protein that prevents a reduction of body temperature by cold shock exposure in not adult but juvenile mice.

A defect in RBM3 has little effect on body weight and feeding behavior

Previous research has suggested that a decline in muscle mass can impair heat production and lead to hypothermia[26]. Conversely, obesity-related fat accumulation can impede heat dissipation and raise body temperature[27]. Consequently, differences in body weight between individuals may represent a crucial factor in regulating body temperature. Thus, we compared the body weight between WT and *Rbm3*$^{-/-}$ mice. We found that the body weight of *Rbm3*$^{-/-}$ mice is similar to that of WT mice (Fig. 3A), indicating that RBM3 is not involved in controlling body weight. Also, it has been suggested that feeding behavior promotes heat production and is associated with increased body temperature[28,29]. We investigated the food intake in WT and *Rbm3*$^{-/-}$ mice. As with body weight, there was no significant difference in decreased food amount (Fig. 3B). These data suggest that RBM3 has little effect on body weight and feeding behavior.

RBM3 regulates the expression level of UCP3 in skeletal muscle

Skeletal muscle and BAT are critical in regulating thermogenesis and body temperature. Within the skeletal muscle, there are two mechanisms for promoting heat generation: shivering and non-shivering. One of the primary candidates for non-shivering machinery is UCP3, which increases heat production through proton gradient. Therefore, we hypothesized that RBM3 regulates the UCP3 expression level. To examine this, we first checked UCP3 expression in the skeletal muscle of WT and *Rbm3*$^{-/-}$ mice at P21. A defect in the Rbm3 gene significantly decreases the mRNA level of UCP3 in an ambient temperature-independent manner (Fig. 4A and 4B), demonstrating that RBM3 controls UCP3 expression level under normal conditions. As cold stimulation promoted a reduction of body temperature in *Rbm3*$^{-/-}$ mice, we investigated the relevance between UCP3 expression and cold stimulation. Contrary to our expectations, there were no significant differences in the cold environment despite a slight reduction of UCP3 level (Fig. 4C). Additionally, the expression of RBM3 was also similar to the control (Fig. 4D). These data suggest that cold shock stimulation does not upregulate UCP3 and RBM3 in juvenile skeletal muscle. Our data provide a possibility that RBM3 may play a significant role in maintaining steady-state temperature stability through the constant expression of UCP3 in the skeletal muscle, but RBM3 does not affect the UCP3-dependent thermogenesis in response to cold stimulation in this tissue.

Cold shock-induced UCP1 expression is decreased in the BAT of *Rbm3*$^{-/-}$ mice.

The next question concerns how cold stimulation affects thermogenesis in juvenile mice. To examine this, we focused on UCP1, another UCP family protein, in the BAT. Because

UCP1 in the BAT plays an important role in thermogenesis by controlling H^+ in mitochondria[4], we first examined whether RBM3 is required for maintaining the UCP1 mRNA level. Consistent with the UCP3 in the skeletal muscle, the UCP1 mRNA was decreased in the BAT of *Rbm3*$^{-/-}$ mice in both room temperature and cold environments (Fig. 5A and 5B), indicating that RBM3 is necessary for maintaining UCP1 mRNA level in the BAT as well as UCP3. Then, we investigated whether cold stimulation modulates the expression of UCP1 and RBM3. Consistent with the previous report, the UCP1 expression in the BAT is increased after cold stimulation[30] (Fig. 5C). We also investigated whether RBM3 expression in the BAT is increased in response to cold stimulation (Fig. 5D). Likewise UCP1, RBM3 expression was increased after cold stimulation, implying that RBM3 may be a regulatory factor that confers resistance to cold environment. Next, we analyzed images of the BAT stained with hematoxylin and eosin (HE). Consistent with the BAT of *Ucp1*$^{-/-}$ mice[31], there were enlarged lipid vacuoles in *Rbm3*$^{-/-}$ mice, but not the number of vacuole size (Fig. 5E to 5H). Taken together, these data suggest that RBM3-dependent UCP1 expression in the BAT is involved in thermogenesis in response to cold shock stimulation.

Discussions

Here, we investigated the role of RBM3 in thermogenesis in mice. We found that RBM3 deficiency causes dispersion of body temperature in juvenile mice, rendering them vulnerable to cold stimuli. In addition, we observed that RBM3 deficiency causes a reduction in the expression of key thermoregulatory genes, UCP1 in the BAT and UCP3 in the skeletal muscle. Moreover, *Rbm3*$^{-/-}$ mice became vulnerable to cold stimulation. Finally, a loss of RBM3 augmented the size of lipid droplets in the BAT. Overall, these findings provide a new mechanism for RBM3-dependent thermoregulation in juvenile mice (Figure 6).

The homeostatic mechanisms responsible for regulating body temperature in mammals are established with growth, which means regulating body temperature during postnatal stages is unstable. Furthermore, a significant deviation from body temperature will result in death, making the maintenance of body temperature during this period critical for sustaining life. However, the mechanisms required to maintain body temperature during this period are still unclear. Previously, it has been reported that RBM3 expression increases after birth in the rat cerebellum but decreases as development progresses, becoming constant at about 6 to 8 weeks after birth[32]. Moreover, we observed that juvenile mice lacking RBM3 show unstable body temperature, while adult mice show normal body temperature (Figure 2). Importantly, the pathological findings of lipid droplets in *Rbm3*$^{-/-}$ BAT were similar to that in *Ucp1*$^{-/-}$ BAT. The proton gradient mediated by the β-oxidation and glycolysis contributes not to ATP synthesis but to heat production through UCP1 activity. Since UCP1 expression is reduced in *Rbm3*$^{-/-}$ mice, fatty acids and glucose might accumulate in lipid droplets and enlarge due to reduced metabolic pathways of β-oxidation and glycolysis. Therefore, these observations suggest that RBM3 is crucial for maintaining body temperature during the postnatal stage, especially in a cold environment.

Recent studies have shown that RBM3 regulates the stability and translation of the target mRNA via direct binding (G[CG][ATG]G[AT][GC]G)[33]. Thus, it is likely that RBM3 acts as the regulatory factor for thermogenesis via modulating target mRNAs in a context-dependent manner. Because we utilized conventional *Rbm3*$^{-/-}$ mice in this study, it remains unclear whether RBM3 regulates UCP1 level through mechanisms limited in the BAT, or whether RBM3 in the nervous system regulates UCP1 through non-tissue autonomous mechanisms. Previous studies have reported that the expression cascade of the *Ucp1* gene is regulated by several transcriptional regulators, including the nuclear receptors PPARγ and PGC-1α[34,35]. We found that the mRNAs of these transcription

factors possess RBM3 binding sequences (Supplementary Table 1), demonstrating that RBM3 may regulate the UCP1 level via binding to the mRNAs of PPARγ and PGC-1α. Furthermore, UCP1 mRNA also has one RBM3 binding site at the 5'-untranslated region (5'-UTR). Hence, it is likely that several mechanisms control the UCP1 expression in the BAT. Also, we have observed that the expression of UCP3 is decreased by the loss of RBM3. However, the expression of RBM3 and UCP3 in skeletal muscle does not change when juvenile mice are exposed to low temperature despite upregulation of PCG-1α by cold shock[35], suggesting that RBM3 is involved in maintaining the expression level of UCP3 only under normal conditions. Although the detailed molecular mechanism has not been clarified, the PGC-1α/PPARβ axis has been reported to regulate UCP3 expression[36]. Given that cold stimulation enhances PGC-1α expression, RBM3-regulated UCP3 level may be independent of PGC-1α/PPARβ axis because UCP3 in the skeletal muscle level was not increased after cold shock in juvenile mice.

Here, we focus on the mechanisms in BAT; however we cannot rule out the possibility that RBM3 in the nervous system influences thermogenesis. In mammals, the hypothalamus, which is a part of the central nervous system (CNS), plays a crucial role in sensing and regulating body temperature. The preoptic area (POA) senses a change in ambient temperature, which is then integrated and transmitted the information to various effectors[37]. Recently, inhibition of GABAergic neurons in the POA has been reported to activate the hypothalamic dorsomedial nucleus (DMH)[38]. The DMH activates a part of the brain, called the rostral raphe pallidus (rRPA), which triggers the release of norepinephrine from sympathetic nerves that are projected from the rRPA. This process enhances the thermogenesis in BAT[39–41]. Apart from this neural circuit, it has been considered that the arcuate nucleus (ARC) and the ventromedial hypothalamus (VMH) in the hypothalamus also play a role in controlling the generation of heat in BAT[42]. So far, we have observed preliminary data whereby RBM3 expression is up-regulated in response to cold stimulation in ARC. Because these nuclei are crucial to regulating thermogenesis in the BAT, upregulation of UCP1 in the BAT after cold stimulation may be regulated by RBM3 in the CNS.

Given that RBM3 in the CNS is important for controlling body temperature, the next question is what the targets of RBM3 are in the CNS. Previously, the neuropeptide Y (NPY) in the DMH has been reported to control the expression of UCP1 mRNA in the BAT[43]. As NPY1r, its receptor protein, has a binding sequence of RBM3 (Supplementary Table 1), it is possible that RBM3 in the CNS regulates the expression of UCP1 in the BAT via NPY1r expression. In addition, *Nrd1*$^{-/-}$ mice exhibit a partially similar phenotype to *Rbm3*$^{-/-}$ mice[44]. Nrd1, a metalloendopeptidase, is secreted to the extracellular space and

regulates the extracellular condition. Indeed, a defect in the *Nrd1* gene decreases the expression level of BAT UCP1 in response to cold stimulation and perturbs the body temperature control[44]. Likewise PGC-1α, Nrd1 mRNA also has the RBM3 binding motif (Supplementary Table.1). Thus, RBM3 may directly regulate Nrd1 expression in the nervous systems, subsequently increasing the expression of UCP1 in the BAT. Furthermore, it has been reported that calsyntenin 3β (CLSTN3β) in the BAT promotes S100β secretion and activates the sympathetic neurons, releasing noradrenaline[45]. The secreted noradrenaline activates the BAT, upregulating the UCP1 level in the BAT. S100β is well known to be a secretory protein from astrocytes and is involved in inflammation[46]. Since we found the biological relevance between astrocyte secretory factors and neuronal RBM3 expression in an *in vitro* culture system, it is interesting that S100β serves as a novel mediator for controlling thermogenesis.

In conclusion, our study has identified RBM3 as a novel factor that controls body temperature under normal conditions and demonstrated its influence on the expression of UCP family proteins. Additionally, we have shown that RBM3 is crucial for maintaining body temperature following cold stimulation. These findings shed light on the previously enigmatic machinery regulating body temperature and provide important insights into the connection between ambient temperature changes and the homeostatic state.

Materials and Methods

Mice

The wild-type (WT) mice were obtained from Japan SLC, Inc. (Shizuoka Japan). The mice were maintained in standard conditions (Mice were housed in a 12 h: 12 h light: dark cycle with lights on at 8 am). All mice used in this study were male mice. All animal experiments were conducted according to the university guidelines for animal care. This study was approved by the Animal Experiment Committee at the University of Tsukuba (the approval numbers: 19-340, 20-438, 21-443, 22-386).

Rbm3$^{-/-}$ mice generation

Rbm3$^{-/-}$ mice of the C57BL/6N strain were generated by *i*-GONAD as previously reported[25]. Briefly, crRNA (Integrated DNA Technologies) was designed with
the upstream-gRNA sequence: 5'-ACCGTACAAAATGACGGGCGTGG-3',
the downstream-gRNA sequence: 5'-ATGTGGCTTGGATATATCAAAGG-3'
to target the intron 1 and intron 5 of the *Rbm3* allele in order to delete the RBM3 protein function. In addition, 200 μM each Alt-R CRISPR Cas9 crRNA and Alt-R CRISPR Cas9 tracrRNA (Integrated DNA Technologies) were annealed at 94°C for 2 min, mixed with 1.0 ug/ml Alt-R S.p. Cas9 Nuclease (Integrated DNA Technologies), and injected into the oviduct at E0.7 of plug-confirmed mice. After injection, 40 V, 5 msec electrical pulses were given three times at 50 msec intervals, followed by 10 V, 50 msec electrical pulses three times at 50 msec intervals, using by gene delivery system NEPA21 (NEPA Gene). In order to fix the mutation in the strain, the F0 founder was mated with WT mice, and the resulting F1 heterozygous male and female mice were crossed to obtain an F2 generation containing homozygous mutants. The established strain was maintained by sibling breeding and used in the current study.

Measurement of rectal temperature, body weight, and food intake

The rectal thermometry of WT and *Rbm3*$^{-/-}$mice (P21 and P60) was measured using a digital thermometer (SATO KEIRYOKI, SK-1260). Briefly, the thermometer was inserted into the rectum, followed by a measurement of the temperature. The thermometry was conducted at 10 am on time. This rectal temperature is corresponding to the core body temperature[47]. This experiment was reproduced at least three times. For cold stimulation, mice were placed in cages without nestlets and left under normal conditions (room temperature) and cold environments (approximately 4~8 ºC) for 3 hours. The body weight of WT and *Rbm3*$^{-/-}$mice (P21) was measured using an electronic balance

(METTLER TOLEDO, BD601). This experiment was conducted at 10 am on time. The food intake of WT and *Rbm3*$^{-/-}$mice (P21) was analyzed by measuring the percent decrease in food for one day. Briefly, the food was put into the cage at 10 am on time. After 24 hours, the rest of the weight of food was measured. Mice were individually housed in cages and placed in standard conditions.

Immunoblot analysis

Samples were collected in homogenization buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA pH8.0, 0.4% TritonX100, 1 mM DTT) and homogenized by DIGITAL HOMOGENIZER (As One International, Inc.). Lysates were centrifuged at 14,000 rpm for 5 min, and were then collected the supernatants. The concentration of samples was measured by the Bradford method (Coomassie Protein Assay Kit, Thermo Fisher). Adjusted samples were mixed with sample buffer (125 mM Tris-HCl pH 6.5, 4% SDS, 10% Glycerol, 0.01% bromophenol blue, 10% 2-mercaptoethanol) and were boiled at 100°C for 3 minutes. Equal amounts of protein (5 μg) were loaded into each well and analyzed by western blot analysis. After SDS-polyacrylamide gel electrophoresis, each sample was transferred to a polyvinylidene difluoride membrane (Pall Corp.). The membranes were blocked in 5% skim milk/TBST for 60 min at room temperature and were then incubated overnight at 4 °C with the primary antibodies [anti-RBM3 antibody (Proteintech, Cat#14363-1-AP, 1:1000) and anti-Tubulin antibody (SIGMA, DM1A, 1:1000)]. After washing with PBS, the membranes were incubated for 1 hour with the peroxidase-conjugated secondary antibodies (SeraCare, 1:20,000).

Quantitative PCR

Brown adipose tissue, skeletal muscle, and hypothalamus of WT or *Rbm3*$^{-/-}$ mice were solved with 500 μl ISOGEN II (NIPPON GENE). The suspension was centrifuged at 12,000 x g for 15 min at 4°C. The colorless upper aqueous phase was collected, and added to 500 μl of 100% isopropanol, and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed in 75% ethanol and re-suspended in RNAase-free water. The reverse transcription was performed on 1 μg of total RNA using the solution containing 100 units ReverTra Ace (TOYOBO), 25 pmol Random Primer (TOYOBO), and 20 nmol dNTPs. The qPCR was performed in triplicate in a 96-well plate (Thermo Fisher Scientific) using THUNDERBIRD SYBR qPCR Mix (TOYOBO) in Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). Cycle threshold (CT) values were normalized to the 5S rRNA value. The relative quantity of the target expression was calculated by $2^{-\Delta\Delta Ct}$ methods

using SDS Software 2.4.2 (Applied Biosystems) with the following calculation. The relative quantity = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct^{target} - Ct^{5S})_{sample} - (Ct^{target} - Ct^{5S})_{reference}$.

Histology

The BAT collected from WT and *Rbm3*$^{-/-}$ littermate mice were perfused with 4% paraformaldehyde in phosphate-buffered saline (4% PFA-PBS). The samples were fixed in the 4% PFA-PBS overnight, and embedded in paraffin. Paraffin sections were then sliced into 2 μm sections and stained with Meyer's hematoxylin and eosin (HE). Tissue specimens were observed using the fluorescence microscope (BZ-9000, Keyence) with 40x (PlanApo 40x/0.95) objective and a charge-coupled device camera (Keyence).

Statistical analysis and image processing

The statistical data were calculated by using GraphPad Prism (GraphPad Software) compared by a two-tailed Student's *t*-test. Adobe Creative Could CC (Photoshop 22.1.0 and Illustrator 25.0.1) (https://www.adobe.com/) and FIJI Image J 2.1.0/1.53c (https://imagej.nih.gov/ij/) were used for all image processing. To quantify the number and size of lipid droplets, we performed the Analyze Particle function using Fiji/ImageJ software. The acquired HE stained slice images were converted to binarized images using the 'Li' method to detect transparent areas. To identify the lipid droplets in contact with each other, the binarized images were processed using the 'Watershed' function. To exclude blood vessels and detect only lipid droplets, the Analyze Particle function was set to detect particles with a size greater than 1 μm^2, a circularity within the range of 0.6-1.0, and exclude those on the edges. To quantify the number of cells, HE stained images were split using the 'Color Deconvolution' function, and hematoxylin channel images were converted to binarized images using the 'RenyiEntropy' method. the Analyze Particle function was set to detect particles with a size greater than 1 μm^2, and exclude those on the edges.

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Figure legends

Figure 1

(A) Schematic diagram of the targeting strategy to eliminate the *Rbm3* gene. The guide RNAs (gRNAs) were designed to target the region between the first and second exons and between the sixth and seventh exons of the *Rbm3* gene (top panel). The waveform data for the sequencing results of PCR products amplified from F0 mice (bottom panel). **(B)** Genotyping analysis was performed on F0 mice with the expected fragment sizes of 2313 bp for WT and 522 bp for *Rbm3*$^{-/-}$ mice. **(C)** Immunoblotting of RBM3 and tubulin in the supernatant of indicated organs derived from WT and *Rbm3*$^{-/-}$ mice littermate mice at P21. The asterisks indicate the non-specific bands.

Figure 2

(A) The core body temperatures of WT and *Rbm3*$^{-/-}$ mice (P60) were measured at room temperature (25°C). n=9 mice respectively, mean ± SEM. *p* value was analyzed by Student's *t*-test. **(B)** The histogram shows the core body temperature in (A). No difference between the two groups. **(C)** The core body temperatures of WT and *Rbm3*$^{-/-}$ mice (P21) were measured at room temperature (25°C). WT; n=17 WT mice, *Rbm3*$^{-/-}$; n=19 mice, mean ± SEM. *p* value was analyzed by Student's *t*-test. **(D)** The histogram shows the core body temperatures in (C). *Rbm3*$^{-/-}$ mice exhibit higher variability in body temperature compared to WT. **(E)** The core body temperatures of WT mice and *Rbm3*$^{-/-}$ mice (P60) were measured before and after a cold stimulation (4°C). WT; n=5 mice, *Rbm3*$^{-/-}$; n=5 mice, mean ± SEM. *p* value was analyzed by Student's *t*-test. **(F)** The core body temperatures of WT mice and *Rbm3*$^{-/-}$ mice (P21) were measured before and after a cold stimulation (4°C). WT; n=8 mice, *Rbm3*$^{-/-}$; n=9 mice, mean ± SEM. *p* value was analyzed by Student's *t*-test.

Figure 3

(A) The body weights of WT and *Rbm3*$^{-/-}$ mice were measured at P21. WT; n=4 mice, *Rbm3*$^{-/-}$; n=4 mice, mean ± SEM. *p* value was analyzed by Student's *t*-test. **(B)** The percentage decrease in food weight due to mouse biting. WT; n=4 mice, *Rbm3*$^{-/-}$; n=4 mice, mean ± SEM. *p* value was analyzed by Student's *t*-test.

Figure 4

(A and B) The mRNA of UCP3 in WT and *Rbm3*$^{-/-}$ skeletal muscles. The RNAs were isolated from WT and *Rbm3*$^{-/-}$ skeletal muscles obtained from P21 mice under the normal

condition (A) and cold stimulation for 3 hours (B). 5S rRNA levels were used for normalization. 3 samples were obtained from each mouse (total n=9 samples from 3 independent mice skeletal muscles), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(C)** The mRNA of UCP3 in WT mice skeletal muscles. The RNAs were isolated from the skeletal muscles obtained from P21 mice with or without cold stimulation for 3 hours. 5S rRNA levels were used for normalization. 2 or 3 samples were obtained from each mouse (total n=14, 15 samples from 5 independent mice skeletal muscles), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(D)** The mRNA of RBM3 in WT mice skeletal muscles. The RNAs were isolated from the skeletal muscles obtained from P21 mice with or without cold stimulation for 3 hours. 5S rRNA levels were used for normalization. 3 samples were obtained from each mouse (total n=14, 15 samples from 5 independent mice skeletal muscles), mean ± SEM. *p* value was analyzed by Student's *t*-test.

Figure 5

(A and B) The mRNA of UCP1 in WT and *Rbm3*$^{-/-}$ BAT. The RNAs were isolated from WT and *Rbm3*$^{-/-}$ BAT obtained from P21 mice under normal condition (A) and cold stimulation for 3 hours (B). 5S rRNA levels were used for normalization. 2 or 3 samples were obtained from each mouse (total n=8,9 samples from 3 independent mice BAT), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(C)** The mRNA of UCP1 in WT mice BAT. The RNAs were isolated from the BAT obtained from P21 mice with or without cold stimulation for 3 hours. 5S rRNA levels were used for normalization. 3 samples were obtained from each mouse (total n= 12 samples from 4 independent mice BAT), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(D)** The mRNA of RBM3 in WT mice BAT. The RNAs were isolated from the BAT obtained from P21 mice with or without cold stimulation for 3 hours. 5S rRNA levels were used for normalization. 3 samples were obtained from each mouse (total n= 9 samples from 3 independent mice BAT), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(E)** Representative HE staining of the BAT obtained from P21 WT and *Rbm3*$^{-/-}$ mice. Scale bar: 50 μm. **(F)** Size of each vacuole in BAT of WT and *Rbm3*$^{-/-}$ mice was measured using the particle analyzer by ImageJ. WT; n=16201, *Rbm3*$^{-/-}$; n=13442. mean ± SEM. *p* value was analyzed by Student's *t*-test. **(G)** The size of the vacuole in BAT of WT and *Rbm3*$^{-/-}$ mice per cell was measured using the particle analyzer by ImageJ. 8 images were obtained from each mouse (n=16 images, 2 independent mice), mean ± SEM. *p* value was analyzed by Student's *t*-test. **(H)** The lipid numbers in BAT of WT and *Rbm3*$^{-/-}$ mice were measured using the particle analyzer by ImageJ. 8 images were obtained from each mouse (n=16 images, 2

independent mice), mean ± SEM. *p* value was analyzed by Student's *t*-test.

Figure 6

The model describing the function of RBM3 involved in thermogenesis in the BAT and skeletal muscle.

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Author contributions

J.N. and F.T. designed research; J.N. performed research; J.N T.T. analyzed data; J.N and F.T. wrote the paper. Study supervision: F.T

Figure 1

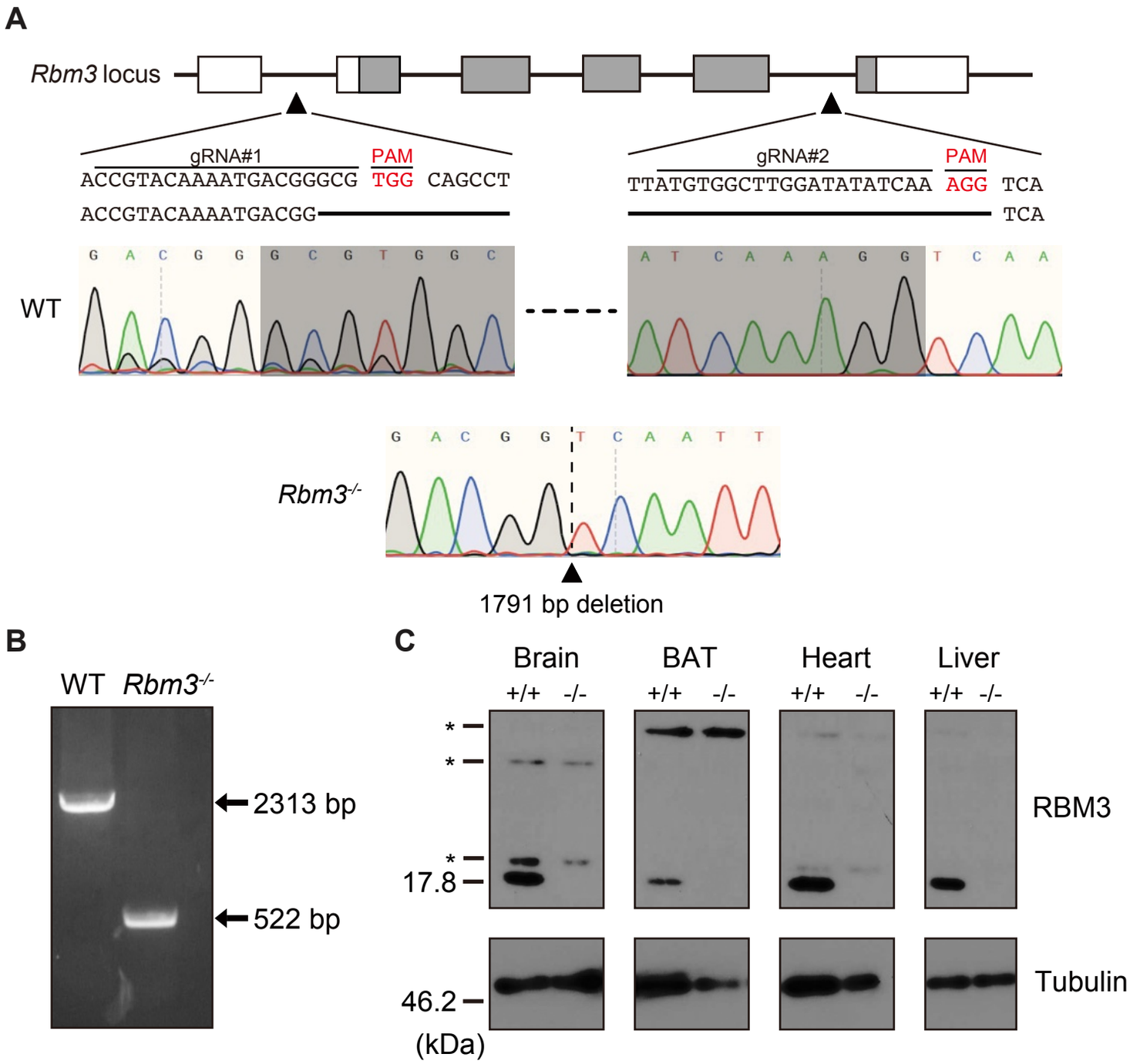

A
Rbm3 locus
gRNA#1
PAM
ACCGTACAAAATGACGGGCG TGG CAGCCT
ACCGTACAAAATGACGG
gRNA#2
PAM
TTATGTGGCTTGGATATATCAA AGG TCA
TCA
WT
Rbm3-/-
1791 bp deletion
B
WT Rbm3-/-
2313 bp
522 bp
C
Brain
BAT
Heart
Liver
+/+ -/-
RBM3
17.8
Tubulin
46.2
(kDa)

Figure 2

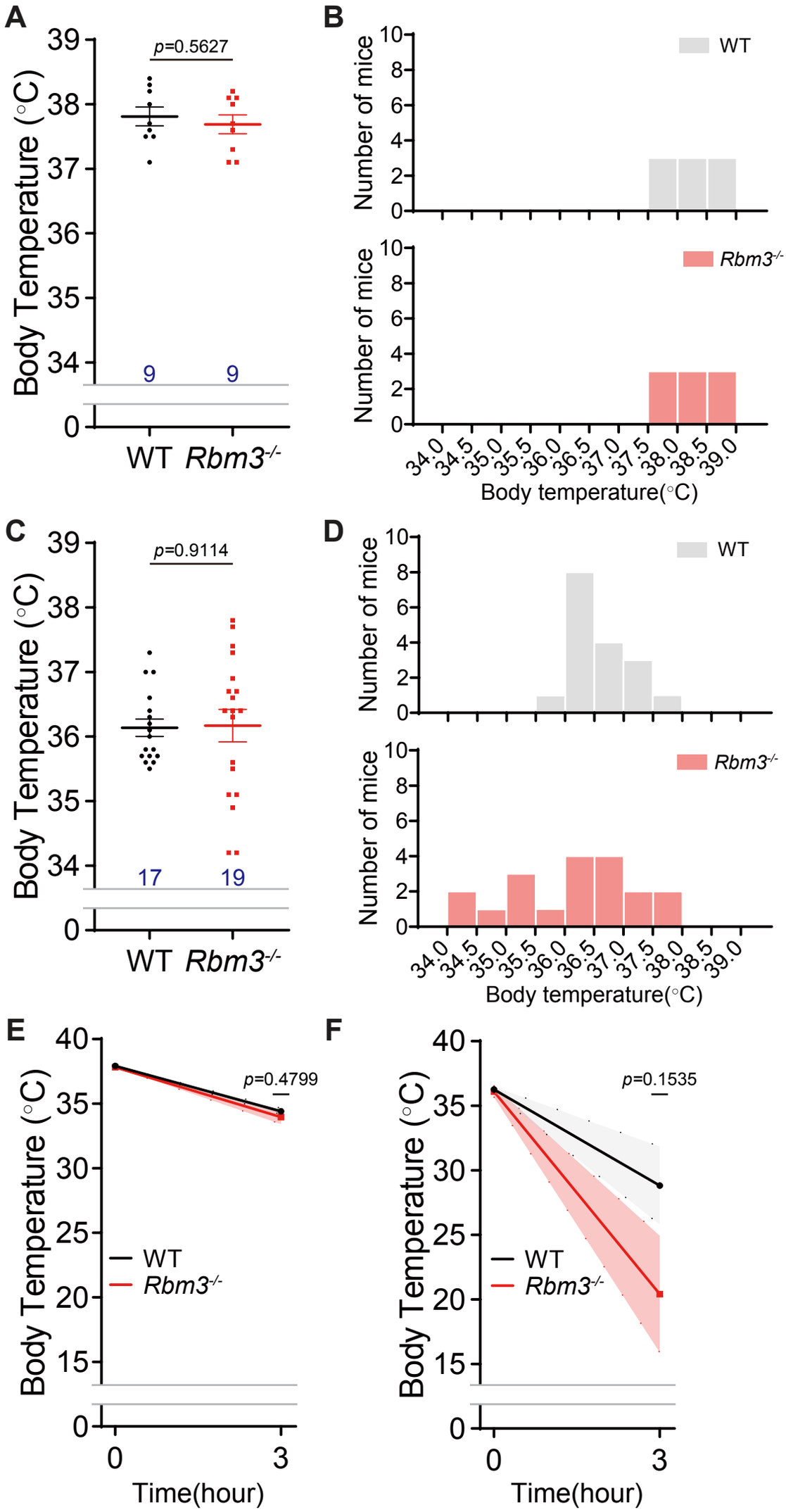

A
Body Temperature (°C)
p=0.5627
9
9
WT
Rbm3-/-
B
Number of mice
WT
Rbm3-/-
34.0
34.5
35.0
35.5
36.0
36.5
37.0
37.5
38.0
38.5
39.0
Body temperature(°C)
C
Body Temperature (°C)
p=0.9114
17
19
WT
Rbm3-/-
D
Number of mice
WT
Rbm3-/-
Body temperature(°C)
E
Body Temperature (°C)
p=0.4799
WT
Rbm3-/-
Time(hour)
F
Body Temperature (°C)
p=0.1535
WT
Rbm3-/-
Time(hour)

Figure 3

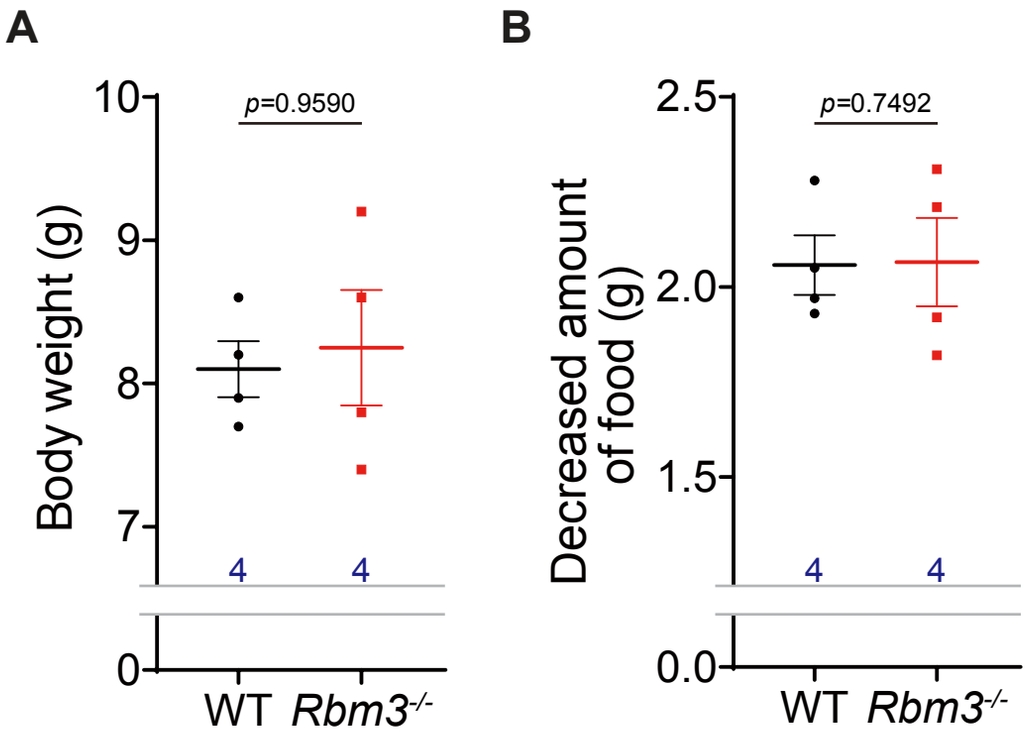

A
B
p=0.9590
Body weight (g)
10
9
8
7
0
4
4
WT
Rbm3-/-
p=0.7492
Decreased amount of food (g)
2.5
2.0
1.5
0.0
4
4
WT
Rbm3-/-

Figure 4

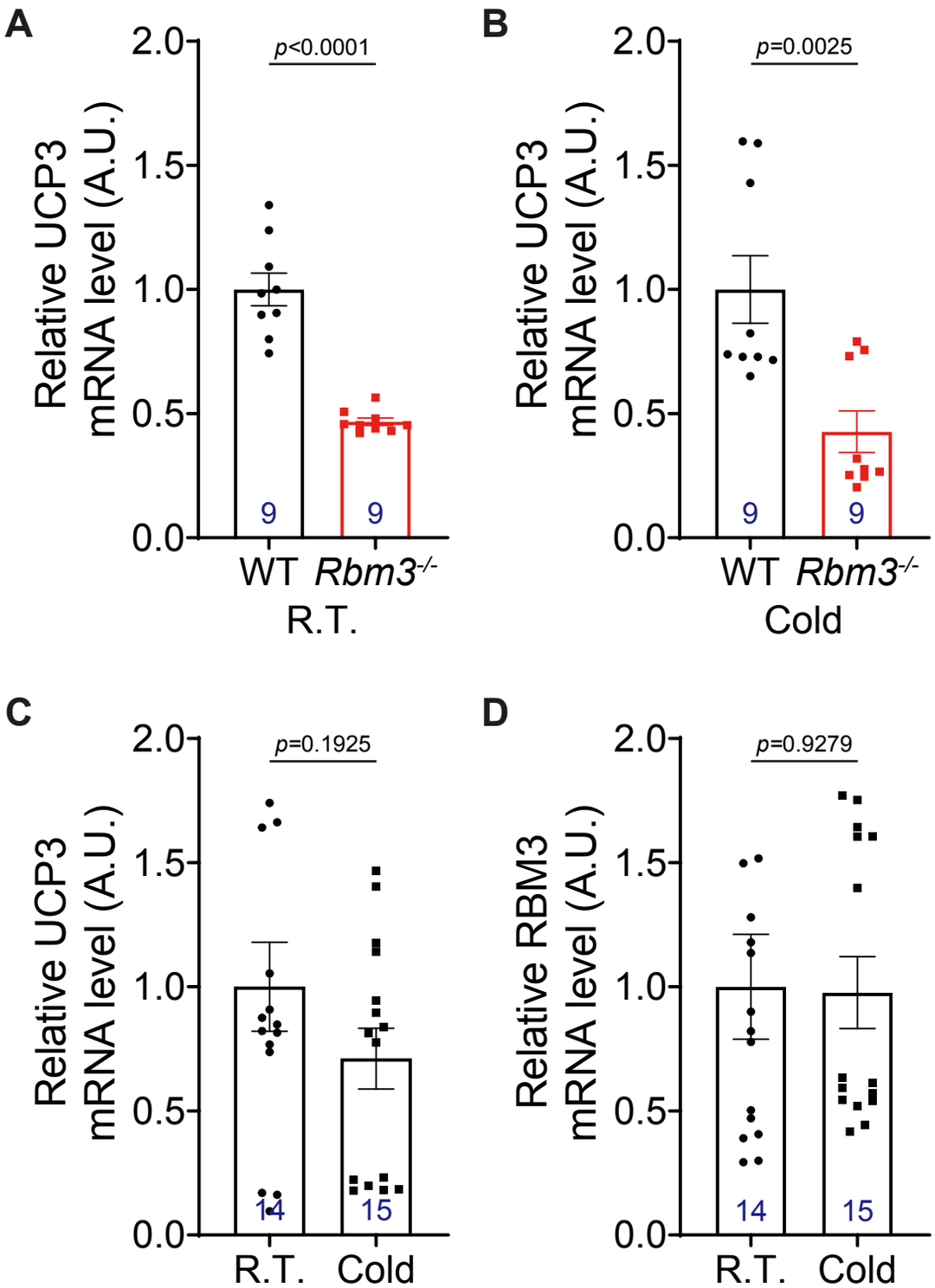

A
p<0.0001
Relative UCP3 mRNA level (A.U.)
2.0
1.5
1.0
0.5
0.0
9
9
WT
Rbm3-/-
R.T.
B
p=0.0025
Relative UCP3 mRNA level (A.U.)
2.0
1.5
1.0
0.5
0.0
9
9
WT
Rbm3-/-
Cold
C
p=0.1925
Relative UCP3 mRNA level (A.U.)
2.0
1.5
1.0
0.5
0.0
14
15
R.T.
Cold
D
p=0.9279
Relative RBM3 mRNA level (A.U.)
2.0
1.5
1.0
0.5
0.0
14
15
R.T.
Cold

Figure 5

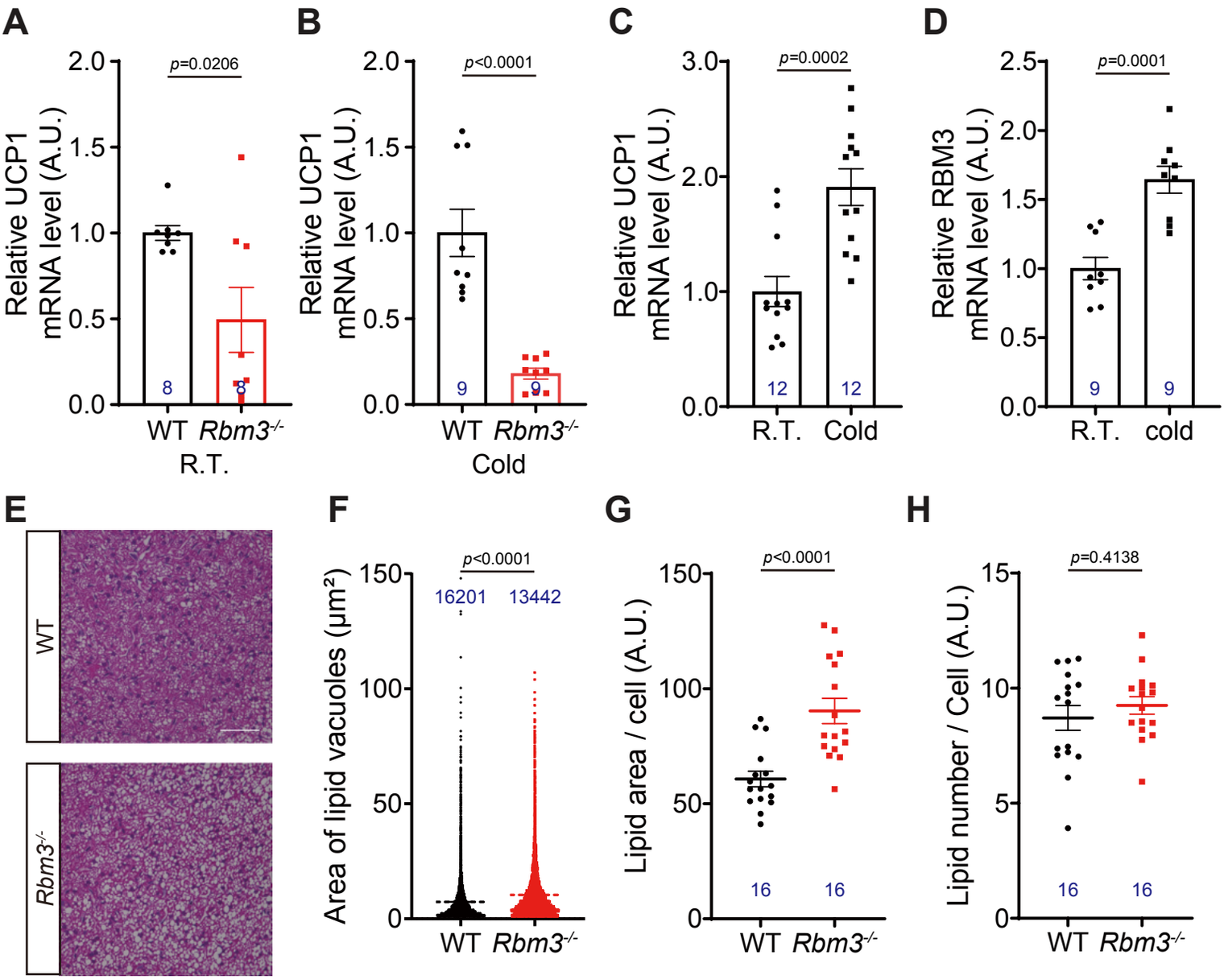
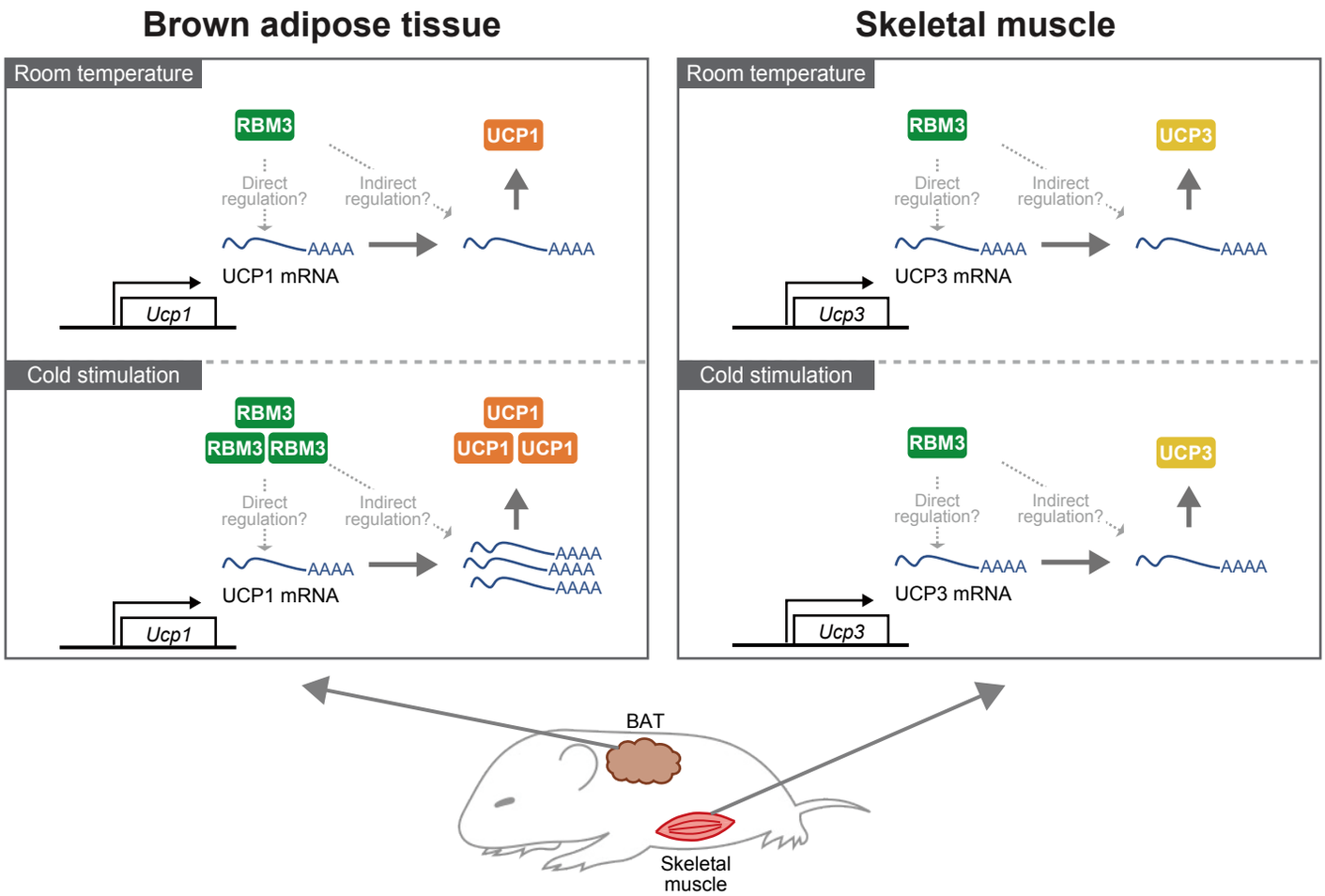

A
B
C
D
E
F
G
H
Relative UCP1 mRNA level (A.U.)
Relative RBM3 mRNA level (A.U.)
p=0.0206
p<0.0001
p=0.0002
p=0.0001
WT
Rbm3-/-
R.T.
Cold
cold
Area of lipid vacuoles (μm²)
Lipid area / cell (A.U.)
Lipid number / Cell (A.U.)
p=0.4138
16201
13442

Figure 6


Brown adipose tissue
Skeletal muscle
Room temperature
RBM3
UCP1
Direct regulation?
Indirect regulation?
AAAA
UCP1 mRNA
Ucp1
Cold stimulation
RBM3
RBM3
RBM3
UCP1
UCP1
UCP1
AAAA
AAAA
AAAA
AAAA
UCP1 mRNA
Ucp1
Room temperature
RBM3
UCP3
Direct regulation?
Indirect regulation?
AAAA
AAAA
UCP3 mRNA
Ucp3
Cold stimulation
RBM3
UCP3
Direct regulation?
Indirect regulation?
AAAA
AAAA
UCP3 mRNA
Ucp3
BAT
Skeletal muscle