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Brown Adipose Tissue and Skeletal Muscle Coordinately Contribute to Thermogenesis in Mice

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eLife Assessment

This is a **useful** paper regarding the roles of brown adipose tissue and skeletal muscle in thermogenesis in mice, with potential significance for the field. The overall approach is innovative but on balance the evidence for the claim is **incomplete**, as cast immobilization, while innovative, is likely stressful, may impact muscle and BAT directly, and imposes an energetic cost of motion on the animal that is not accounted for. Further experiments are also needed to directly assess the role of adipose-derived BCAAs in thermogenesis. The authors have done a good job of textually editing their manuscript to clarify the findings and limitations of the study.

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Abstract

Endotherms increase the rate of metabolism in metabolic organs as one strategy to cope with a decline in temperature of the external environment. However, an additional major contributor to maintenance of body temperature in a cold environment is contraction-based thermogenesis in skeletal muscle. Here, we show that impairment of hind limb muscle contraction by cast immobilization induced a loss of function of skeletal muscle and activated brown adipose tissue (BAT) thermogenesis as a compensatory mechanism. BAT utilizes free branched-chain amino acids (BCAAs) derived from skeletal muscle as an energy substrate for thermogenesis, and interleukin-6 released by skeletal muscle stimulates BCAAs production in muscle for support of BAT thermogenesis. Additionally, this thermoregulatory system between BAT and skeletal muscle may also play an important role in response to cold temperatures or acute stress. Our findings suggest that BAT and skeletal muscle cooperate to maintain body temperature in endotherms.

Introduction

Endotherms rely on the regulation of heat generation by metabolism in tissues of the body to maintain a stable core body temperature in the face of a fluctuating temperature of the external environment and thereby to ensure optimal physiological function.[1] Small mammals adjust their metabolic rate to cope with temperature fluctuations and to support their survival during seasonal acclimation. Some mammalian species are able to lower their metabolic rate and enter a state of torpor or hibernation in order to conserve energy and ensure survival.[2] However, small mammals that do not hibernate must increase their rate of metabolism, including obligatory thermogenesis in metabolic organs such as the liver and skeletal muscle as well as regulatory thermogenesis (shivering or nonshivering thermogenesis) in skeletal muscle and brown adipose tissue (BAT), in order to maintain thermal homeostasis in a cold environment.[1,3,4]

Under normothermic conditions, core body temperature is determined by basal metabolic organs such as the brain, heart, liver, and skeletal muscle. [1] Skeletal muscle is the largest weight-bearing organ in humans and consumes a substantial amount of energy, even at rest. Shivering is the major source of heat production in acute cold exposure before sufficient amount of UCP1 (uncoupling protein 1) can be recruited in BAT.[1,5,6]. Shivering is gradually replaced by UCP1 dependent thermogenesis and nonshivering thermogenesis is necessary for long-term adaptation to a cold environment. [5] Skeletal muscle and BAT thermogenesis can compensate in part each other to maintain core body temperature is widely recognaized[5–8], and recent study suggests that suppression of BAT thermogenesis has been found to promote thermogenesis in skeletal muscle.[8]

While skeletal muscle is the critical metabolic organ, it also stores an abundance of amino acids as energy substrates in the form of muscle proteins.[9] Skeletal muscle also has the plasticity for adapting to environmental conditions and shows metabolic flexibility in response to the substrate requirements of other organs and thereby contributes to the maintenance of energy balance.[9,10] Activation of BAT themogenesis is associated with increased lipid and glucose catabolism.[11–13] Succinate and branched-chain amino acids (BCAAs) have also been found to support BAT thermogenesis, however.[14–16] Although succinate may be provided by muscle contraction,[14,17] the primary source of BCAAs for BAT thermogenesis has remained unclear. Indeed, mitochondrial BCAAs catabolism in brown adipocytes promotes systemic BCAAs clearance. Skeletal muscle may therefore also respond to the energy demands of BAT.

Whereas body temperature and energy metabolism in metabolic organs are closely related, how they are regulated in a coordinated manner is poorly understood. Several endocrine factors alter metabolism in abdominal organs.[18] In particular, interleukin (IL)–6 is an endocrine factor that plays multiple roles in the response of the body to infection, exercise, and stress as well as in inflammation.[19–21] IL-6 induces various fever responses, including nonshivering thermogenesis in BAT, by promoting prostaglandin E2 (PGE2) synthesis in central vascular endothelial cells.[22] On the other hand, IL-6 directly affects energy metabolism in various organs, including skeletal muscle, liver, and adipose tissue.[23–27] These multiple roles suggest that IL-6 is a key molecule linking thermogenesis and energy metabolism.

Here we show that a loss of skeletal muscle function by cast immobilization in mice activates a compensatory thermoregulatory system dependent on BAT thermogenesis. We also show that free BCAAs derived from skeletal muscle support BAT thermogenesis, and we identify IL-6 as a key regulator of this system. Our findings reveal the importance of skeletal muscle as a source of amino acids and uncover a previously unrecognized thermoregulatory system mediated by BAT and skeletal muscle metabolism.

Results

Cast immobilization activates a compensatory thermoregulatory system

We first examined the role of skeletal muscle in endothermic homeostasis by performing an acute cold tolerance test at 4°C for 4 h in mice with both hind limbs immobilized in casts. We confirmed that ~50% of systemic skeletal muscle was immobilized (Table S1), and that the immobilization induced skeletal muscle atrophy within 3 to 5 days (**Figure 1_figure supplement 1A–1C**). Systemic locomotor activity was decreased during dark phase in cast immobilized mice compared to control mice (Figure1_ figure supplement 1D and 1E). Hence, the core body temperature showed a tended to decrease in cast-immobilized mice compared to control mice under room temperature (**Figure 1_figure supplement 1F**). Mice with both hind limbs immobilized for 7 days showed a significantly lower core body temperature after cold exposure compared with control mice (**Figure 1A**). Cast immobilization for 24 h also induced cold intolerance with preservation of muscle mass (**Figure 1B** and **Figure 1_figure supplement 1A-1C**). Expression of *Ucp1* mRNA in interscapular BAT (iBAT) was significantly increased after cold exposure in control mice, whereas those expression were not induced by cold exposure in cast immobilized mice (**Figure 1_figure supplement 1G**). Although the amount of *Sln* (sarcolipin) mRNA in immobilized muscle was not increased by acute cold exposure, expression of *Ucp2*, *Ucp3* and *Ppargc1a* (PGC-1α) were significantly increased in control mice but not in cast-immobilized mice (Figure 1_fugure supplement 1H-1K). To investigate whether cold intolerance depends on the immobilized muscle mass, we examined the change in core body temperature in mice with unilateral immobilization. Systemic locomotor activity during cold exposure was similar for mice with unilateral or bilateral immobilization and for control mice (**Figure 1_figure supplement 1L**). However, the decrease in body temperature induced by cold exposure in mice with unilateral immobilization was attenuated compared with that in mice subjected to bilateral immobilization (**Figure 1C**). These findings suggested that immobilization of skeletal muscle suppresses thermogenesis in this organ independently of systemic locomotor activity.

To investigate the mechanism underlying the maintenance of core body temperature in mice with cast immobilization, we examined the effects of such immobilization on skeletal muscle and BAT. Metabolomic profiling of the immobilized muscle revealed decreases in the amounts of fumaric acid and malic acid within 1 day of cast immobilization (Figure 1_fugure supplement 2A). The amount of *Ucp3* mRNA increased transiently in immobilized muscle before returning to baseline levels, whereas that of *Ucp2* mRNA was not affected until 5 days of cast immobilization (**Figure 1_figure supplement 2B and 2C**).

Sarcolipin signaling has been shown to promote mitochondrial biogenesis and oxidative metabolism via activation of Ca^{2+}- and calmodulin-dependent protein kinase II (CaMKII) and PGC-1α in skeletal muscle.[28] However, we found that expression of the *Sln, Camk2a, Ppargc1a* (PGC-1α), and *Tfam* (transcription factor A, mitochondrial) genes was not increased in immobilized muscle (**Figure 1_figure supplement 2D-2G**). These findings thus suggested that nonshivering thermogenesis was induced minimally in cast-immobilized skeletal muscle. On the other hand, expression of UCP1 at the mRNA and protein levels as well as the abundance of PGC-1α, a transcriptional cofactor that promotes the expression of thermogenesis-related genes, showed transient increases in iBAT after cast immobilization (**Figures 1E**–**1G**). Small and multilocular lipid droplets were apparent in adipocytes of iBAT within 24 h of cast immobilization (**Figure 1_figure supplement 2H**). However, mice subjected to cast immobilization showed no increase in food consumption or systemic activity levels (**Figure 1_figure supplement 1D and 1E** and **2I**). These results indicated that the suppression of muscle thermogenesis by cast

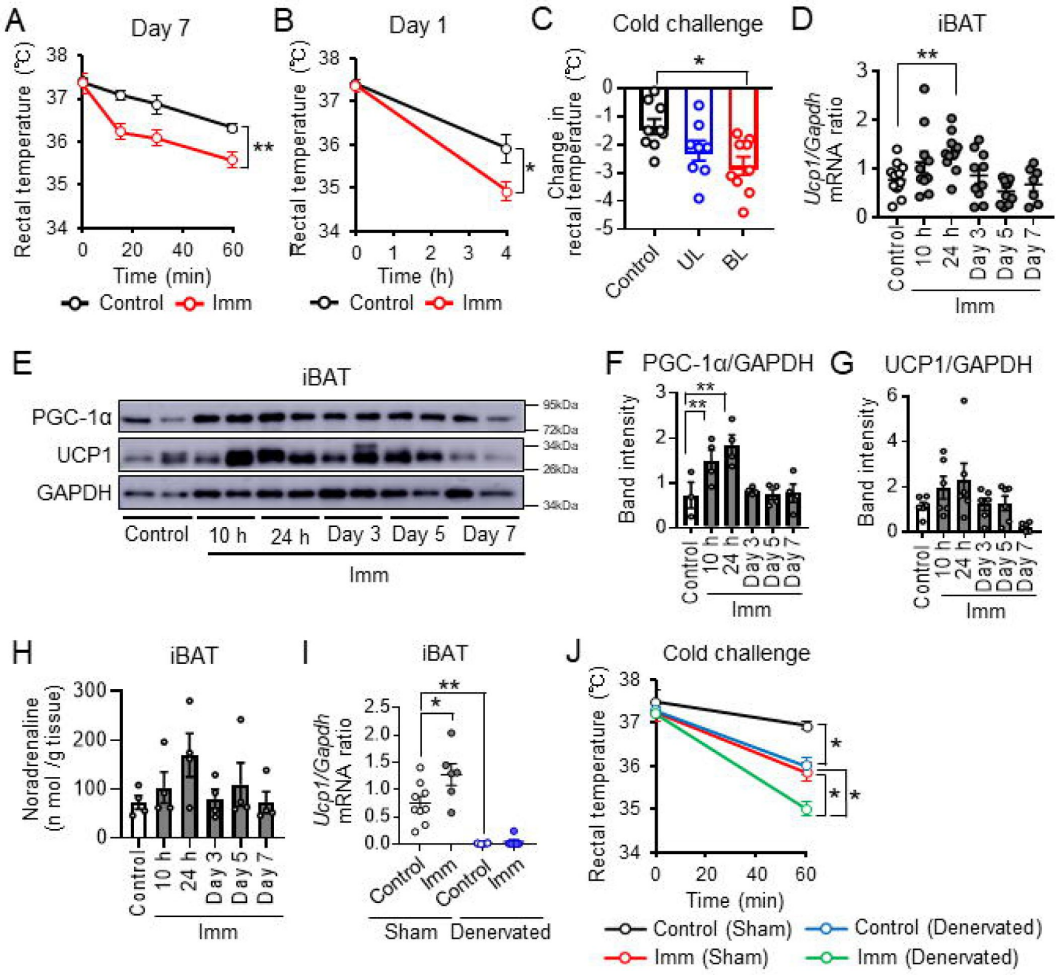

Figure 1.

Cast immobilization induces BAT thermogenesis via sympathetic activation.

(A) Time course of rectal core body temperature of cast-immobilized (gypsum, n = 6) and control (n = 5) mice subjected to a cold (4°C) challenge test. The test was performed 7 days after bilateral immobilization of hind limbs. (B) Rectal core body temperature of cast-immobilized (n = 5) and control (n = 5) mice during cold (4°C) exposure at 24 h after bilateral cast immobilization. (C) Decline in rectal temperature of cast-immobilized and control (n = 9) mice after cold (4°C) exposure for 4 h performed 2 h after unilateral (UL, n =8) or bilateral (BL, n = 9) immobilization. (D) Reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis of the *Ucp1/Gapdh* mRNA abundance ratio in iBAT of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 7 to 13 per group). (E–G) Immunoblot analysis of PGC-1α (n = 3 or 4 per group) and UCP1 (n = 6 per group) in iBAT of control mice and mice subjected to bilateral cast immobilization for the indicated times. GAPDH was examined as a loading control. Representative blots (E) and densitometric quantitation of the PGC- 1α/GAPDH (F) and UCP1/GAPDH (G) band intensity ratios are shown. (H) Noradrenaline concentrations in iBAT of cast-immobilized (n = 4) and control (n = 4) mice after subsequent biteral cast immobilization. (I) RT and real-time PCR analysis of the *Ucp1/Gapdh* mRNA abundance ratio in iBAT subjected to surgical denervation or sham surgery, either in control mice or in mice at 24 h after subsequent bilateral cast immobilization (n = 6–9 per group). (J) Rectal core body temperature of mice as in (I) subjected to cold (4°C) exposure for 1 h (n = 3 or 4 per group). All quantitative data are means ± SEM. *p < 0.05, **p < 0.01 as determined by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction (A, B, I and J), by one-way ANOVA followed by Tukey's post hoc test (C), by Dunnett's test (D, F, G and H), or by the unpaired t test (I). See also **Figure 1_figure supplement 1** and **2**, and Table S1.

immobilization induces BAT thermogenesis without an increase in food consumption or systemic activity levels. Cast immobilization thus preferentially activated nonshivering thermogenesis in BAT, not in skeletal muscle.

Cast immobilization promotes BAT thermogenesis via sympathetic activation

We next explored the mechanism underlying the activation of BAT thermogenesis by cast immobilization. We first focused on the sympathetic nervous system, a general inducer of BAT thermogenesis,[29] and found that the norepinephrine concentration in iBAT was tended to increase transiently after 24 h of cast immobilization (**Figure 1H**). We then subjected mice to sympathetic denervation of iBAT. Expression of *Ucp1* mRNA in iBAT was significantly attenuated by such denervation in mice with or without subsequent cast immobilization (**Figure 1I**). Whereas adipocytes of iBAT showed small and multilocular lipid droplets after 7 days of cast immobilization in sham-operated mice, iBAT of mice subjected to surgical denervation was hypertrophied and did not show such lipid droplets (**Figure 1_figure supplement 2J**). Moreover, mice with denervated iBAT manifested a lower core body temperature compared with sham-operated mice after cold exposure at 4°C for 1 h, and cast immobilization further increased the extent of hypothermia in the denervated mice (**Figure 1J**). These results suggested that BAT thermogenesis is activated via sympathetic nerves to maintain core body temperature in cast-immobilized mice.

Cast immobilization alters systemic metabolic dynamics associated with BAT thermogenesis

We next examined the metabolic dynamics of iBAT as well as systemic metabolic changes in mice subjected to cast immobilization. Previous study suggests that acute cold exposure on mice increases the free amino acids concentration and carbohydrate metabolites such as the glycolysis pathway, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle in BAT.[30] Consistent with previous findings, metabolomics analysis revealed increases in the amounts of carbohydrate metabolites and amino acids in iBAT that were apparent as early as 10 h after cast immobilization, and several glucose and amino acid metabolic pathways were significantly activated (**Figure 2_figure supplement 1A**).Increases in these metabolites after cast immobilization were not apparent in mice with iBAT denervation (**Figures 2A** and **2B**). Brown adipocytes incorporate fatty acids and various other energy substrates to initiate thermogenesis and contribute to the maintenance of core body temperature.[31] We found that expression of the genes for fatty acid (**Figure 2C**) and succinate (**Figure 2D**) transporters in iBAT was induced by cast immobilization and that such induction was prevented by iBAT denervation. The concentration of succinate was also increased in iBAT of cast-immobilized mice in a manner sensitive to iBAT denervation (**Figure 2E**). Consistent with these changes in metabolite levels, oxygen consumption and systemic lipid utilization were increased by cast immobilization in intact mice (**Figures 2F** and **2G**) but not in those subjected to iBAT denervation (**Figure 2_figure supplement 1B and 1C**).

The weight of epididymal white adipose tissue (eWAT) and hepatic glycogen content were reduced by cast immobilization (**Figures 2H** and **2I**). Expression of the genes for glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver increased gradually after cast immobilization, peaking at day 5 (**Figure 2_figure supplement 2A and 2B**), suggesting that gluconeogenesis was slowly activated in the cast- immobilized mice. Metabolomic profiling of the liver revealed marked increases in the amounts of 3-phosphoglyceric acid and 2-phosphoglyceric acid within 1 day of cast immobilization (**Figure 2_figure supplement 2C**), suggesting that glycerol influx was also enhanced in the liver. Together, these results thus indicated that cast immobilization affects metabolic dynamics in the liver and WAT, and that these changes might occur after the activation of BAT thermogenesis.

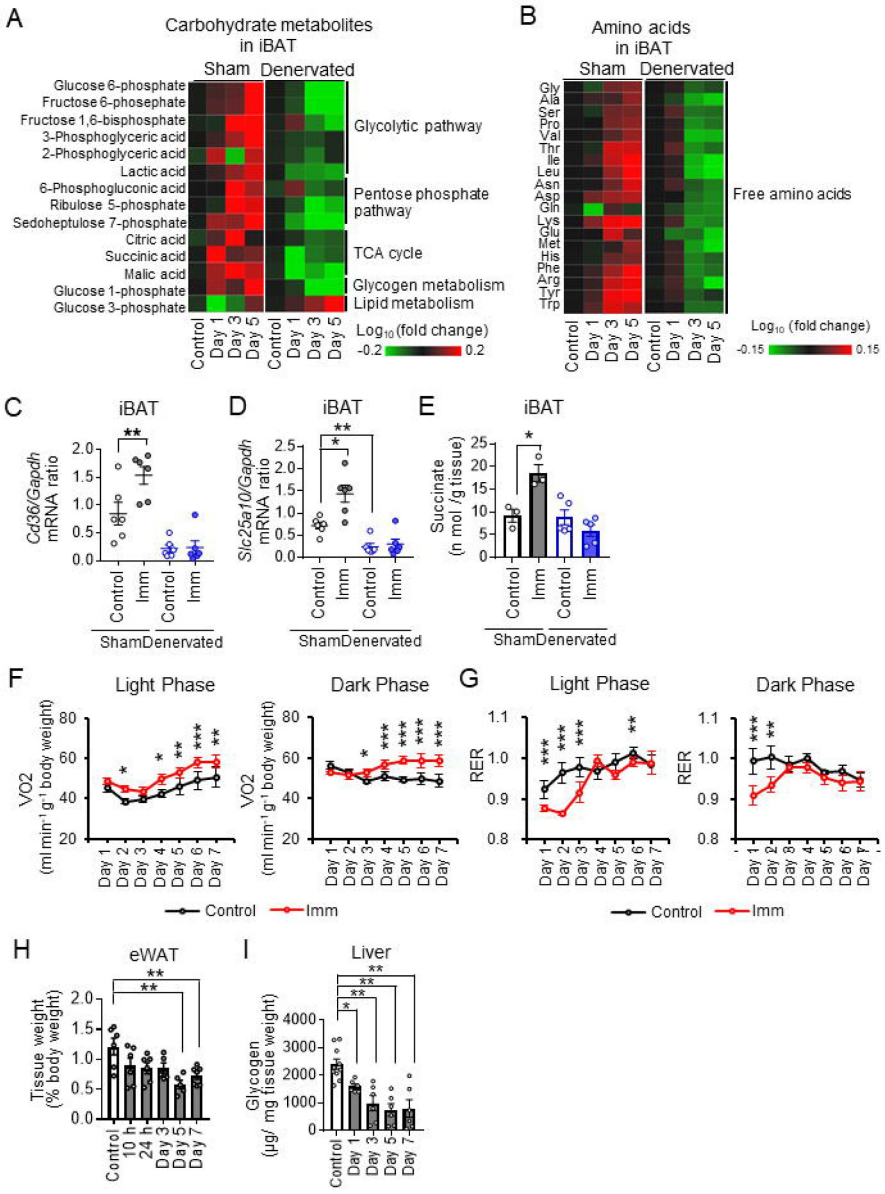

Figure 2.

Cast immobilization alters systemic metabolic dynamics associated with BAT thermogenesis.

(A and B) Concentrations of carbohydrate metabolites (A) and amino acids (B) in sham-operated or denervated iBAT of mice without (control) or at the indicated times after bilateral cast immobilization. The metabolomics data are presented as heat maps corresponding to the $\log_{10}$ value of fold change in cast- immobilized mice relative to the corresponding control mice and are means of four or five mice in each group. TCA, tricarboxylic acid cycle. (C and D) RT and real-time PCR analysis of mRNA abundance for the fatty acid transporter CD36 and the succinate transporter SLC25A10 in sham-operated or denervated iBAT of control mice or mice subjected to bilateral cast immobilization for 24 h (n = 6 per group). (E) Succinate content of sham-operated or denervated iBAT of control mice or mice subjected to bilateral cast immobilization for 24 h (n = 3–5 per group). (F and G) Oxygen consumption rate (VO_2) and respiratory exchange ratio (RER), respectively, for control mice and mice subjected to bilateral cast immobilization for 7 days (n = 6 per group). (H) Weight of eWAT for control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 5–7 per group). (I) Hepatic glycogen content for control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6–10 per group). Date in (C) through (I) are means ± SEM. *p < 0.05, **p < 0.01 as determined by one-way ANOVA followed by Tukey's post hoc test (C–E), by two-way ANOVA followed by Tukey's post hoc test or the unpaired post hoc t test with Bonferroni's correction (F and G), or by Dunnett's test (H and I). See also **Figure 2_figure supplement 1** and **2**.

The serum concentration of noradrenaline was increased within 1 day of cast immobilization (**Figure 2_figure supplement 2D**), but these metabolic changes induced by cast immobilization were not associated with by an increase in the serum concentration of corticosterone or in expression of the gene for corticotropin-releasing hormone (CRH) in the hypothalamus (**Figure 2_figure supplement 1E and 1F**). These results suggest that cast immobilization may activates BAT thermogenesis and systemic metabolic changes through lower body temperature resulting from suppression of muscle thermogenesis rather than stress.

Free amino acids are transferred from skeletal muscle to BAT and the liver for energy homeostasis

We next focused on metabolism of amino acids in BAT and skeletal muscle in mice after cast immobilization. As shown above, amino acid concentrations in iBAT were increased by cast immobilization in a manner sensitive to denervation of iBAT (**Figure 2B** and **Figure 2_figure supplement 1A**). The amounts of free amino acids were also increased in both soleus and extensor digitorum longus (EDL) muscles of cast-immobilized mice (**Figure 3A**), whereas those in serum and the liver were not increased by cast immobilization (**Figure 3B**). Consistent with previous findings,[32] we found that gene expression for the ubiquitin ligases Atrogin-1 and MuRF-1 was increased during the early phase (days 1–3) of cast immobilization (**Figure 3_figure supplement 1A and 1B**). We then examined gene expression profiles for solute carrier (SLC) transporters that mediate the transport of amino acids across cell membranes as well as for mitochondrial BCAA catabolic enzymes (BCAT2, branched-chain aminotransferase 2; BCKDHA, branched-chain keto acid dehydrogenase E1 subunit α) in iBAT, liver, and soleus. Expression of the genes for SLC1A5 (a sodium-dependent glutamate transporter) and SLC38A2 (a sodium-coupled neutral amino acid transporter) was significantly increased in iBAT after cast immobilization for 10 h (**Figure 3_figure supplement 1C–1H**). In the liver, expression of the gene for SLC38A2, which is upregulated by extracellular amino acid deprivation,[33,34] and of that for BCKDHA, which is thought to be upregulated under fasting conditions to oxidize BCAAs for gluconeogenesis,[35,36] was significantly increased by cast immobilization (**Figure 3_figure supplement 1I–1M**). On the other hand, gene expression for amino acid transporters (SLC1A5, SLC7A5, SLC38A2) and BCAAs catabolic enzymes (BCAT2, BCKDHA) in soleus was significantly decreased after cast immobilization (**Figure 3_figure supplement 1N–1S**). Expression of the gene for SLC43A1, which is upregulated by food deprivation and is thought to transport amino acids from cells to the external environment,[37] was significantly increased in soleus by cast immobilization (**Figure 3_figure supplement 1Q**).

We next investigated amino acid incorporation in tissues by administration of [^{3}H]leucine into the tail vein of mice. Incorporation of [^{3}H]leucine was not increased in skeletal muscle of mice subjected to cast immobilization for 1 or 3 days (data not shown), whereas it was significantly increased in iBAT, liver, and kidney of those subjected to cast immobilization for 1 day (**Figure 3C**). Gene expression for SLC25A44, a mitochondrial BCAAs transporter that contributes to BAT thermogenesis,[15] was increased in iBAT at 24 h after cast immobilization (**Figure 3D** and **Figure 3_figure supplement 2A-2C**). However, the early increases in expression of the genes for SLC25A44, SLC1A5, and SLC38A2 induced by cast immobilization in iBAT of intact mice were prevented by denervation of iBAT (**Figure 3D** and **Figure 3_figure supplement 1D–1J**). Our results thus suggested that free amino acids are utilized as an energy substrate in BAT and the liver rather than in skeletal muscle of cast-immobilized mice.

Cast immobilization upregulates IL-6 gene expression in BAT and skeletal muscle

We next set out to identify regulatory molecules that might contribute to the maintenance of body temperature and to the systemic metabolic changes in cast-immobilized mice. Gene expression analysis revealed that the IL-6 gene tended to be induced earlier and to a greater extent in iBAT

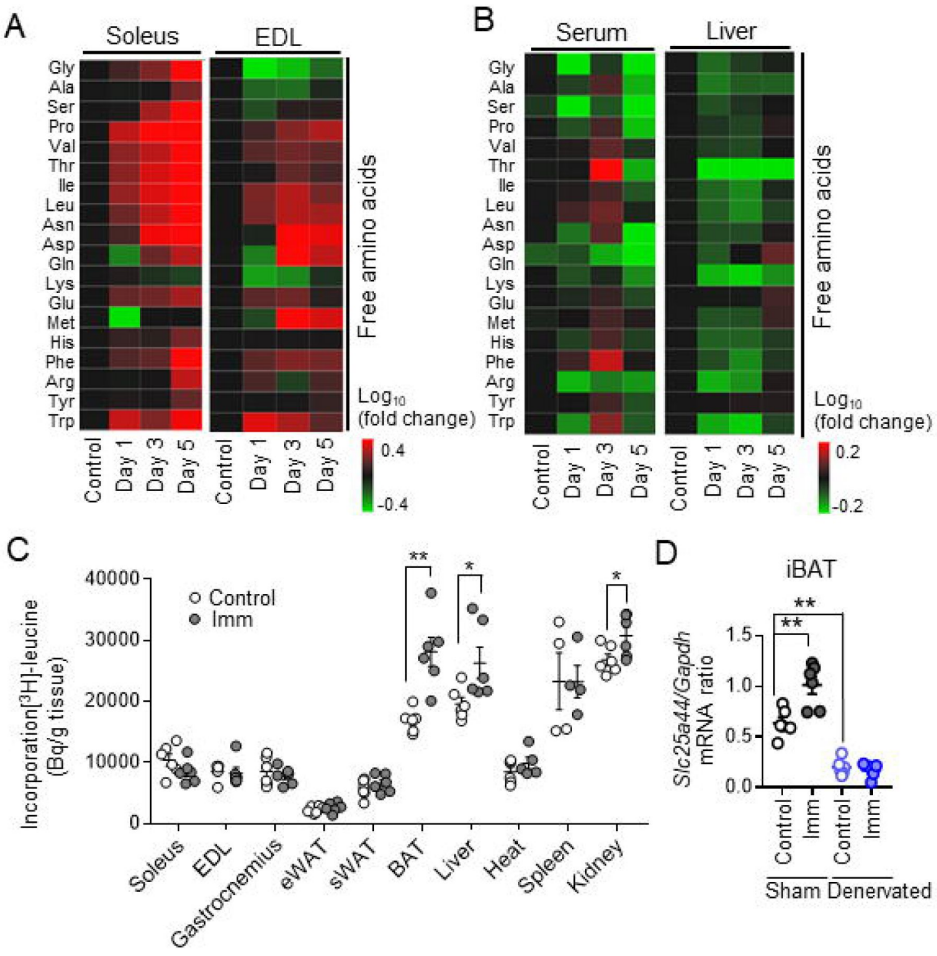

Figure 3.

Free amino acids are transferred from skeletal muscle to BAT and the liver for maintenance of energy homeostasis.

(A and B) Metabolomics analysis of amino acid concentrations in soleus and EDL muscles (A) as well as in serum and the liver (B) of control mice and mice subjected to bilateral cast immobilization for the indicated times. Results are presented as heat maps of the $\log_{10}$ value of fold change for cast-immobilized mice relative to control mice and are means of three mice per group. (C) Organ-specific [^{3}H]leucine uptake in control mice and mice subjected to bilateral cast immobilization for 24 h (n = 4–6 per group). sWAT, subcutaneous WAT. (D) RT and real-time PCR analysis of SLC25A44 gene expression in sham- operated or denervated iBAT of control mice and mice subjected to subsequent bilateral cast immobilization for 24 h (n = 6 per group). Data in (D) and (E) are means ± SEM. *p < 0.05, **p < 0.01 as determined by the unpaired t test (C) or by one-way ANOVA followed by Tukey's post hoc test (D). See also **Figure 3_figure supplement 1** and **2**.

and soleus than were genes for other cytokines including tumor necrosis factor–α (TNF-α), monocyte chemoattractant protein–1 (MCP-1), IL-1β, IL-10, and IL-15; for batokines including fibroblast growth factor 21 (FGF21) and bone morphogenetic protein 8B (BMP8B); or for myokines including FGF21 and irisin (**Figures 4A**–**4D** and **Figure 4_figure supplement 1A–1N**). Whereas the increase in the amount of *Il6* mRNA was apparent as early as 6 to 10 h in iBAT, that in soleus was more gradual (**Figures 4C** and **4D**). IL-6 has been found to be released by various organs, including adipose tissue and skeletal muscle, in response to inflammation, stress, or exercise.[18–21] The abundance of *Il6* mRNA in liver, eWAT, or spleen was not affected by cast immobilization (**Figure 4_figure supplement 1O–1Q**). Expression of the genes for serum amyloid A3 (*Saa3*) and suppressor of cytokine signaling 3 (*Socs3*), both of which are regulated by IL-6 signaling, was also increased in iBAT and soleus at the same time as was that of *Il6* (**Figures 4E**–**4H**). Expression of these genes was also increased in the liver or eWAT at 10 or 24 h after cast immobilization (**Figures 4I**–**4L**). However, expression of *Il6* was not induced by cast immobilization in denervated iBAT (**Figures 4M**). The serum concentration of IL-6 was significantly increased from 10 h after cast immobilization (**Figure 4N**), whereas that of TNF-α was not altered in cast-immobilized mice (**Figure 4_figure supplement 1R**). Together, these results suggested that cast immobilization induces *Il6* expression in iBAT and skeletal muscle, and that induction of *Il6* expression by cast immobilization in iBAT is dependent on the sympathetic nervous system.

IL-6 affects energy metabolism in BAT and skeletal muscle of cast- immobilized mice

We investigated the role of IL-6 in the skeletal muscle–BAT thermoregulatory system with the use of subjected IL-6 knockout (KO) mice to cast immobilization. Expression of *Ucp1* in iBAT was significantly increased at 24 h after cast immobilization in both WT and IL-6 KO mice (**Figure 5_figure supplement 1A**). However, core body temperature of cast-immobilized IL-6 KO mice was decreased to a greater extent by cold exposure at 4°C for 4 h compared with that of cast-immobilized WT mice (**Figure 5A**). Furthermore, unlike that in WT mice, oxygen consumption was not increased in IL-6 KO mice after cast immobilization (**Figure 5_figure supplement 1B and 1C**).

Metabolomics analysis revealed that the amounts of carbohydrate metabolites including glucose 1-phosphate, glucose 6-phosphate, and phosphoenolpyruvic acid in iBAT were higher for IL-6 KO mice than for WT mice and were not increased substantially by cast immobilization in the mutant mice (**Figure 5B**). Whereas the concentrations of amino acids such as Ser, Asn, Lys, Met, His, Phe, and Tyr in iBAT of WT mice were increased markedly at 24 h after cast immobilization, such was not the case for IL-6 KO mice (**Figure 5C**). Expression of the genes for SLC25A44, BCKDHA, and CD36 in iBAT was higher for IL-6 KO mice than for WT mice, but expression of the genes for SLC25A44 and CD36 in iBAT of IL-6 KO mice was not increased further at 24 h after cast immobilization (**Figures 5D**–**5F**). Incorporation of [^{3}H]leucine was also not increased in iBAT of IL-6 KO mice after cast immobilization for 24 h (**Figure 5G**). In addition, adipocytes with small and multilocular lipid droplets were observed in iBAT of IL-6 KO mice with or without cast immobilization (**Figure 5_figure supplement 1D**). The respiratory exchange ratio (RER) of WT mice was significantly decreased in both the light and dark phases for the first 5 days of cast immobilization, whereas similar differences were apparent only for the first 2 days in IL-6 KO mice (**Figure 5_figure supplement 1E and 1F**). IL-6 has been shown to promote lipolysis and fatty acid oxidation in WAT.[26] We found that, unlike in WT mice, cast immobilization did not result in a loss of eWAT mass in IL-6 KO mice (**Figure 5_figure supplement 1G**). Collectively, these data suggested that utilization of energy substrates was not substantially altered by cast immobilization in IL-6 KO mice.

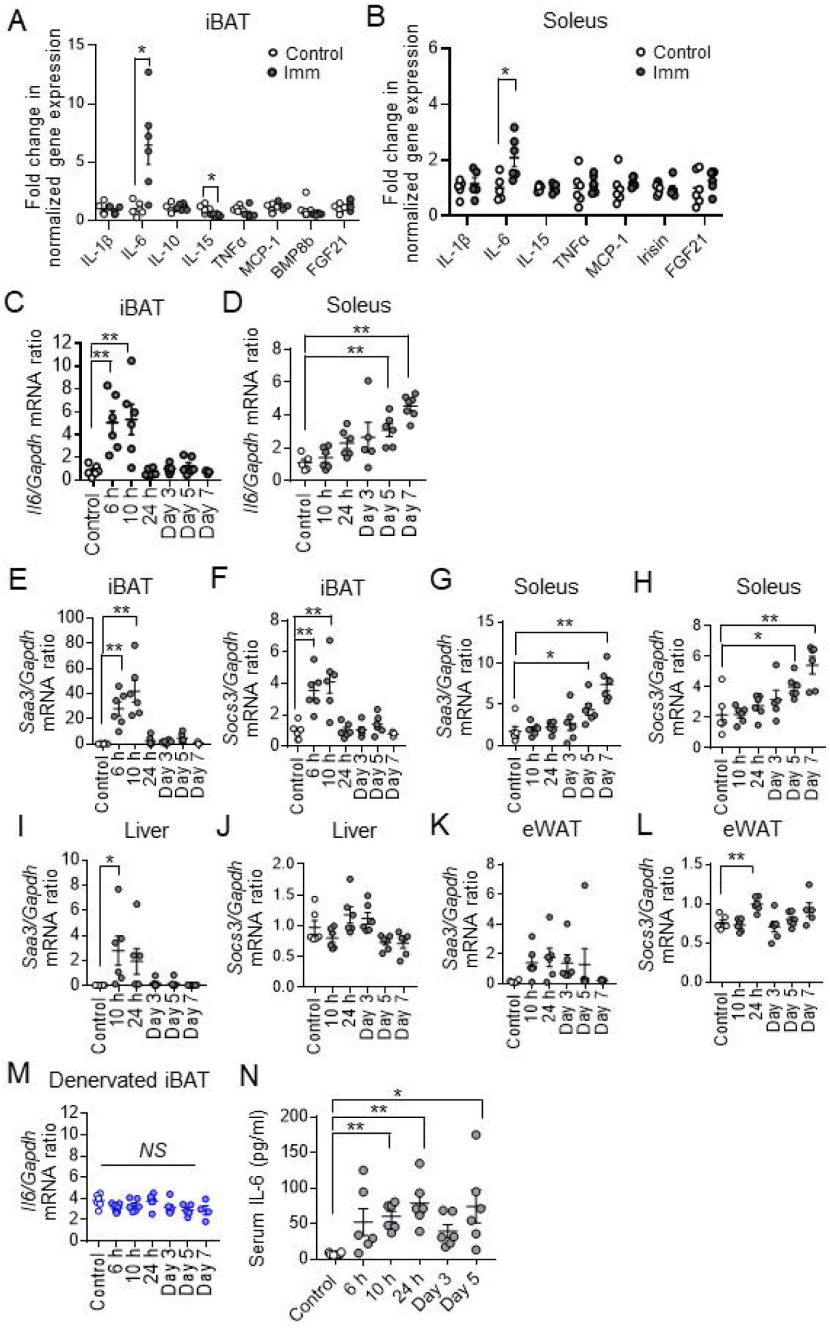

Figure 4.

Cast immobilization upregulates *Il6* expression in BAT and skeletal muscle.

(A and B) RT and real-time PCR analysis of IL-1β, IL-6, IL-10, IL-15, TNF-α, MCP-1, BMP8B, FGF21, and irisin gene expression in iBAT (A) or soleus (B) of control mice or mice at 10 h (iBAT) or 24 h (soleus) after bilateral cast immobilization (n = 5 or 6 per group). (C and D) RT and real-time PCR analysis of *Il6* expression in iBAT (C) and soleus (D) of control mice and mice at the indicated times after bilateral cast immobilization (n = 3–7 per group). (E–L) RT and real-time PCR analysis of *Saa3* (E, G, I, and K) and *Socs3* (F, H, J, and L) mRNA abundance in iBAT (E and F), soleus (G and H), liver (I and J), and eWAT (K and L) of control mice and mice at the indicated times after bilateral cast immobilization (n = 3–7 per group) (M) RT and real-time PCR analysis of *Il6* expression, respectively, in denervated iBAT of control mice or mice at the indicated times after bilateral cast immobilization (n = 4-7 per group). (N) Serum IL-6 concentration in control mice or mice at the indicated times after bilateral cast immobilization (n = 6 per group). All data are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by the unpaired t test (A and B) or by Dunnett's test (C–N). See also **Figure 4_figure supplement 1**.

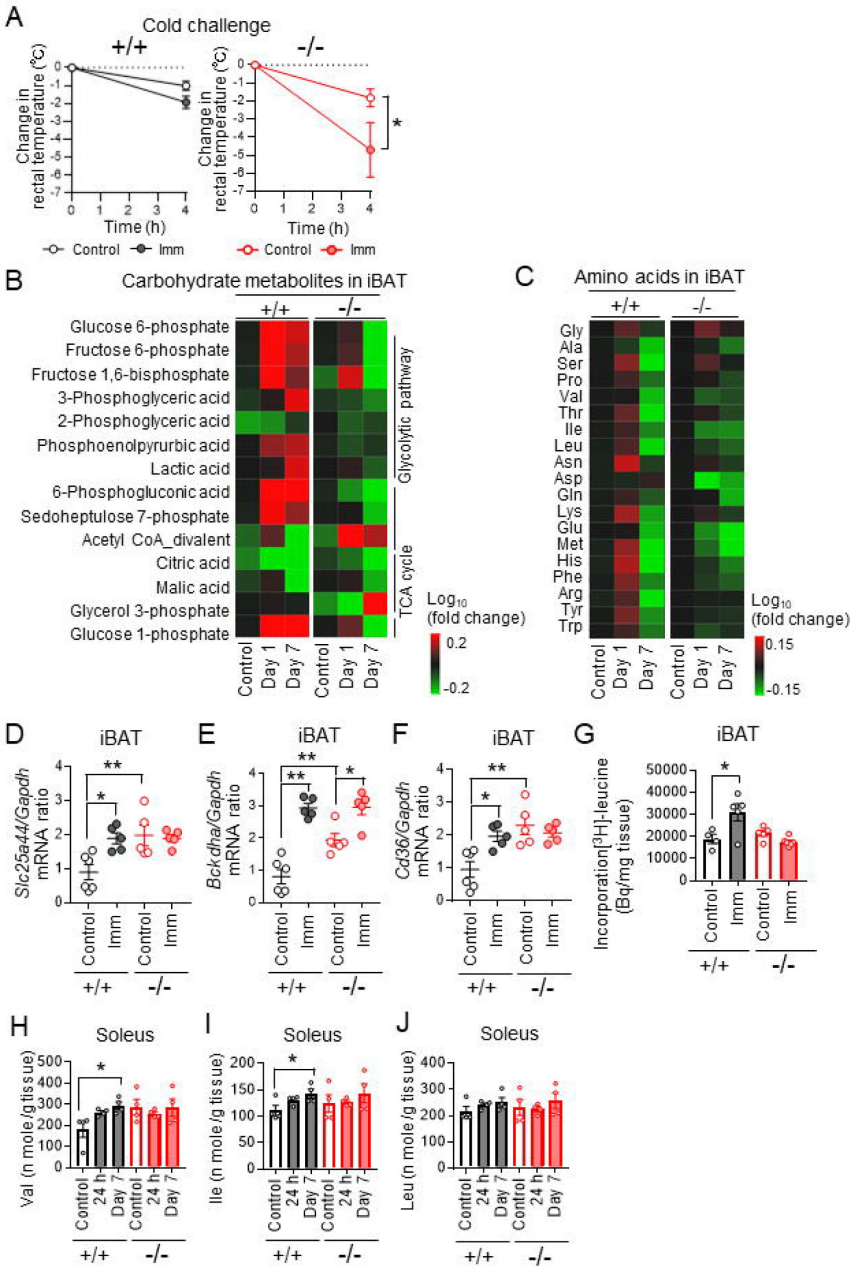

Figure 5.

IL-6 affects energy metabolism in BAT and skeletal muscle of cast-immobilized mice.

(A) Change in rectal body temperature during cold exposure at 4°C for 4 h for WT (n = 4 or 5) and IL-6 KO (n = 4 or 5) mice that had been subjected (or not) to bilateral cast immobilization for 24 h. (B and C) Concentrations of carbohydrate metabolites (B) and amino acids (C) in iBAT of IL-6 KO (–/–) and WT (+/+) mice without (control) or with bilateral cast immobilization for the indicated times. Results are presented as heat maps of the $\log_{10}$ value of the fold change relative to control WT mice and are means of four mice in each group. (D–F) RT and real-time PCR analysis of the expression of SLC25A44 (D), BCKDHA (E), and CD36 (F) genes in iBAT of IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 24 h (n = 5 or 6). (G) Incorporation of [^{3}H]leucine in iBAT of IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 24 h (n = 4 or 5 per group). (H–J) BCAA (Val, Ile, and Leu, respectively) concentrations in soleus of IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 1 or 7 days (n = 4 per group). Data in (A) and (D) through (J) are means ± SEM. *p < 0.05, **p < 0.01 as determined by one-way ANOVA followed by Tukey's post hoc test (D–G), by Dunnett's test (H–J), or by two-way ANOVA followed by Tukey's post hoc test (A). See also **Figure 5_figure supplement 1**.

The amounts of BCAAs in soleus were significantly increased after cast immobilization in WT mice but not in IL-6 KO mice (**Figures 5H**–**5J**). Serum BCAA concentrations were decreased after cast immobilization in both WT and IL-6 KO mice (**Figure 5_figure supplement 1H–1J**). These findings suggested that deficiency of IL-6 impairs the maintenance of core body temperature and alters energy metabolism in BAT and skeletal muscle in mice subjected to cast immobilization.

Administration of IL-6 increases BCAA concentrations in skeletal muscle of cast-immobilized IL-6 KO mice

We examined whether administration of exogenous IL-6 might increase amino acid concentrations in skeletal muscle and BAT of IL-6 KO mice with cast immobilization. Administration of IL-6 restored the increase in the amounts of amino acids including BCAAs in soleus of IL-6 KO mice with cast immobilization (**Figures 6A**–**6C** and **Figure 6_figure supplement 1A**). Treatment with IL-6 also increased expression of *Saa3* and *Socs3* (**Figure 6_figure supplement 1B and 1C**) as well as the amounts of BCAAs (**Figure 6D**) in cultured mouse C2C12 myotubes. However, the amounts of carbohydrate metabolites in iBAT of cast-immobilized IL-6 KO mice were not increased by IL-6 administration (**Figure 6_figure supplement 1D**). In contrast to its effects on soleus, administration of IL-6 also did not increase the amounts of amino acids in iBAT of IL-6 KO mice with cast immobilization (**Figure 6_figure supplement 1A and 1E**). In addition, exogenous IL-6 did not increase the expression of UCP1, SLC25A44, and BCKDHA genes in iBAT of these mice (**Figure 6_figure supplement 1F–1H**). On the other hand, administration of IL-6 ameliorated the cold intolerance of IL-6 KO mice with cast immobilization (**Figure 6E**). We therefore examined whether IL-6 might increase core body temperature via the sympathetic nervous system with the use of C57BL/6J mice with denervated iBAT. Core body temperature of cold-exposed mice with cast immobilization and denervated iBAT was not significantly increased by administration of IL-6 (**Figure 6F**).

We also found that the core body temperature of IL-6 KO mice was lower than that of WT mice after cold exposure at 4°C for 4 h (**Figure 6_figure supplement 1I**) whereas the amount of *Ucp1* mRNA in iBAT was significantly increased for IL-6 KO mice compared with WT mice at room temperature (**Figure 6_figure supplement 1J**). We then investigated the possible direct effects of IL-6 on energy metabolism in BAT with the use of cultured mouse brown adipocytes. Expression of the SLC25A44, BCKDHA, and UCP1 genes in the brown adipocytes were not increased by treatment with IL-6 (**Figure 6_figure supplement 1K–1M**). In contrast, treatment with the β_3-adrenergic receptor agonist CL316 243 increased the expression of these genes, and this upregulation was suppressed by treatment with IL-6 (**Figure 6_figure supplement 1K–1M**). Long-term administration of CL316 243 in mice was previously shown to increase expression of *Ucp1*, *Slc25a44*, and *Bckdha* in BAT.[16] We found that administration of CL316 243 for 7 days significantly increased the amounts of *Ucp1*, *Slc25a44*, and *Bckdha* mRNAs in iBAT of IL-6 KO mice but not in that of WT mice (**Figure 6_figure supplement 1N–1P**).

Together, these data suggested that IL-6 may suppress β-adrenergic receptor– mediated thermogenesis and BCAA metabolism in brown adipocytes.

Nevertheless, IL-6 induces fever response through stimulation of the central[21,22] and uptake of energy-rich BCAA delivered to BAT from skeletal muscle may facilitate a net induction of BAT thermogenesis.

Skeletal muscle–derived IL-6 increases BCAA concentrations in skeletal muscle for BAT thermogenesis

Given that our data suggested that an elevated blood concentration of IL-6 contributes to maintenance of body temperature in cast-immobilized mice in a manner dependent on the sympathetic nervous system (**Figures 6E** and **6F**), we sought to determine the main source of

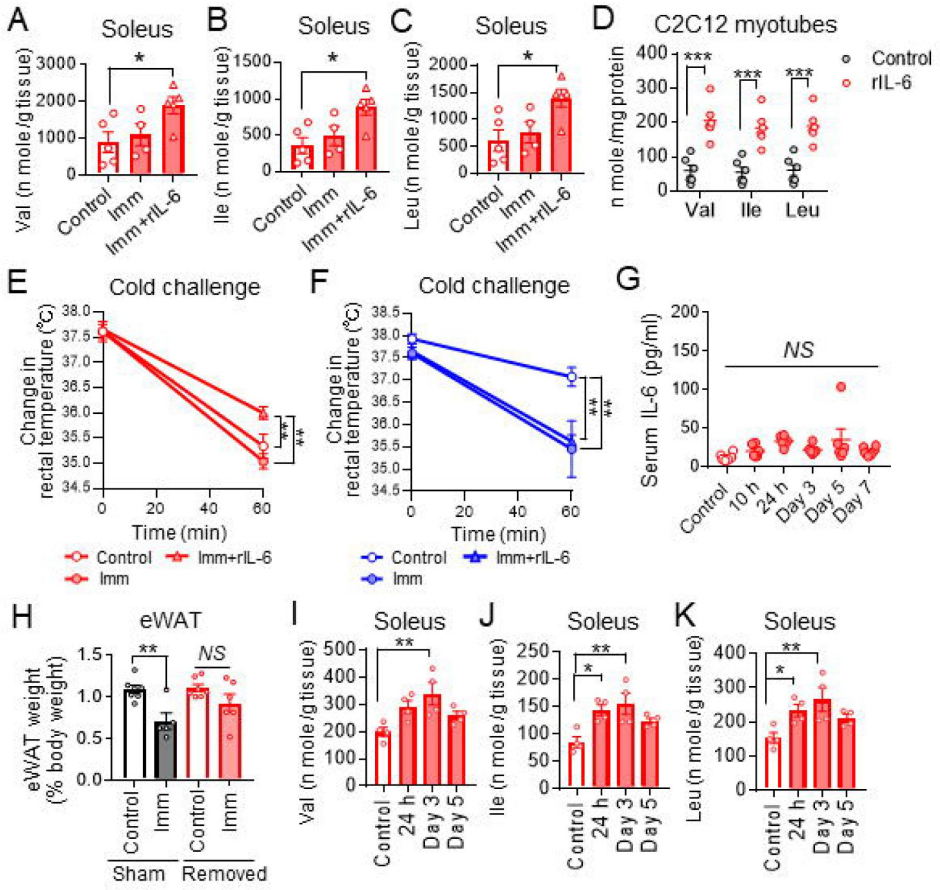

Figure 6.

Skeletal-muscle-derived IL-6 increases BCAA concentrations in skeletal muscle for support of BAT thermogenesis.

(A–C) Concentrations of BCAAs (Val, Ile, and Leu, respectively) in the soleus of control IL-6 KO mice and at 3 h after intraperitoneal administration of recombinant IL-6 (rIL-6, 400 ng) or vehicle in IL-6 KO mice that had been subjected to bilateral cast immobilization for 24 h (n = 4 or 5 per group). (D) BCAA concentrations in C2C12 myotubes incubated in the absence or presence of rIL-6 (50 ng/ml) for 1 h (n = 6 independent experiments) . (E) Rectal core body temperature during cold exposure at 4°C for 1 h for control IL-6 KO mice and for IL-6 KO mice subjected to bilateral cast immobilization for 24 h and injected intraperitoneally with rIL-6 (400 ng) or vehicle 30 min before cold challenge (n = 5 per group). The white circles represent control mice, the red circles indicate cast-immobilized mice, and the red triangles represent cast- immobilized mice that were treated with recombinant IL-6 (rIL-6). (F) Rectal core body temperature during cold exposure at 4°C for 1 h for C57BL/6J mice with denervated iBAT subjected or not to bilateral cast immobilization and treatment with rIL-6 or vehicle as in (E) (n = 4 per group). (G) Serum IL-6 concentration of mice subjected to surgical removal of iBAT and then subjected (or not, control) to bilateral cast immobilization for the indicated times (n = 4-6 per group). The white circles represent control mice, the blue circles indicate C57BL/6J mice with denervated iBAT, and the blue triangles denote C57BL/6J mice with denervated iBAT that were treated with recombinant IL-6 (rIL-6). (H) Weight of eWAT in mice subjected to surgical removal of iBAT (or to sham surgery) followed (or not, control) by bilateral cast immobilization for 7 days (n = 5-7 per group). (I–K) Concentrations of BCAAs (Val, Ile, and Leu, respectively) in soleus of mice subjected to surgical removal of iBAT and then subjected (or not, control) to bilateral cast immobilization for the indicated times (n = 4 per group). All data are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by Dunnett's test (A–C and G and I-K), or by the unpaired t test (D) or by one-way ANOVA followed by Tukey's post hoc test (H), or by two-way ANOVA followed by the post hoc paired/unpaired t test with Bonferroni's correction (E and F). See also **Figure 6_figure supplement 1** and **2**.

circulating IL-6 in cast-immobilized mice. We first focused on the acute expression of the IL-6 gene apparent in iBAT after cast immobilization (**Figure 4C**), and we subjected mice to surgical removal of iBAT. The serum IL-6 concentration of such mice was not increased after cast immobilization (**Figure 6G**). We also found that the reduction in eWAT mass after cast immobilization in C57BL/6J mice was suppressed by surgical removal of iBAT (**Figure 6H**). These results suggested that BAT-derived IL-6 induces lipolysis in WAT.

We further investigated the effects of BAT-derived IL-6 on skeletal muscle. Whereas the serum concentration of IL-6 in mice depleted of iBAT was not affected by cast immobilization (**Figure 6G**), the concentrations of BCAAs in soleus were increased after cast immobilization in such mice (**Figures 6I**–**6K**). The amount of *Slc43a1* mRNA in soleus of mice depleted of iBAT was significantly increased by cast immobilization (**Figure 6_figure supplement 2A**). Gene expression for other amino acid transporters (such as SLC1A5 and SLC7A5) and for BCAA catabolic enzymes (BCAT2 and BCKDHA) in soleus also showed a similar pattern of changes after cast immobilization in iBAT- depleted mice (**Figure 6_figure supplement 2B–2E**) as in intact mice (**Figure 3_figure supplement 1L**, 1M, 1P, and 1Q). Moreover, cast immobilization for 7 days induced similar losses of both soleus and gastrocnemius mass in mice with or without removal of iBAT (**Figure 6_figure supplement 2F** and Table S2). These data thus suggested that IL-6 secretion in an autocrine or paracrine manner may regulate BCAA metabolism in skeletal muscle independently of IL- 6 derived from iBAT.

Acute cold exposure or restraint stress also induced IL-6 from skeletal muscle to supply BCAA for BAT thermogenesis

We further found that expression of *Il6* was also increased in not immobilized fore limb muscles (biceps brachii muscle; BBM) after cast immobilization (**Figure 7_figure supplement 1A**). Expression of *Saa3* and *Socs3* were increased in BBM at the same time as was that of *Il6* after cast immobilization (**Figure 7_figure supplement 1B and 1C**). Furthermore, gene expression for the ubiquitin ligases Atrogin-1 and MuRF-1 in BBM was increased during the early phase of cast immobilization (**Figure 7_figure supplement 1D** and 1E). BCAA concentrations in BBM were also increased after cast immobilization (**Figure 7_figure supplement 1F-1H**). These data suggest that cast immobilization stimulates BCAA metabolites in not immobilized fore limb muscles as well as immobilized muscle.

Finally, we investigated whether the thermoregulatory system through amino acid metabolism in the interaction between BAT and skeletal muscle also plays an important role in other conditions, such as cold temperature and restraint stress. Acute cold exposure increased expression of *Il6* only in iBAT and skeletal muscle in mice (**Figure 7A**), and expression of *Saa3* and *Socs3* were increased in soleus (Figures 7_figure supplement 1I and 1J). Gene expression for the ubiquitin ligases Atrogin-1 and MuRF-1 was not significantly increased in soleus (**Figure 7_figure supplement 1K and 1L**), but both soleus and gastrocnemius mass were decreased by cold exposure at 4°C for 4 h (**Figure 7_figure supplement 1M and 1N**). Furthermore, the amounts of valine in soleus were significantly increased by acute cold exposure in WT mice but not in IL-6 KO mice (**Figures 7B**-**7D** and **Figure 7_figure supplement 1O**). BCAA concentrations in soleus were also increased after cold exposure at 4°C for 4 h in iBAT-depleted mice (**Figure 7_figure supplement 1P**).

Restraint stress for 6 h on mice increased expression of *Ucp1* in iBAT (**Figure 7_figure supplement 2A**), and the amounts of *Il6* mRNA was increased in iBAT and skeletal muscle (**Figures 7E** and **7F**). Expression of *Saa3* and *Socs3* were tended to increase in soleus (Figures 7_figure supplement 2B and 2C). Skeletal muscle mass was not decreased by restraint stress for 6 h (**Figure 7_figure supplement 2D-2F**), but gene expression for the ubiquitin ligases Atrogin-1 and MuRF-1 were increased after restraint stress (**Figure 7_figure supplement 2G and 2H**). BCAA concentrations in soleus were tended to increased by restraint stress in WT mice but not in IL-6 KO

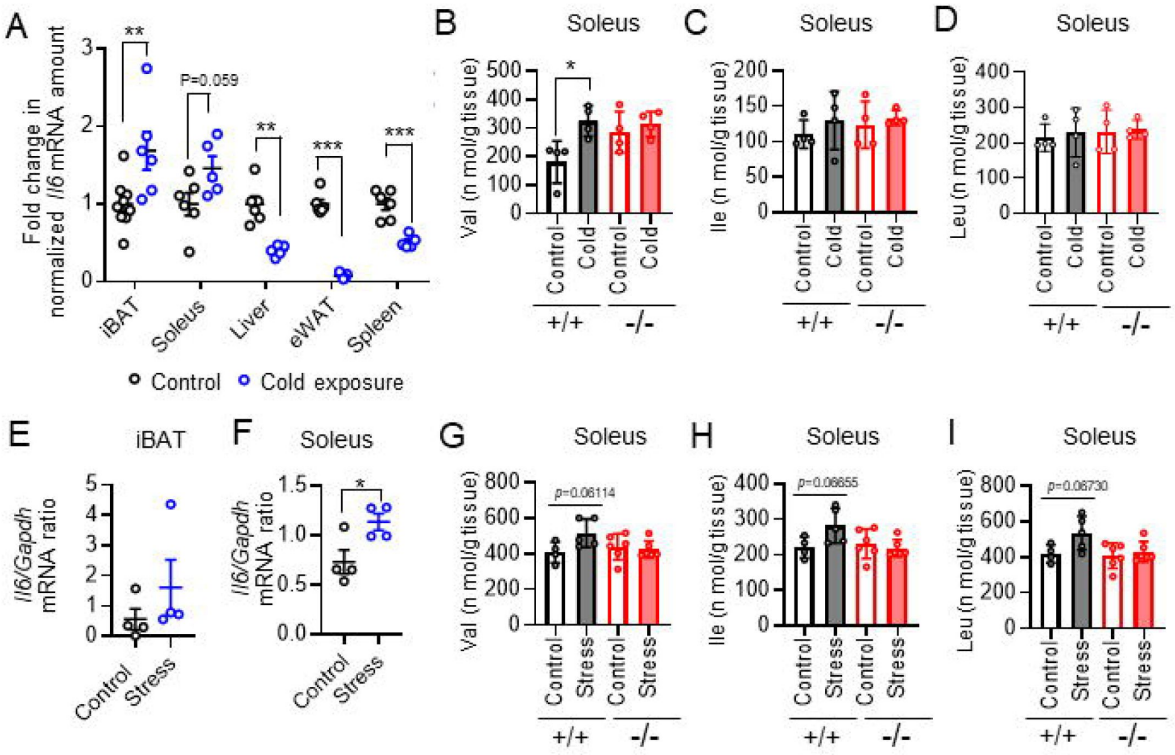

Figure 7.

Acute cold exposure or restraint stress also induced IL-6 from skeletal muscle to supply BCAA for BAT thermogenesis.

(A) RT and real-time PCR analysis of *Il6* expression in iBAT, soleus, liver, eWAT, and spleen of mice subjected (or not) to cold (4°C) exposure for 4 h (control, n = 6–9; cold exposure, n = 5 or 6). (B–D) Concentrations of BCAAs (Val, Ile, and Leu, respectively) in soleus of WT and IL-6 KO mice subjected (or not) to cold exposure at 4°C for 4 h (n = 4 per group). (E and F) RT and real-time PCR analysis of *Il6* expression in iBAT (E) and soleus (F) of control mice and mice subjected to restraint stress for 6 h (n = 4 per group). (G–I) Concentrations of BCAAs (Val, Ile, and Leu, respectively) in soleus of WT and IL-6 KO mice subjected (or not) to restraint stress for 6 h (n = 4 per group). All data are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by the unpaired t test (A–I). See also **Figure 7_figure supplement 1** and **2**.

mice (**Figures 7G**-**7I** and **Figure 7_figure supplement 2I**). Together, thermoregulatory system through amino acid metabolism in skeletal muscle and BAT may be activated under cold temperature and acute stress.

Discussion

We have shown here that cast immobilisation leads to a passive loss of skeletal muscle function, resulting in failure to maintain adequate core body temperature in a cold environment. Cast immobilization also activated BAT thermogenesis via the sympathetic nervous system and triggered systemic changes in energy metabolism associated with BAT thermogenesis. Furthermore, we found that free BCAAs derived from skeletal muscle serve as substrates for energy metabolism in BAT, and that skeletal muscle–derived IL-6 promotes this provision of BAT with amino acids from muscle. In addition, this thermoregulatory system between BAT and skeletal muscle may also be activated in response to cold temperature or acute stress.

A first aim of our study was to investigate the role of skeletal muscle in thermogenesis. In this study, mice with impairment of hind limb muscle contraction by cast immobilization were used as a model for a loss of muscle function. Exercise-induced muscle contraction generates large amounts of heat which dependents on hydrolysis of ATP, and skeletal muscle, as the first organ to be recruited for thermogenesis, plays an important role in maintenance of body temperature in endotherms.[38] Muscle thermogenesis can account for up to 90% of systemic oxygen consumption during periods of maximal recruitment of muscle contraction, such as during exercise or an intense bout of shivering.[38,39] We found that cast immobilization decreased the amounts of metabolites in TCA cycle and suppressed expression of mitochondrial-related genes from the early stage of immobilization, suggesting that metabolic rate is rapidly decreased in immobilized muscle. On the other hands, thermoregulatory system in endotherms cannot be explained by thermogenesis based on muscle contraction alone, with nonshivering thermogenesis being required as a component of the ability to tolerate cold temperatures in the long term[1]. Our results now show that expression of the gene for sarcolipin, a key regulator of the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) and an important mediator of muscle nonshivering thermogenesis, was not increased in muscle after cast immobilization. In contrast, we have now shown that UCP3 gene expression in muscle was transiently increased during the early stage of cast immobilization. Although the importance of UCP3 for mitochondrial energy metabolism is well established,[40,41] its physiological function as a mitochondrial uncoupler remains unclear.[42,43] In addition, we found that several genes induced by cold stimulation in skeletal muscle were not increased in cast-immobilized mice. Our results thus show that metabolic thermogenesis in skeletal muscle is crucial for mammals, and that cast immobilization increases cold intolerance, even in BAT-enriched mammals such as rodents. Although our study showed that cold intolerance in mice was observed after just 2 h of cast immobilization, these results could also be attributed not only to loss of skeletal muscle function but also to stress, decreased calorie reserves, or reduced systemic locomotor activity.

In contrast to skeletal muscle, BAT thermogenesis was activated via the sympathetic nervous system under room temperature even when skeletal muscle was immobilized. It is a well-recognized fact that BAT and skeletal muscle function in an orchestrated manner to maintain core body temperature in endotherms[5–7]. A bit surprisingly, the capacity of the BAT itself was insufficient to maintain stable body temperature during acute cold exposure. Our results also suggest that the increase in noradrenaline concentrations in BAT or in blood are transient, suggesting that the activation of sympathetic neve activity after cast immobilization is also transient. Consequently, the expression of thermogenic genes and metabolic changes in BAT may also have been induced to peak after 24 h of cast immobilization. In addition, it has been reported that long-term cast immobilization increases nonshivering thermogenesis in immobilized muscle.[44] Our results show that the expression of *Ucp2* and *Sln* were increased in immobilized

muscle after 7 days of cast immobilization. These data suggest that the maintenance of core body temperature by BAT thermogenesis may be an important system during short-term cast immobilization.

We also found that changes in systemic energy metabolism induced by cast immobilization were associated with the activation of BAT thermogenesis. Activation of BAT results in the tissue becoming a high consumer of a variety of energy substrates including lipids, glucose, BCAAs, succinate, and lactate.[31] Previous findings reported that mitochondrial BCAA catabolism in brown adipocytes promotes systemic BCAA clearance, suggesting that BCAAs may be supplied to BAT from other organs during BAT thermogenesis. Our results suggest that skeletal muscle is a source of free amino acids for BAT thermogenesis or hepatic gluconeogenesis. In response to metabolic demands imposed by starvation, exercise, or cold exposure, for example, skeletal muscle manifests metabolic flexibility in order to meet the demands of other organs and is an important determinant of metabolic homeostasis.[9,10] In contrast, the reduction in metabolic rate may contribute to protein conservation and maintain skeletal muscle mass under hibernation.[9] Our findings now provide important insight into the role of skeletal muscle as a source of amino acids. However, a recent study also suggests that a highly active proteolysis system in the heart provides substantial amounts of amino acids for distribution to other organs via the bloodstream.[45] The heart may therefore be another source of free BCAAs for BAT thermogenesis in cast-immobilized mice.

We found that muscle-derived IL-6 directly increased the abundance of BCAAs in skeletal muscle after cast immobilization. Previous studies have shown that excessive IL-6 induces amino acid catabolism in skeletal muscle,[46,47] and that IL-6 is one of the factors responsible for the induction of muscle atrophy in cast-immobilized mice.[48] IL-6 is a secreted factor induced by several mechanisms including muscle contraction, intracellular calcium signaling, and inflammation.[20,49] However, inflammation associated with macrophage infiltration is not thought to be induced in the early stages of muscle atrophy[32], and thus inflammation-induced IL-6 expression may therefore not contribute to the changes in amino acid metabolism in cast-immobilized muscle. In addition, whereas intracellular calcium concentrations may increase after muscle immobilization for 2 weeks,[44] they appear to decrease in response to short-term cast immobilization.[48] Cast immobilization induced *Il6* expression may therefore also not be regulated by calcium signaling. Upregulation of IL-6 expression by cast immobilization was previously shown to be mediated by a Piezo1-KLF15 axis,[48] whereas our observation that *Il6* expression were increased in non immobilized fore limb muscles. Furthermore, acute cold exposure and short-term restraint stress tended to increase *Il6* expression in skeletal muscle, suggesting that muscle-derived IL-6 for thermoregulatory system may also be regulated by the central nervous system. A recent study showed that motor circuits modulate the production of neutrophil-induced chemokines in skeletal muscle after acute stress.[50] The regulation of IL-6 production in muscle by the central nervous system warrants further investigation.

The central nervous system tightly controls core body temperature through integrates information about external temperature, humidity, and thermal sensation to induce an adaptive response.[1] Disappointingly, our study was inconclusive as to whether the trigger for BAT thermogenesis after cast immobilization was hypothermia associated with loss of skeletal muscle function or stress. However, we found that acute cold exposure and short-term restraint stress may also recruits substrate supply from skeletal muscle for BAT thermogenesis. Possibly, thermoregulatory system through amino acid metabolism in skeletal muscle and BAT may also be an important metabolic strategy even in the case of fever response induced by stress or infection. Furthermore, inflammation, infection, and acute stress trigger rapid increases in the circulating IL-6 concentration[21,51] and induce a fever response by promoting PGE2 synthesis. Mitochondrial BCAA oxidation in BAT was recently shown to be increased to support thermogenesis induced by PGE2.[16] We found that BAT- derived IL-6 increased blood IL-6 levels after cast immobilization

and administration of exogenous IL-6 ameliorated cold intolerance in cast- immobilized IL-6 KO mice via the sympathetic nervous system. It may modulate thermal and energy homeostasis through the fever response and metabolic regulation in multiple organs.

In conclusion, we have shown that cast immobilization induced thermogenesis in BAT that was dependent on the utilization of free amino acids derived from skeletal muscle, and that muscle-derived IL-6 stimulated BCAA metabolism in skeletal muscle. Although some effects, such as activation of BAT thermogenesis and changes in energy metabolic dynamics, observed with cast immobilization were modest in magnitude, thermoregulatory system through amino acid metabolism in skeletal muscle and BAT may be an important metabolic strategy even under cold temperature or acute stress. Our findings may provide new insights into the significance of skeletal muscle as a large reservoir of amino acids in the regulation of body temperature. In addition, given that circulating levels of IL-6 or BCAAs are associated with obesity and diabetes,[52,53] IL-6–mediated BCAA metabolism in skeletal muscle and BAT may also be associated with muscle atrophy in metabolic diseases. Further investigation of IL-6–dependent amino acid metabolism in BAT and skeletal muscle may inform the development of preventive or therapeutic interventions for some forms of muscle atrophy.

Limitations of the study

In our cast immobilization strategy, we were unable to determine which components of muscle thermogenesis are actually inhibited by cast immobilization, and what is the relative heat contribution. This study does not experiment that directly test whether BCAAs derived from adipose tissue are used for thermogenesis, which would require more robust tracing experiments. In addition, given that rodents are BAT-enriched mammals, whether BAT thermogenesis is similarly activated in humans after skeletal muscle immobilization remains to be investigated. Our study also did not determine the mechanism underlying the induction of *Il6* expression in skeletal muscle by cast immobilization. In addition, whereas muscle-derived IL-6 may stimulate protein catabolism in immobilized muscle, the mechanism by which IL-6 increases BCAA concentrations in skeletal muscle remains unclear. In further investigation, RNA-seq profiling of BAT and muscle tissues should be used to rigorously determine the mechanisms of BCAA metabolism stimulated by muscle-derived IL-6.

Methods

Mice

All experiments were approved by the Animal Care and Use Committee of Tokushima University and were conducted in accordance with the guidelines for the care and use of animals approved by the Council of the Physiological Society of Japan. Every effort was made to minimize animal suffering and to reduce the number of animals used in the experiments. All mice were housed at a constant room temperature of 23° ± 1°C and with a 12-h-light/12-h-dark cycle (lights on at 8.00 a.m.). They were fed standard nonpurified chow (Oriental Yeast) and had free access to both food and water. Our study examined male mice because male animals exhibited less variability in phenotype. Male mice at 9 to 13 weeks of age were studied. The mice were randomly assigned to experimental groups at the time of purchase or weaning. C57BL/6J mice were obtained from Japan SLC (Shizuoka, Japan), and IL-6 KO mice (B6;129S2- Il6<TM1KOPF>) from RIKEN BRC through the National Bioresource Project of MEXT/AMED.[52] The IL-6 KO mice were maintained on the C57BL/6J background.

Cells

The mouse myoblast cell line C2C12 was obtained from American Type Culture Collection (CRL-1772™) and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose (#D6429, Sigma) and supplemented with 10% fetal bovine serum (535-94155, BioSera) and 1% penicillin-streptomycin (15140-122, Gibco). After the cells had achieved 95% to 100% confluence, the culture medium was changed to DMEM containing 25 mM glucose and supplemented with 2% horse serum (16050-130, Thermo Fisher Scientific) and the cells were cultured for 5 days to promote their differentiation from myoblasts into myotubes. The myotubes were washed with phosphate-buffered saline (PBS) and then incubated in DMEM containing 25 mM glucose and supplemented with 1% bovine serum albumin (A8806-5G, Sigma) and 1% penicillin-streptomycin for 16 h before exposure to recombinant mouse IL-6 (rIL-6) at 50 ng/ml (575702, BioLegend) or vehicle (PBS) in the same medium for 1 h. The treated cells were washed with PBS or 5% mannitol solution and collected for RNA or metabolite extraction.

Mouse preadipocytes were obtained from Dr. Takeshi Yoneshiro the University of Tohoku, Tohoku, Japan[54] and were maintained in DMEM containing 25 mM glucose and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. After the cells had achieved 95% to 100% confluence, they were induced to differentiate into brown adipocytes by incubation in the same medium that was also supplemented with 1 nM triiodothyronine (45006-44, Nacalai Tesque), insulin (093-06473, Wako) at 5 μg/ml, dexamethasone (11107-51, Nacalai Tesque) at 2 μg/ml, 0.5 mM isobutylmethylxanthine (15879, Sigma), and 125 μM indomethacin (19233-51, Nacalai Tesque). After culture for 2 days, the medium was replaced with DMEM containing 25 mM glucose and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and insulin (5 μg/ml), and the cells were maintained in this medium with medium replenishment every 2 days. The cells were fully differentiated at 6 to 7 days after the induction of differentiation. For experiments, the brown adipocytes were washed with PBS at 7 days after differentiation initiation and then incubated in DMEM containing 25 mM glucose and supplemented with 1% bovine serum albumin and 1% penicillin-streptomycin for 12 h before exposure to rIL-6 (50 ng/ml), 1 μM CL316 243 (Ab144605, Abcam), both agents, or vehicle (PBS). The treated cells were collected after 24 h for RNA extraction.

Cast immobilization

Mice were subjected to bilateral or unilateral cast immobilization of hind limbs, and unfixed mice were studied as controls. In brief, mice were lightly anesthetized with inhalational isoflurane (MSD Animal Health), and the hind limbs were fixed in a natural position, with care taken to avoid skin irritation and congestion. After casting, the mice were housed individually with free access to standard mouse chow and water. The mice out of cast immobilization were excluded from experiments. In all experiments, mice were killed by cardiac puncture after food deprivation for 3 h and tissue samples were collected.

Proportion of immobilized skeletal muscle weight for cast-immobilized mice was obtained by extirpation skeletal muscle of the posterior cervical region and measuring wet tissue weights.

Surgical denervation of iBAT

Surgical denervation of iBAT was performed on 9-week-old male mice as described previously.[55] Animals were anesthetized by intraperitoneal injection of domitor (Zenoaq) at a dose of 0.75 mg/kg, midazolam (Wako) at 4 mg/kg, and butorphanol tartrate (Meiji Seika Pharma) at 5 mg/kg, and the incision site was shaved and then disinfected with 0.05% chlorhexidine gluconate (5% Hibitane, Sumitomo Pharma). A midline skin incision was made in the interscapular region to expose both iBAT pads, the nerve fibers (five nerves per pad) were identified and cut with sterile

scissors, and the incision was closed with sutures. For sham surgery, a skin incision was made and the medial side was gently exposed. All mice were then individually housed in clean cages at room temperature and were monitored daily. Cast immobilization was performed 1 week after surgery.

Surgical removal of iBAT

Surgical removal of iBAT was performed on 11-week-old male mice, with cast immobilization being performed 1 week after surgery. Animals were anesthetized and the incision site was shaved and disinfected as described for iBAT denervation. A midline skin incision was made in the interscapular region, all iBAT was removed, and the incision was closed with sutures. For sham surgery, a skin incision was made and the medial side was gently exposed. All mice were then housed individually in clean cages at room temperature.

Acute cold tolerance test

Core body temperature was measured with a Homeothermic Monitor (BWT- 100A, BRC) by gentle insertion of a thermal probe into the rectum of the mouse. After recording of baseline body temperature at room temperature, animals were placed in a cold chamber at 4°C for up to 4 h without access to food.

Tissue was then collected and snap-frozen.

Acute Restraint Stress

Mice were confined in 50-mL tubes with several ventilation holes of about 0.5 cm in diameter on the sides, and were restrained for 6 hours without access to food. Mice restrained in tubes were maintained in a state in which they could hardly move their bodies. In all experiments, mice were killed by cardiac puncture and tissue samples were collected.

Treatment of mice with CL316 243 or rIL-6

IL-6 KO mice with cast immobilization for 24 h were subjected to intraperitoneal administration of 400 ng of rIL-6 (575702, BioLegend) in saline or of saline alone. The mice were maintained sedentary for 3 h without access to food before collection of tissue samples. Acute exposure to a cold environment chamber was initiated 30 min after intraperitoneal treatment with rIL-6 (400 ng) or saline in IL-6 KO mice or in C57BL/6J mice with denervated iBAT that had been subjected to cast immobilization for 24 h. IL-6 KO mice with denervated iBAT were treated intraperitoneally with CL316 243 (Ab144605, Abcam) at a dose of 0.1 mg/kg, rIL-6 (400 ng), both agents, or saline vehicle, and tissue was collected 3 h later. For long-term treatment of IL-6 KO mice with CL316 243, the animals were injected intraperitoneally with the drug (0.1 mg/kg per day) or saline for 7 days. Tissue was collected at 3 h after drug treatment on the last day.

Oxygen consumption and RER measurement

Mice were acclimated to the analysis cage for 3 days before the start of metabolic measurements. They were housed individually with free access to food and water. Analysis of respiratory gases was performed with a mass spectrometer (ARCO-2000, ARCO System). Measurements were performed for seven consecutive days after cast immobilization.

Locomotor activity recording

The activity level of mice was assessed with an infrared activity monitor (ACTIMO-100N, Shin Factory). A cage containing one mouse was placed inside the activity monitor with infrared beams at 20-mm intervals. Mouse movements were counted every 0.5 s for 24 h. Recording was performed for seven consecutive days after cast immobilization.

RNA isolation and RT and real-time PCR analysis

Total RNA was extracted from mouse tissues and cells with the use of RNAiso (#9109, Takara Bio) and subjected to RT with the use of a TaKaRa PrimeScript II 1st Stand cDNA Synthesis Kit (Takara Bio). The resulting cDNA was subjected to real-time PCR analysis with Fast SYBR Green Master Mix (3485612, Applied Biosystems) in a Step One Plus Real-Time PCR System (Applied Biosystems). The abundance of target mRNAs was normalized by that of *Gapdh* mRNA. The PCR primers are listed in Table S3.

Protein extraction and immunoblot analysis

Total protein was extracted from mouse tissues by homogenization in a lysis solution consisting of 1 M Tris-HCL (pH 7.4), 0.1 M EDTA, 1% Nonidet P-40, 5 mM sodium pyrophosphate, and phosphatase (25955-24, Nacalai Tesque) and protease (07575-51, Nacalai Tesque) inhibitor cocktails. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4°C, and the resulting supernatant was assayed for protein concentration with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and then subjected to SDS-polyacrylamide gel electrophoresis on a 10% acrylamide/4% bisacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride membrane, which was then exposed to 5% dried skim milk for 1 h at room temperature before incubation with primary antibodies including those to PGC-1α (A12348, Abclonal), to UCP1 (sc-293418, Santa Cruz Biotechnology), and to GAPDH (CAB932Hu01, Cloud-Clone) for 16 h at 4°C. Immune complexes were detected with horseradish peroxidase– conjugated secondary antibodies (458, MBL; 7076P2, Cell Signaling Technology), enhanced chemiluminescence reagents (170-5061, Bio-Rad Labotatories, Inc.), and an Amersham Imager 600 instrument (GE Healthcare). Band intensity was quantified with ImageJ software.

Histology

BAT was fixed in formalin, sectioned, and stained with hematoxylin-eosin.

Metabolomics analysis

Targeted profiling of amino acid metabolites was performed by capillary electrophoresis–time-of-flight mass spectrometry (CE-TOF/MS) with an Agilent 7100 CE system coupled to an Agilent 6230 TOF mass spectrometer (Agilent Technologies) according to previously described methods.[30] The collected metabolite data were subjected to enrichment analysis using Metaboanalyst 6.0 (*https://www.metaboanalyst.ca*). Pathway enrichment was tested using the SMPDB databases.

Measurement of noradrenaline

Tissue samples were homogenized in methanol containing internal standards ((±)-Norepinephrine-D6 hydrochloride solution, Sigma-Ardrich). The mixture was centrifuged at 4 °C, and the aqueous fraction was isolated and centrifuged through an cosmonice filter W (06543-04, nacalai tesque). Noradrenaline content of the supernatants was measured by high-performance liquid chromatography (SHIMAZU) with a reversed-phase column (Cadenza CD-C18 MF 100×2 mm 3 µm, Imtakt) and tandem mass spectrometry (MS/MS) (API 3200, AB SCIEX). Data are analyzed using AnalystLauncher (AB SCIEX).

Measurement of [^{3}H]leucine uptake

Mice were deprived of food for 3 h before intravenous injection of L-[4,5- ^{3}H(*N*)]leucine (NET1166, PerkinElmer) at a dose of 1 µCi/g. They were then maintained in a resting state for 40 min until blood and tissue collection, with perfusion being performed before tissue isolation. Isolated tissue was cut into small pieces, which were then placed in a round-bottom polypropylene tube before the addition of 500 µl of distilled water, 150 µl of 30% hydrogen peroxide (Wako), and 150 µl of 2 M

KOH (1310-58-3, Hayashi Pure Chemical) in 2- propanol (000-64783, Kishida Chemical) and incubation at 65°C for 2 h. The tissue lysate was neutralized with 30% acetic acid, and 1 ml of liquid scintillation solubilizer (Soluen-350, PerkinElmer) was added to the sample before incubation at 50°C for 5 h and the further addition of 5 ml of liquid scintillation cocktail (Hionic-Flour, PerkinElmer). Leucine uptake in each organ was quantified by measurement of radioactivity with a scintillation counter (LSC- 7400, Hitachi).

Hepatic glycogen assay

The liver was homogenized in ice-cold citrate buffer (pH 4.2) containing NaF (2.5 g/l), the homogenate was centrifuged at 14,000 × *g* for 5 min at 4°C, and the resulting supernatant was assayed for glycogen with an EnzyChrom Glycogen Assay Kit (BioAssay Systems).

ELISAs

Blood samples were collected from the heart of mice that had been anesthetized by intraperitoneal injection of domitor (Zenoaq) at a dose of 0.75 mg/kg, midazolam (Wako) at 4 mg/kg, and butorphanol tartrate (Meiji Seika Pharma) at 5 mg/kg after food deprivation for 3 h, and they were centrifuged at 7500 × *g* for 10 min at 4°C to isolate serum. Serum IL-6 and TNF-α concentrations were determined with enzyme-linked immunosorbent assay (ELISA) kits (Legend Max Mouse IL-6 ELISA Kit, BioLegend, and Mouse TNF Alpha Uncoated ELISA, Invitrogen, respectively). Serum noradrenaline was extracted with the use of a cis-diol–specific affinity gel, acylated, enzymatically converted, and then measured with an ELISA kit (Noradrenaline Research ELISA, ImmuSmol). Serum corticosterone was determined with a Corticosterone ELISA Kit (Enzo Life Sciences).

Statistical analysis

Data are presented as means ± SEM unless indicated otherwise and were analyzed with statistical software (JMP or Statcel–The Useful Add-in Forms on Excel 4th ed.). Comparisons between two groups were performed with the paired or unpaired t test, as appropriate. Those among more than two groups were performed with Dunnett's test, by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test, or by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction. A two-tailed p value was calculated for all experiments, and a value of <0.05 was considered statistically significant.

Supplementary Figure Legends

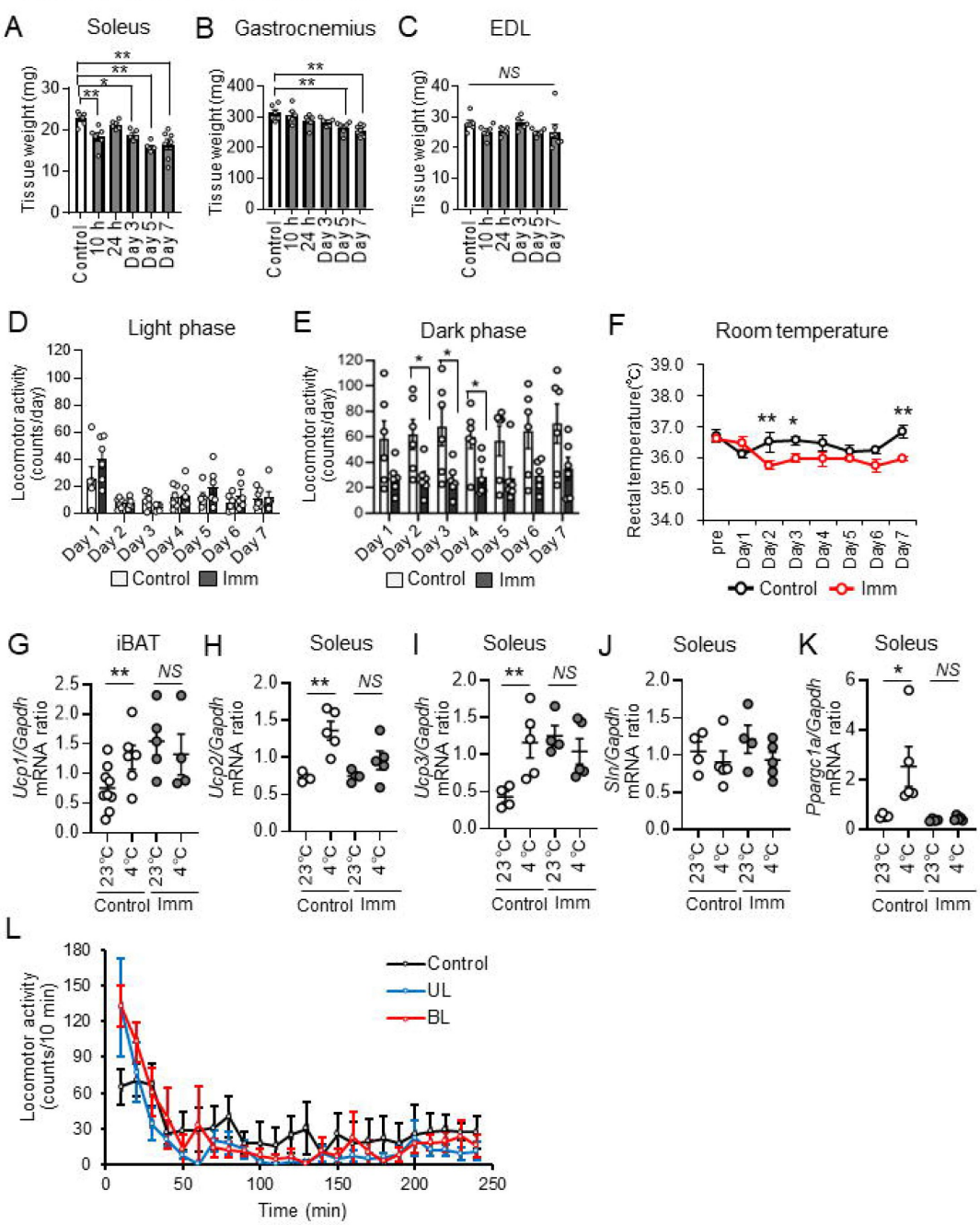

Figure 1_figure supplement 1.

(A–C) Weight of individual soleus (A), gastrocnemius (B), and extensor digitorum longus (EDL) muscles of control mice and mice subjected to bilateral cast immobilization of hind limbs for the indicated times (n = 5-7 per group). (D and E) Locomotor activity during light (D) and dark (E) phases for control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (F) Rectal core body temperature of cast-immobilized (n = 6) and control (n = 4) mice at room temperature after bilateral cast immobilization. (G) RT and real-time PCR analysis of the expression of UCP1 genes in iBAT of control and cast-immobilized mice subjected (or not) to cold exposure at 4°C for 4 h (n = 4-9 per group). (H-K) RT and real-time PCR analysis of *Ucp2*, *Ucp3*, *Sln* and *Ppagc1a* expression in iBAT of control and cast-immobilized mice subjected (or not) to cold exposure at 4°C for 4 h (n = 4-9 per group). (L) Locomotor activity during cold (4°C) exposure for 4 h determined for control mice and mice subjected to unilateral (UL) or bilateral (BL) cast immobilization (n = 5 per group). These data were obtained in conjunction with those in Figure1C. All quantitative data are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by Dunnett's test (A-C) or by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction (D-F and L), or by the unpaired t test (G-K)

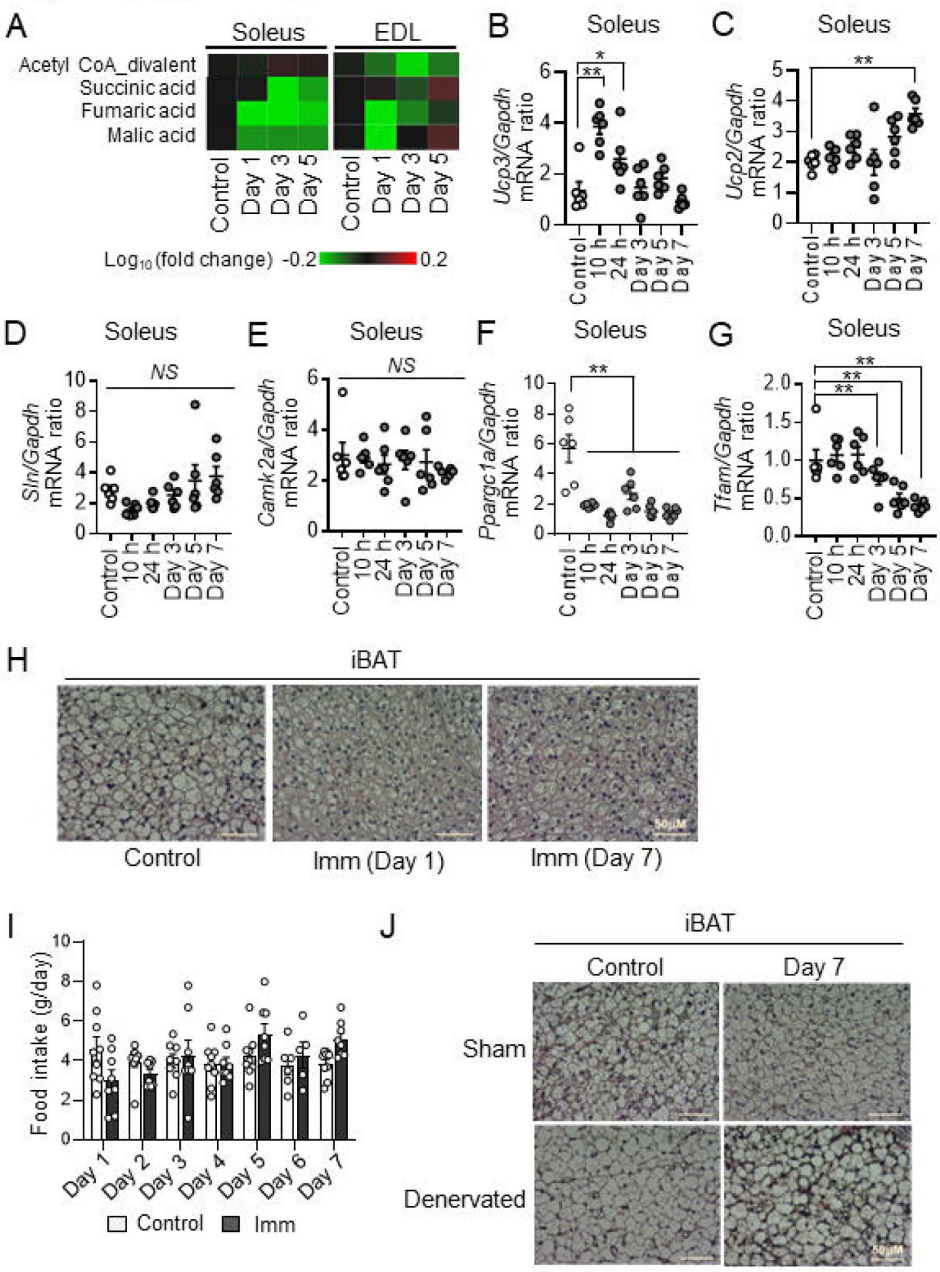

Figure 1_figure supplement 2.

(A) Concentrations of carbohydrate metabolites (acetyl CoA divalent, succinic acid, fumaric acid and malic acid) in soleus and EDL of control mice and mice subjected to bilateral cast immobilization for the indicated times. Results are presented as heat maps of the $\log_{10}$ value of fold change for cast-immobilized mice relative to control mice and are are shown individually for three mice of each group. (B and C) RT and real-time PCR analysis of *Ucp3* and *Ucp2* expression, respectively, in soleus of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 5-6 per group). (D and E) RT and real-time PCR analysis of *Sln* and *Camk2a* expression, respectively, in soleus of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (F and G) RT and real-time PCR analysis of *Ppargc1a*, and *Tfam* expression, respectively, in soleus of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (H) Hematoxylin-eosin staining of surgically denervated or sham-operated iBAT from control mice or mice subjected to bilateral cast immobilization for 7 days. Scale bar, 50 μm. (I) Food intake of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6–9 per group). (J) Hematoxylin-eosin staining of iBAT of control mice and mice subjected to bilateral cast immobilization for 1 or 7 days. Scale bar, 50 μm. All quantitative data other than those in (A, H and J) are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by Dunnett's test (B-G) or by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction (I).

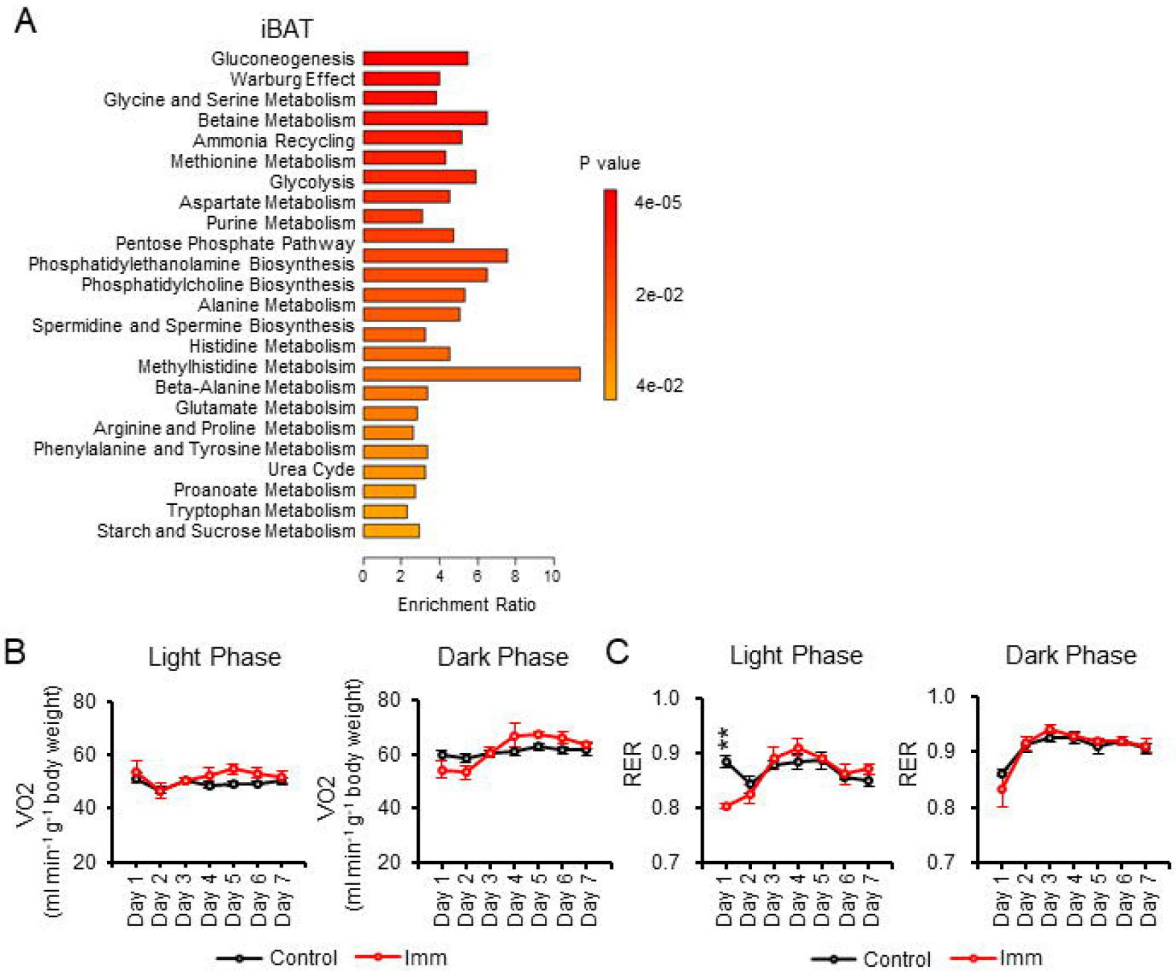

Figure 2_figure supplement 1.

(A) Pathway analysis of metabolites upregulated in iBAT by cast immobilization for 10 h. (B and C) Oxygen consumption rate (VO_2) and RER, respectively, for mice with denervated iBAT subjected (or not) to bilateral cast immobilization for 7 days (n = 3 per group). Quantitative data other than in (A) are means ± SEM. *p < 0.05, **p < 0.01, as determined by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction (B and C).

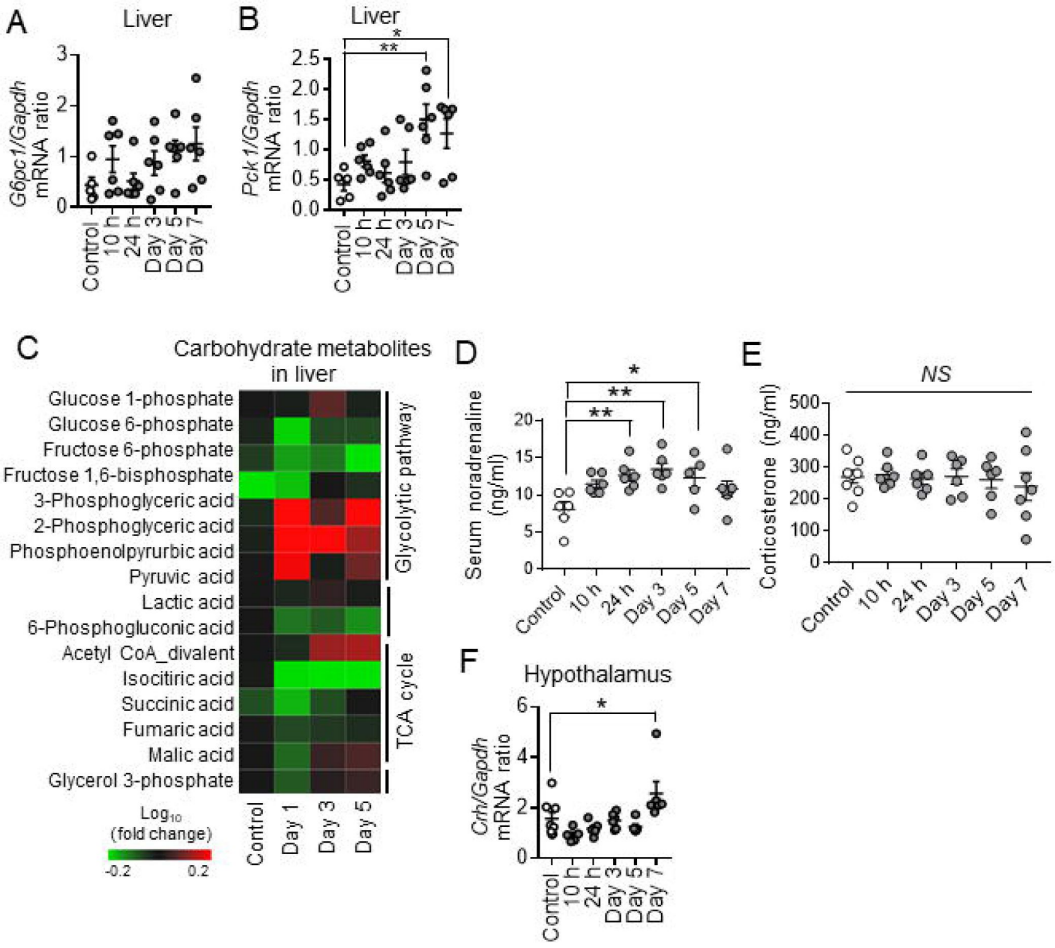

Figure 2_figure supplement 2.

(A and B) RT and real-time PCR analysis of G6Pase (*G6pc1*) and PEPCK (*Pck1*) gene expression in the liver of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 5 or 6 per group). (C) Concentrations of carbohydrate metabolites in the liver of control mice and mice subjected to bilateral cast immobilization for the indicated times. The metabolomics data are presented as heat maps corresponding to the $\log_{10}$ value of fold change in cast-immobilized mice relative to control mice. Results are means of three mice per group. (D) Serum concentration of noradrenaline in control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 5–7 per group). (E) Serum concentration of corticosterone for control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6–8 per group). (F) RT and real-time PCR analysis of *Crh* expression in the hypothalamus of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 5–8 per group). Quantitative data other than in (C) are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by Dunnett's test (A, B and D-F).

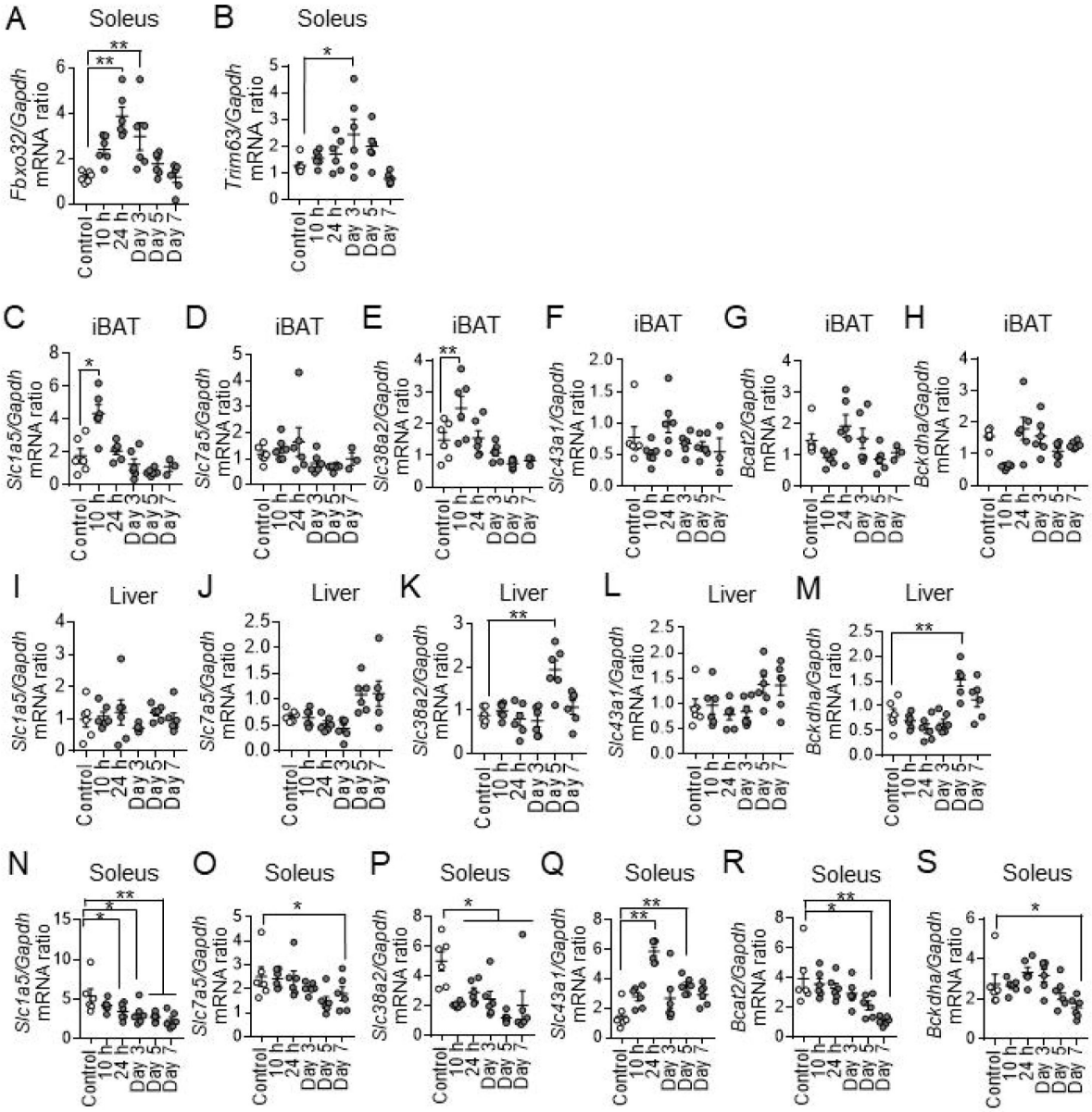

Figure 3_figure supplement 1.

(A and B) RT and real-time PCR analysis of *Fbxo32* (Atrogin-1) and *Trim63* (MuRF-1) expression in the soleus muscle of control mice and mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (C–H) RT and real-time PCR analysis of SLC1A5, SLC7A5, SLC38A2, SLC43A1, BCAT2, and BCKHDA gene expression, respectively, in iBAT of control mice or mice subjected to bilateral cast immobilization for the indicated times (n = 3–6 per group). (I–M) RT and real-time PCR analysis of gene expression for SLC1A5, SLC7A5, SLC38A2, SLC43A1, and BCKHDA, respectively, in the liver of control mice or mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (N–S) RT and real-time PCR analysis of gene expression for SLC1A5, SLC7A5, SLC38A2, SLC43A1, BCAT2, and BCKHDA in soleus of control mice or mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). All data are means ± SEM. *p < 0.05, **p < 0.01 as determined by Dunnett's test.

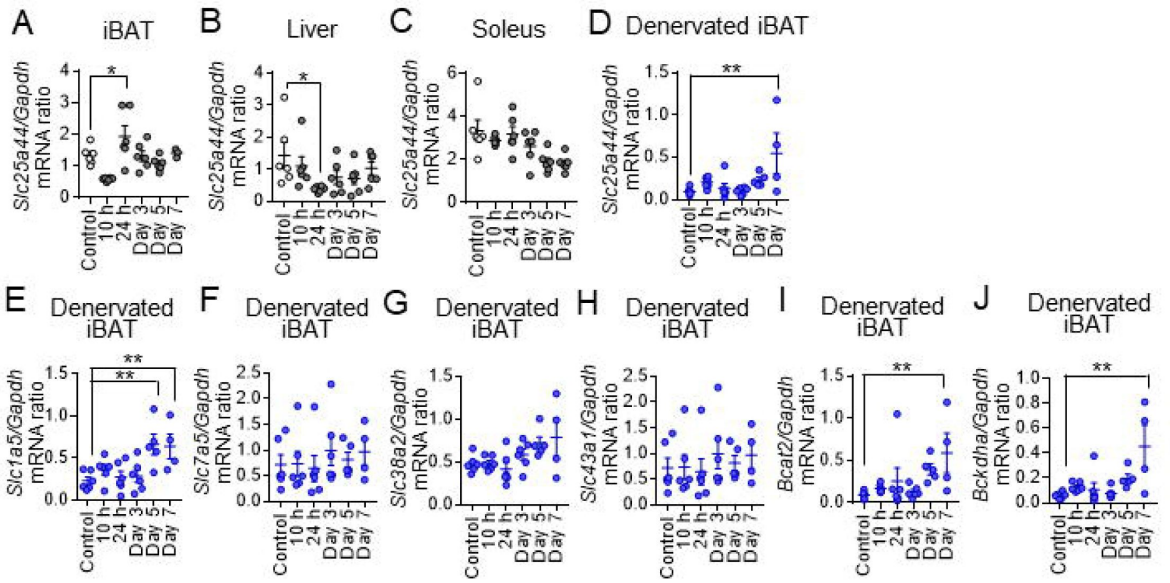

Figure 3_figure supplement 2.

(A–C) RT and real-time PCR analysis of SLC25A44 gene expression in iBAT (A), liver (B), and soleus (C) of control mice or mice subjected to bilateral cast immobilization for the indicated times (n = 6 per group). (D–J) RT and real-time PCR analysis of gene expression for SLC25A44, SLC1A5, SLC7A5, SLC38A2, SLC43A1, BCAT2, and BCKDHA, respectively, in denervated iBAT of control mice or mice subjected to bilateral cast immobilization for the indicated times (n = 4–6 per group). All data are means ± SEM. *p < 0.05, **p < 0.01 as determined by Dunnett's test.

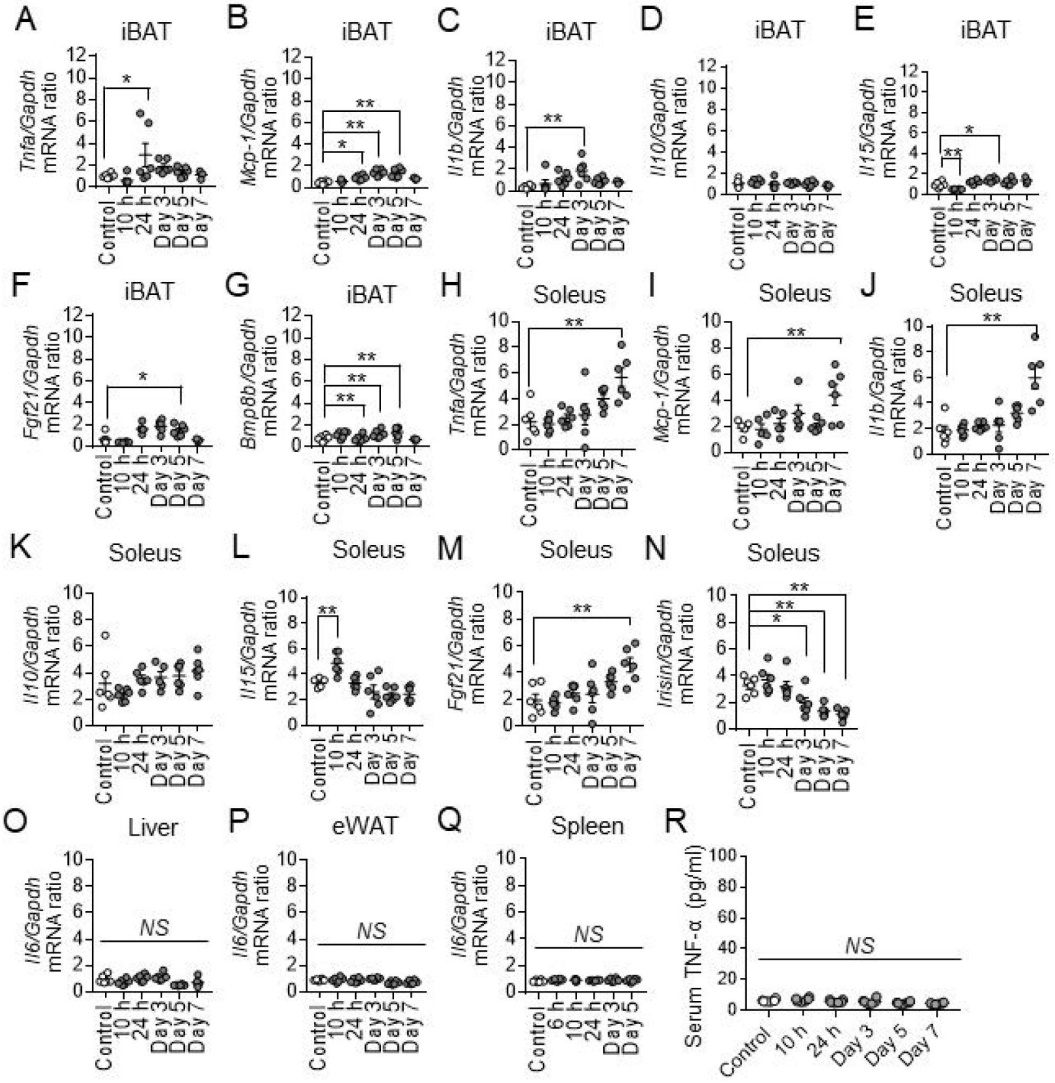

Figure 4_figure supplement 1.

(A–G) RT and real-time PCR analysis of TNF-α, MCP-1, IL-1β, IL-10, IL-15, FGF21, and BMP8B gene expression, respectively, in iBAT of control mice and mice at the indicated times after bilateral cast immobilization (n = 3–6 per group). (H–N) RT and real-time PCR analysis of TNF-α, MCP-1, IL-1β, IL-10, IL-15, FGF21, and irisin gene expression, respectively, in soleus of control mice and mice at the indicated times after bilateral cast immobilization (n = 5 or 6 per group). (O–Q) RT and real-time PCR analysis of *Il6* expression in liver (O), eWAT (P), and spleen (Q) of control mice and mice at the indicated times after bilateral cast immobilization (n = 5-6 per group). (R) Serum concentration of TNF-α in control mice and mice at the indicated times after bilateral cast immobilization (n = 6 per group). All data are means ± SEM. *p < 0.05, **p < 0.01, NS (not significant) as determined by Dunnett's test.

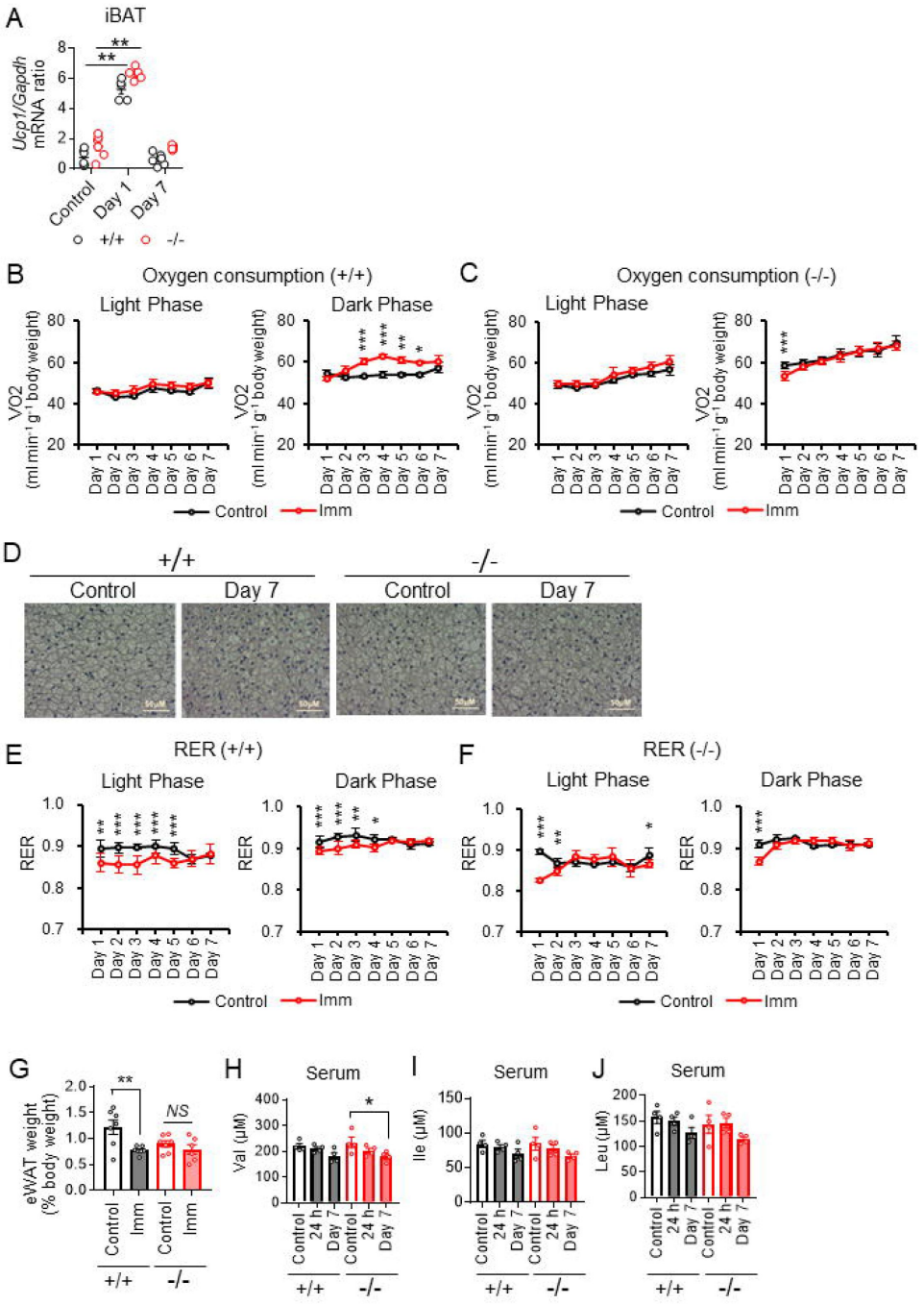

Figure 5_figure supplement 1.

(A) RT and real-time PCR analysis of *Ucp1* mRNA abundance in iBAT of WT and IL-6 KO mice without (control) or with bilateral cast immobilization for 1 or 7 days (n = 5 or 6 per group). (B and C) Oxygen consumption rate for WT (B) and IL-6 KO (C) mice subjected (or not) to bilateral cast immobilization for 7 days (n = 4 per group). (D) Hematoxylin-eosin staining of iBAT from IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 7 days. Scale bar, 50 μm. (E and F) RER for WT (E) and IL-6 KO (F) mice subjected (or not) to bilateral cast immobilization for 7 days (n = 4 per group). (G) Weight of eWAT in WT (+/+) and IL-6 KO (-/- mice subjected (or not, control) to bilateral cast immobilization for 7 days (n = 5-7 per group). (H–J) Serum concentrations of BCAA (Val, Ile, and Leu, respectively) concentrations of IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 1 or 7 days (n = 4 per group). All data other than those in (F) are means ± SEM. *p < 0.05, **p < 0.01 as determined by one-way ANOVA followed by Tukey's post hoc test (A and G) or by two-way ANOVA followed by Tukey's post hoc test or the post hoc paired/unpaired t test with Bonferroni's correction (B-C and E-F), or by Dunnett's test (H-I).

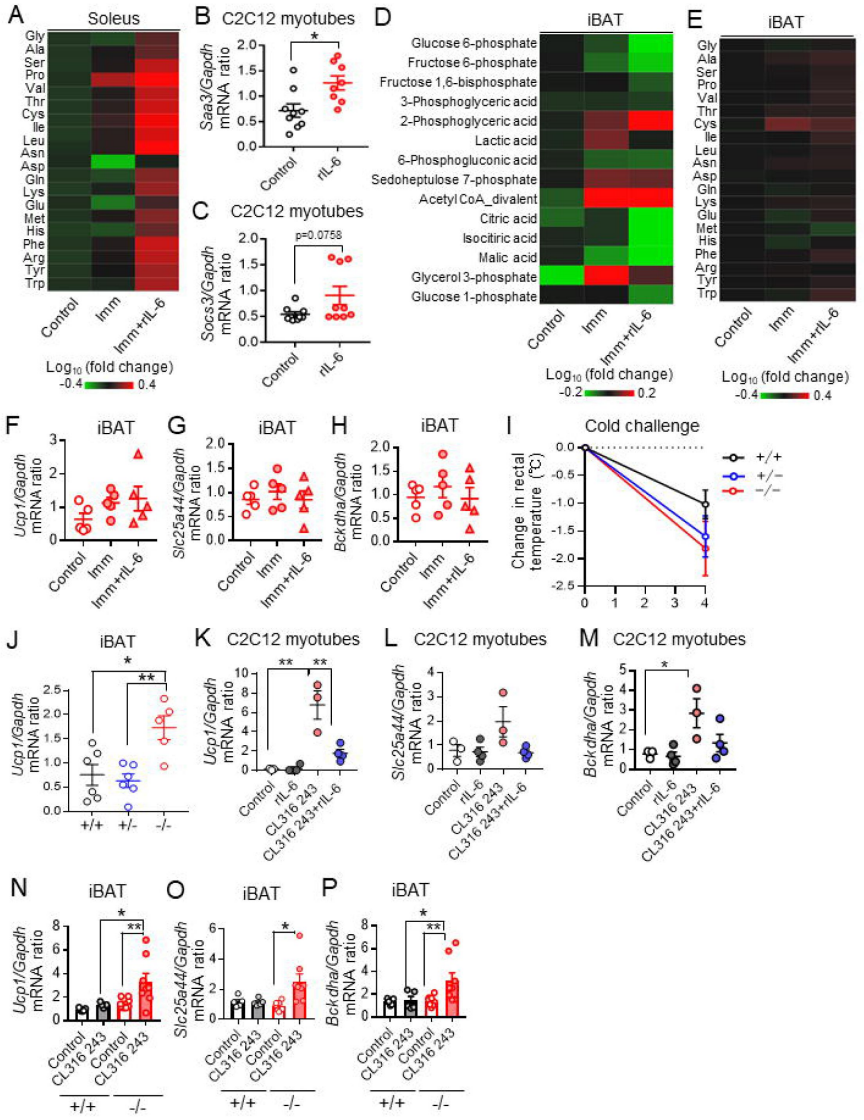

Figure 6_figure supplement 1.

(A) Amino acid concentrations in soleus of control IL-6 KO mice and at 3 h after intraperitoneal administration of recombinant IL-6 (rIL-6, 400 ng) or vehicle in IL- 6 KO mice that had been subjected to bilateral cast immobilization for 24 h. Results are presented as heat maps of the $\log_{10}$ value of the fold change relative to control mice and are means of four or five mice in each group. (B and C) RT and real-time PCR analysis of *Saa3* (B) and *Socs3* (C) mRNA abundance in C2C12 myotubes incubated in the absence or presence of rIL-6 (50 ng/ml) for 1 h (n = 8 or 9 independent experiments). (D) Amounts of carbohydrate metabolites in iBAT of mice as in (A). Results are presented as heat maps of the $\log_{10}$ value of the fold change relative to control mice and are means of four mice in each group. (E) Amino acid concentrations in iBAT of mice as in (A). Results are presented as heat maps of the $\log_{10}$ value of the fold change relative to control mice and are means of four or five mice in each group. (F–H) RT and real-time PCR analysis of UCP1 (F), SLC25A44 (G), and BCKDHA (H) gene expression in iBAT of mice as in (A) (n = 5 per group). (I) Change in rectal core body temperature of mice of the indicated *Il6* genotypes during cold exposure at 4°C for 4 h (+/+ and –/–, n = 5; +/–, n = 6). (J) RT and real-time PCR analysis of *Ucp1* mRNA abundance in iBAT of mice of the indicated *Il6* genotypes (n = 5 or 6 per group). (K–M) RT and real-time PCR analysis of UCP1 (K), SLC25A44 (L), and BCKDHA (M) gene expression in cultured brown adipocytes incubated in the absence or presence of rIL-6 (50 ng/ml) or CL316 243 (1 μM) for 24 h (n = 3 or 4 independent experiments). (N–P) RT and real-time PCR analysis of UCP1 (N), SLC25A44 (O) and BCKDHA (P) gene expression in iBAT of WT (+/+, n = 5 per group) or IL-6 KO (–/–, n = 8 per group) mice after intraperitoneal treatment with CL316 243 (0.1 mg/kg per day) or vehicle for 7 days. All data other than those in (A),(D) and (E) are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by the unpaired t test (B and C), by one-way ANOVA followed by Tukey's post hoc test (F–H and J-P), or by two-way ANOVA followed by the post hoc paired/unpaired t test with Bonferroni's correction (I).

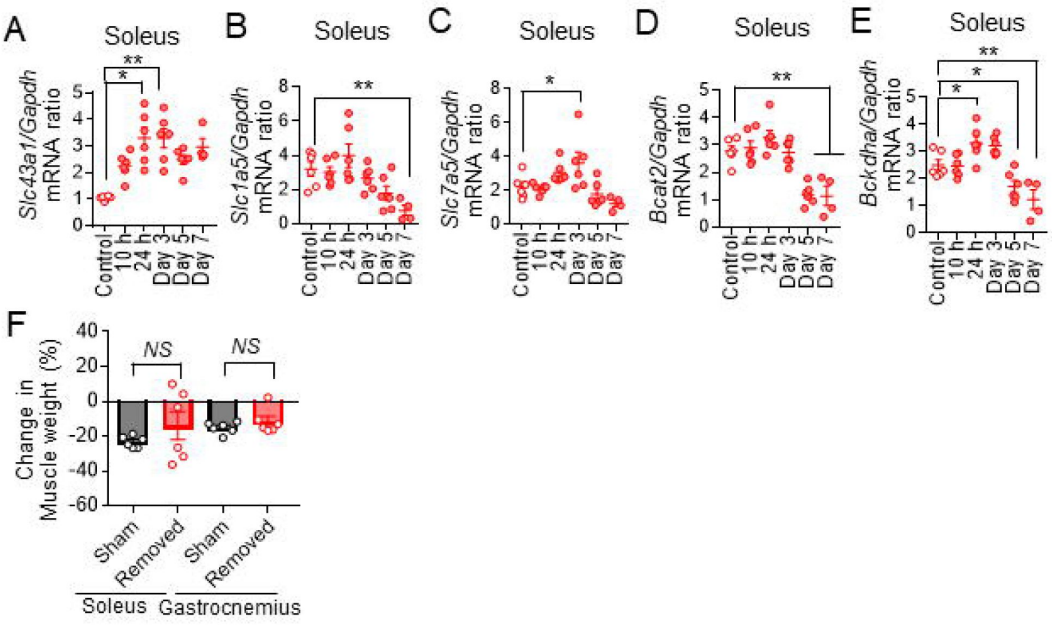

Figure 6_figure supplement 2.

(A-E) RT and real-time PCR analysis of SLC43A1, SLC1A5, SLC7A5, BCAT2, and BCKDHA gene expression, respectively, in soleus of iBAT-depleted mice subjected (or not, control) to bilateral cast immobilization for the indicated times (n = 4–7 per group). (F) Change in soleus and gastrocnemius muscle weights of iBAT-depleted or sham-operated mice subjected to bilateral cast immobilization for 7 days (n = 6 per group). All data are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by the unpaired t test (F) or by Dunnett's test (A-E). See also Tables S2.

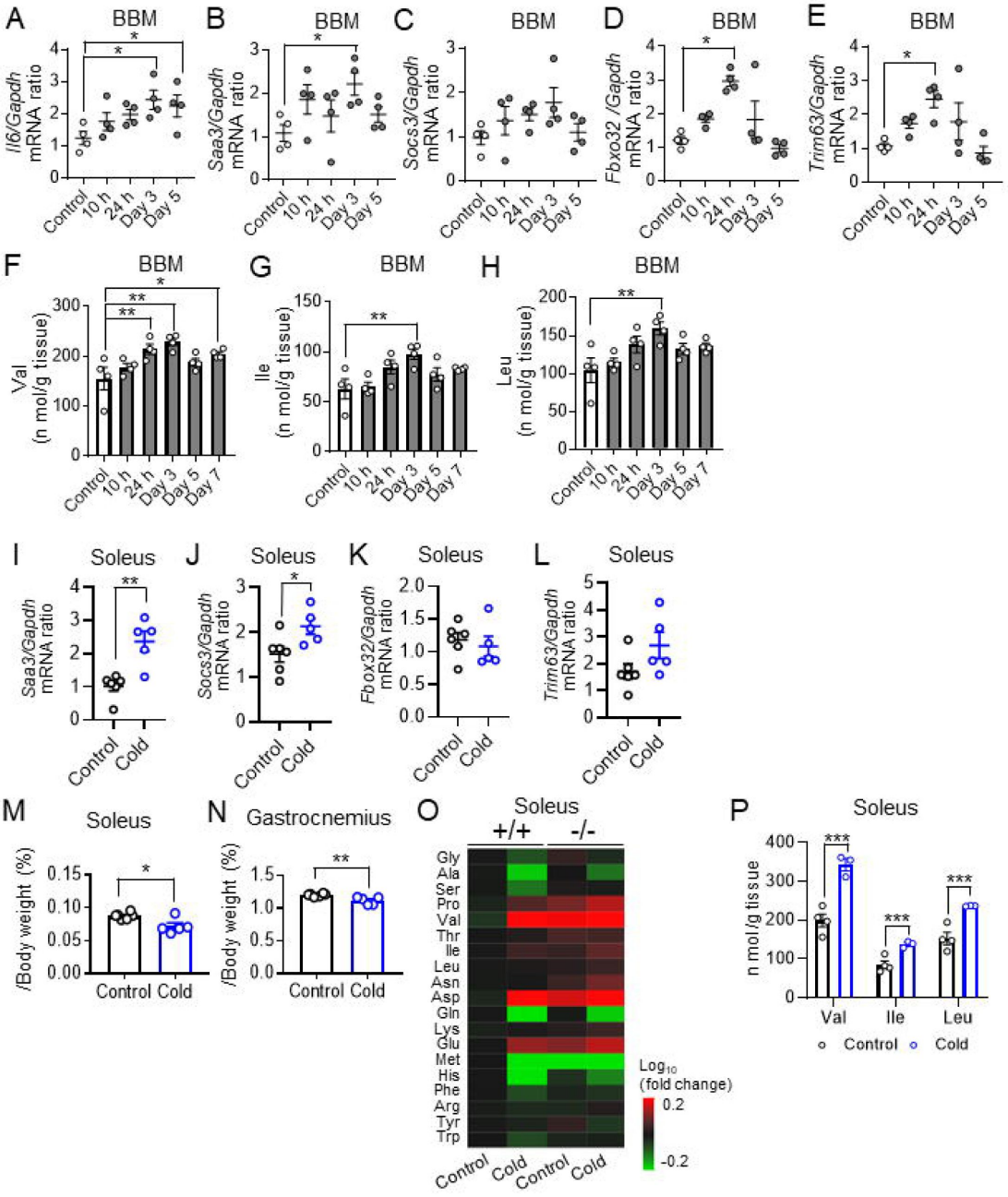

Figure 7_figure supplement 1.

(A-E) RT and real-time PCR analysis of *Il6*, *Saa3*, *Socs3*, *Fbxo32* and *Trim63* mRNA abundance in BBM of mice subjected (or not, control) to bilateral cast immobilization for the indicated times (n = 4 per group). (F-H) BCAA (Val, Ile, and Leu, respectively) concentrations in BBM of mice subjected (or not, control) to bilateral cast immobilization for the indicated times (n = 4 per group). (I and J) RT and real-time PCR analysis of *Saa3* (I) and *Socs3* (J) mRNA abundance in soleus of mice subjected (or not) to cold (4°C) exposure for 4 h (control, n = 6; cold exposure, n = 5). (K and L) RT and real-time PCR analysis of *Fbox32* (K) and *Trim63* (L) mRNA abundance in soleus of mice subjected (or not) to cold (4°C) exposure for 4 h (control, n = 6; cold exposure, n = 5). (M and N) Weight of individual soleus (M) and gastrocnemius (N) muscles of control mice and mice subjected to. cold exposure at 4°C for 4 h (n = 5 or 6 per group). (O) Amino acid concentrations in soleus of WT (+/+) and IL-6 KO (–/–) mice subjected (or not) to cold exposure at 4°C for 4 h. Results are presented as heat maps of the $\log_{10}$ value of fold change relative to WT control mice and are means of four mice in each group. (P) BCAA concentrations in soleus of iBAT-depleted mice subjected (or not, control) to cold exposure at 4°C for 4 h (n = 3 or 4 per group). All data other than in (O) are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by the unpaired t test (I-N and P) or by Dunnett's test (A-H).

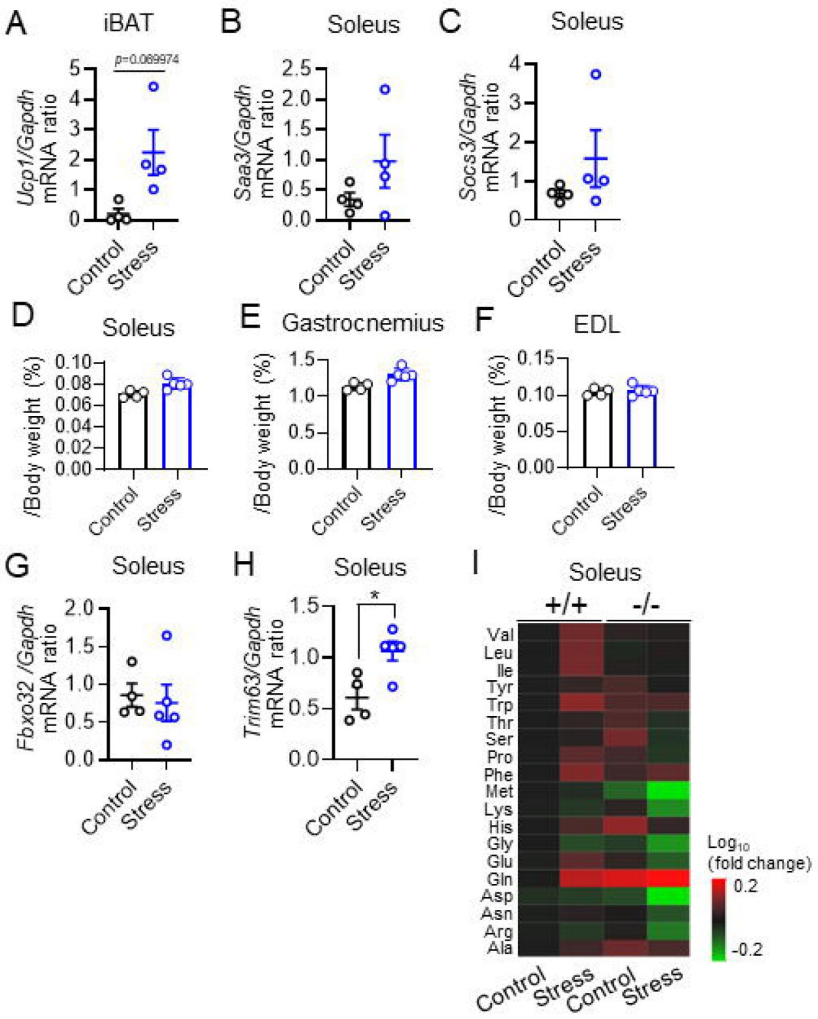

Figure 7_figure supplement 2.

(A) RT and real-time PCR analysis of *Ucp1* mRNA abundance in iBAT of mice subjected (or not) to restraint stress for 6 h (n = 4 per group). (B and C) RT and real-time PCR analysis of *Saa3* (B) and *Socs3* (C) mRNA abundance in soleus of mice subjected (or not) to restraint stress for 6 h (n = 4 per group). (D-F) Weight of soleus (D), gastrocnemius (E) and EDL (F) in mice subjected to restraint stress for 6 h (n = 4 per group). (G and H) RT and real-time PCR analysis of *Fbox32* (G) and *Trim63* (H) mRNA abundance in soleus of mice subjected (or not) to restraint stress for 6 h (n = 4 per group). (I) Amino acid concentrations in soleus of WT (+/+) and IL-6 KO (–/–) mice subjected (or not) to restraint stress for 6 h. Results are presented as heat maps of the $\log_{10}$ value of fold change relative to WT control mice and are means of four mice in each group. All data are means ± SEM. *p < 0.05 as determined by the unpaired t test (A-H).

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Additional information

Auther Contributions

Y.I.-M., T.S., S.F., M.T., M.S. and M.T. conducted the experiments; R.T. and H.S. designed the experiments; Y.I.-M., R.T., and H.S. wrote the manuscript; Y.O.-O., T.Y., K.N., and M.K. provided conceptual advice on data interpretation.

Additional files

Supplementary information

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Reviewer #1 (Public review):

Summary:

Heat production mechanisms are flexible, depending on a wide variety of genetic, dietary and environmental factors. The physiology associated with each mechanism is important to understand, since loss of flexibility associates with metabolic decline and disease.

The phenomenon of compensatory heat production has been described in some detail in publications and reviews, notably by modifying BAT-dependent thermogenesis (for example by deleting UCP1 or impairing lipolysis, cited in this paper).

These authors chose to eliminate exercise as an alternative means for maintaining body temperature. To do this, they cast either one or both mouse hindlimbs.

This paper is set up as an evaluation of a loss of function of muscle on the functionality of BAT. However, the authors show that cast immobilization (CI) does not work as a (passive) loss of function, instead this procedure produces a dramatic gain of function.

It does not test the hypothesis as stated, instead it adds an extraneous variable, which is that the animal is put under enormous stress, inducing b-adrenergic effectors, increased oxygen consumption, and IL6 expression in a variety of tissues, together with commensurate cachectic effects on muscle and fat. The BAT is stressed by this procedure, becoming super-induced but relatively poor functioning. This is an inaccurate experimental construct, and the paper is therefore full of wrong conclusions.

Within hours and days of CI, there is massive muscle loss (leading to high circulating BCAAs), and loss of lipid reserves in adipose and liver. The lipid cycle that maintains BAT thermogenesis is depleted and the mouse is unable to maintain body temperature.

I cannot agree with these statements in the Discussion -

"We have here shown that cast immobilization suppressed skeletal muscle thermogenesis, resulting in failure to maintain core body temperature in a cold environment."

• This result could also be attributed to high stress and decreased calorie reserves. Note also: CI suppresses 50% locomoter activity, but the actual work done by the mouse carrying bilateral casts is not taken into account (how heavy are they?). Presumably other muscles in the mouse body are compensating to allow the mouse to drag itself to the food source, to maintain food consumption, which remarkably, is unchanged. Is the demand for heat even the same when the mouse is wrapped in gypsum?

I cannot be convinced that this approach (CI) can be interpreted at all in terms of organ communication during thermogenic challenge. This paper describes instead the resilience and adaptation of mouse physiology in the face of dragging around hind limb casts.

From Rebuttal:

"On the other hand, the experiment shown in Fig.1C involved acute cold exposure of mice 2 h after cast immobilization. This result suggests that, even before the depletion of energy stores by immobilization of skeletal muscle, cast immobilization may cause cold intolerance in mice."

Since the mice are in acute recovery from the anesthetic, there can be no conclusions drawn about thermogenesis. Isoflurane is a great way to depress body temperature (http://www.ncbi.nlm.nih.gov/pubmed/12552204), and the recovery time is not known.

"In addition, as the reviewer suggests, cast immobilization may result in BAT thermogenesis and cachectic effects on muscle and fat. However, circulating corticosterone concentrations and hypothalamic CRH gene expression are not significantly altered after cast immobilization (Figure 2_figure supplement 2D-F)."

The absence of positive results from your stress assays does not exclude stress as the primary source of the results. These mice are not proceeding as normal with their lives - they are learning whole new behaviors in order to stay fed and watered.

https://doi.org/10.7554/eLife.99982.2.sa2

Reviewer #2 (Public review):

Summary:

In this study, the authors identified a previously unrecognized organ interaction where limb immobilization induces thermogenesis in BAT. They showed that limb immobilization by cast fixation enhances the expression of UCP1 as well as amino acid transporters in BAT, and amino acids are supplied from skeletal muscle to BAT during this process, likely contributing to increased thermogenesis in BAT. Furthermore, the experiments with IL-6 knockout mice and IL-6 administration to these mice suggest that this cytokine is likely involved in the supply of amino acids from skeletal muscle to BAT during limb immobilization.

Strengths:

The function of BAT plays a crucial role in the regulation of an individual's energy and body weight. Therefore, identifying new interventions that can control BAT function is not only scientifically significant but also holds substantial promise for medical applications. The authors have thoroughly and comprehensively examined the changes in skeletal muscle and BAT under these conditions, convincingly demonstrating the significance of this organ interaction.

Weaknesses:

Through considerable effort, the authors have demonstrated that limb-immobilized mice exhibit changes in thermogenesis and energy metabolism dynamics at their steady state. However, The impact of immobilization on the function of skeletal muscle and BAT during cold exposure has not been thoroughly analyzed.

Comments on revisions:

The authors appropriately responded to the reviewers' recommendations made during the previous round of peer review.

https://doi.org/10.7554/eLife.99982.2.sa1

Author response:

The following is the authors' response to the current reviews.

Response to eLife Assessment:

We sincerely appreciate your recognition of the novelty and potential significance of our study, and we are grateful for your constructive and valuable comments.

With regard to your concern that cast immobilization (CI) may itself act as a stressor—potentially influencing skeletal muscle, brown adipose tissue (BAT), and locomotor energy expenditure—we fully recognize this as a highly important issue. In our study, we sought to interpret the findings in light of oxygen consumption and activity data; however, it is inherently difficult to disentangle systemic stress responses and the increased energetic costs associated with CI. We have therefore revised the manuscript to explicitly acknowledge this point as a limitation, and to identify it as a subject for future investigation.

We also greatly value your suggestion concerning the potential involvement of branched-chain amino acids (BCAAs) derived from adipose tissue in BAT thermogenesis. While our present work primarily focused on muscle-derived amino acids, previous studies have reported that impaired BCAA catabolism in white adipose tissue (WAT) is associated with

elevated circulating BCAA levels and metabolic dysfunction [1]. Thus, the possibility that adipose tissue contributes to the BCAA pool used by BAT cannot be disregard. We fully agree that directly addressing this possibility would be highly valuable, and in future work we plan to locally administer isotope-labeled BCAAs into skeletal muscle or adipose tissue and analyze their contribution to circulating BCAA levels and BAT utilization. Although such experiments could not be performed within the timeframe of this resubmission, we have explicitly stated this limitation in the revised manuscript.

In summary, we have revised the text to acknowledge the limitations highlighted in your comments and to better clarify future research directions. We believe these revisions more accurately position our current study within the broader context. Once again, we are deeply grateful for your recognition of the originality of our work and for your constructive guidance in refining it.

Response to Reviewers:

We sincerely appreciate the reviewers' thoughtful evaluations and constructive comments, and we are grateful for their recognition of the novelty and significance of our study.

Response to Reviewer 1:

We thank the reviewer for the detailed and thoughtful comments regarding the potential systemic effects of CI, including stress responses, energy balance, and tissue wasting. These factors are indeed critical when interpreting our findings, and we agree that CI is not merely a passive loss-of-function model but also introduces stress-related influences.

The principal aim of our study was to investigate the "physiological compensatory mechanisms" that are triggered by loss of muscle function induced by CI. Although CI inevitably elicits systemic metabolic alterations—including stress-related responses—our study is, to our knowledge, the first to demonstrate that a compensatory thermogenic pathway, mediated by the supply of amino acids from skeletal muscle to BAT, is activated under such conditions. We regard this as the central novelty of our work, and it is consistent with the reviewer's observation that CI results in a "gain of function."

Our intention is not to exclude stress as a contributing factor. Rather, we emphasize that under physiological stress conditions requiring BAT thermogenesis—such as reduced energy stores or decreased heat production from skeletal muscle—amino acid supply from muscle to BAT is induced. Importantly, this mechanism is not unique to CI, as we have confirmed similar metabolic crosstalk under acute cold exposure.

At the same time, we acknowledge that our current data do not allow us to conclude that "stress is not a primary driver" of BAT thermogenesis induced by CI. Chronic stress induced by CI appeared to be limited in our study (Fig. 2_figure supplement 2), but we cannot fully exclude stress-related effects. Accordingly, we now describe the potential triggers of BAT thermogenesis in the manuscript as either decreased body temperature due to muscle functional loss or stress, explicitly noting in the Discussion that stress and reductions in energy reserves may both contribute, as the reviewer suggested. We also modified the original overstatement that "suppression of muscle thermogenesis induces hypothermia," and now limit the description to the observed phenomenon that "CI-induced restriction of muscle activity leads to reduced cold tolerance," while recognizing that multiple factors—including stress, substrate availability, and BAT functional capacity—may underlie this effect.

We further appreciate the reviewer's comment regarding the energetic burden imposed by CI. The cast weighed less than 2 g (5–10% of body weight), and thus increased locomotor costs cannot be excluded. However, locomotor activity during the dark phase was reduced by approximately 50%, making the net energetic effect difficult to quantify. In the manuscript, we now present oxygen consumption data and restrict our description to "an increase in

oxygen consumption per body weight." Moreover, as food intake remained almost unchanged compared with controls, the animals appear to have compensated for additional energetic demands, supporting the interpretation that the observed effects were not solely attributable to starvation.

We also find the reviewer's suggestion—that CI induces BAT overactivation but impairs its functional capacity—extremely important. Indeed, although CI increased thermogenic gene expression in BAT, body temperature maintenance was impaired. We interpret this reduction in thermoregulation as reflecting decreased heat production from skeletal muscle; however, as the reviewer noted, under prolonged CI, depletion of energy stores could further prevent BAT from fully exerting its thermogenic function.

We have clarified in the revised Discussion that BAT activation under CI is transient, and that long-term outcomes may be influenced by contributions from other thermogenic organs, and that we recognize the impact of energy depletion as an important issue to be addressed in future studies. We also agree that detailed analyses of metabolic changes and BCAA dynamics following prolonged CI will be an important next step.

Regarding the reviewer's concern about potential anesthesia effects on acute cold exposure experiments, we confirmed that body temperature had returned to baseline one hour before testing, and that mice displayed spontaneous feeding and grooming behaviors, which suggested adequate recovery. Moreover, the differences observed compared with sham-anesthetized controls support our interpretation that the results reflect CI-specific effects. Nonetheless, we acknowledge this potential confounding factor as an additional limitation.

Response to Reviewer 2:

We thank the reviewer for the constructive comments and clear summary of our findings. We fully agree that the impact of immobilization on skeletal muscle and BAT function under cold exposure represents a key future direction. In the present study, we performed acute cold exposure following short-term immobilization and assessed UCP1 expression and metabolic changes in BAT. However, we acknowledge that we did not fully examine coordinated functional adaptations between skeletal muscle and BAT under cold stress. In particular, how skeletal muscle–derived amino acid supply and IL-6–dependent mechanisms operate during cold exposure remains unresolved. We have therefore noted this explicitly as a limitation and highlighted it as a focus for future work. Going forward, we plan to investigate muscle–BAT metabolic crosstalk and IL-6 signaling in detail under cold conditions to clarify whether the observed responses are specific to CI or represent more general physiological adaptations.

(1) Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem. 2010;285(15):11348-56. doi:10.1074/jbc.M109.075184.

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Heat production mechanisms are flexible, depending on a wide variety of genetic, dietary, and environmental factors. The physiology associated with each mechanism is important to understand since loss of flexibility is associated with metabolic decline and disease. The phenomenon of compensatory heat production has been described in some detail in publications and reviews, notably by modifying BAT-dependent thermogenesis (for example by deleting UCP1 or impairing lipolysis, cited in this paper). These authors chose to eliminate exercise as an alternative means of maintaining body temperature. To*

> *do this, they cast either one or both mouse hindlimbs. This paper is set up as an evaluation of a loss of function of muscle on the functionality of BAT.*
>
> *Strengths:*
>
> *The study is supported by a variety of modern techniques and procedures.*
>
> *Weaknesses:*
>
> *The authors show that cast immobilization (CI) does not work as a (passive) loss of function, instead, this procedure produces a dramatic gain of function, putting the animal under considerable stress, inducing b-adrenergic effectors, increased oxygen consumption, and IL6 expression in a variety of tissues, together with commensurate cachectic effects on muscle and fat. The BAT is put under considerable stress, super-induced but relatively poor functioning. Thus within hours and days of CI, there is massive muscle loss (leading to high circulating BCAAs), and loss of lipid reserves in adipose and liver. The lipid cycle that maintains BAT thermogenesis is depleted and the mouse is unable to maintain body temperature.*
>
> *I cannot agree with these statements in the Discussion:*
>
> *"We have here shown that cast immobilization suppressed skeletal muscle thermogenesis, resulting in failure to maintain core body temperature in a cold environment."*
>
> *This result could also be attributed to high stress and decreased calorie reserves. Note also: CI suppresses 50% of locomotor activity, but the actual work done by the mouse carrying bilateral casts is not taken into account.*

We appreciate the reviewer's suggestion. We thank you for raising this issue. As the reviewers suggest, we also consider that cold intolerance resulting from cast immobilization may be attributed to high stress levels, decreased calorie reserves, or reduced systemic locomotor activity. Indeed, reductions in the weight of visceral adipose tissue weight and increases in lipid utilization were observed in the early phase of cast immobilization (Fig.2G and 2F). This suggests that the depletion of calorie reserves induced by stress may affect cold intolerance in cast immobilized mice (Fig.1A-1B). On the other hand, the experiment shown in Fig.1C involved acute cold exposure of mice 2 h after cast immobilization. This result suggests that, even before the depletion of energy stores by immobilization of skeletal muscle, cast immobilization may cause cold intolerance in mice. In addition, as the reviewer suggests, cast immobilization may result in BAT thermogenesis and cachectic effects on muscle and fat. However, circulating corticosterone concentrations and hypothalamic CRH gene expression are not significantly altered after cast immobilization (Figure 2_figure supplement 2D-F). This raises questions about the contribution of stress to the changes in the systemic energy metabolism in this model. As such, we responded to the reviewers' comments by revising this statement at the beginning of the 'Discussion' section and adding a discussion on pages 16, in addition to the existing discussion on pages 17–18.

Furthermore, to respond as best we could to the reviewer's comments, we performed additional experiments using the restraint stress model (Figure 7). We found that short-term restraint stress may recruit substrate supply from skeletal muscle for BAT thermogenesis via Il6 gene expression. Based on these data, we speculate that the interaction between BAT and skeletal muscle amino acid metabolism may operate under various physiological stress conditions, including infection and exercise, as well as skeletal muscle immobilization, stress, and cold exposure. This interaction may play a significant role in regulating body temperature and energy metabolism. We are currently investigating the effects of sympathetic activation on skeletal muscle amino acid metabolism and systemic

thermoregulation via IL-6 secretion from skeletal muscle using a new model. These data will be reported in a subsequent report.

> *"Thermoregulatory system in endotherms cannot be explained by thermogenesis based on muscle contraction alone, with nonshivering thermogenesis being required as a component of the ability to tolerate cold temperatures in the long term."*
>
> *This statement is correct, and it clearly showcases how difficult it is to interpret results using this CI strategy. The question to the author is- which components of muscle thermogenesis are actually inhibited by CI, and what is the relative heat contribution?*

We appreciate raising this important issue. This study required the measurements of skeletal muscle temperature and electromyography in mice with cast immobilization, but we were unable to perform these measurements. We have therefore described the reviewers suggest on page 18 as limitations of this study.

In our additional experiments, we found that several genes that are usually activated in skeletal muscle during cold exposure are repressed in mice with cast immobilization (Figure 1_figure supplement 1_G-1K). Skeletal muscle is an important thermogenic organ. Although the role of the sarcolipin gene in non-shivering thermogenesis is well understood, the primary regulator of thermogenesis in metabolism and shivering remains unclear. In Future, we would like to use models in which key signals for energy metabolism are inhibited, such as muscle-specific PGC-1α-deficient mice and muscle-specific AMPK-deficient mice, to clarify important factors in skeletal muscle heat thermogenesis. We expect this approach to enable us to analyze the relative thermal contributions of each component of the heat production process in skeletal muscle, which has proven difficult in immobilized muscle models.

> *This conclusion is overinterpreted:*
>
> *"In conclusion, we have shown that cast immobilization induced thermogenesis in BAT that was dependent on the utilization of free amino acids derived from skeletal muscle, and that muscle-derived IL-6 stimulated BCAA metabolism in skeletal muscle. Our findings may provide new insights into the significance of skeletal muscle as a large reservoir of amino acids in the regulation of body temperature".*
>
> *In terms of the production of the article - the data shown in the heat maps has oddly obscure log10 dimensions. The changes are minimal, approx. 1.5x increase/decrease and therefore significance would be key to reporting these data. Fig.3C heatmap is not suitable. What are the 6 lanes to each condition? Overall, this has little/no information.*
>
> *Rather than cherry-picking for a few genes, the results could be made more rigorous using RNA-seq profiling of BAT and muscle tissues.*

We agree that this is an important point. Indeed, our model of skeletal muscle immobilization reveals only modest changes in metabolomics and gene expression analysis. We consider this to be a weakness of the study. However, the interactive thermogenic system that we discovered between skeletal muscle and BAT may also function under other conditions, such as acute stress and cold exposure. We should investigate this further in future models involving such dramatic metabolic changes. In fact, it has been shown that the levels of several metabolites are significantly altered in BAT after acute cold exposure.[1] Therefore, we have corrected the conclusion of this section, as stated on page 18, and added it. We also performed an enrichment analysis on the metabolomics data in BAT following cast immobilization and included the results in Figure 2_figure Supplement 1A. In addition, we excluded the heatmap from Fig. 3C of the pre-revision manuscript, as advised by the reviewer. Although we excluded the results in Figure 3C, we consider Figure 3_figure supplement_1 to be sufficient for the text.

In addition, we agree with the reviewer's remarks on our gene expression analysis. In this study, we were unable to examine RNA-seq profiling of BAT and muscle tissue. Therefore, we have described this as a limitation of the study on page 20. However, we are interested in investigating the effect of IL-6 derived from skeletal muscle on RNA-seq profiling of skeletal muscle and BAT. We will conduct future RNA-seq analyses of BAT and skeletal muscle, using models of skeletal muscle immobilization, acute cold exposure and restraint stress.

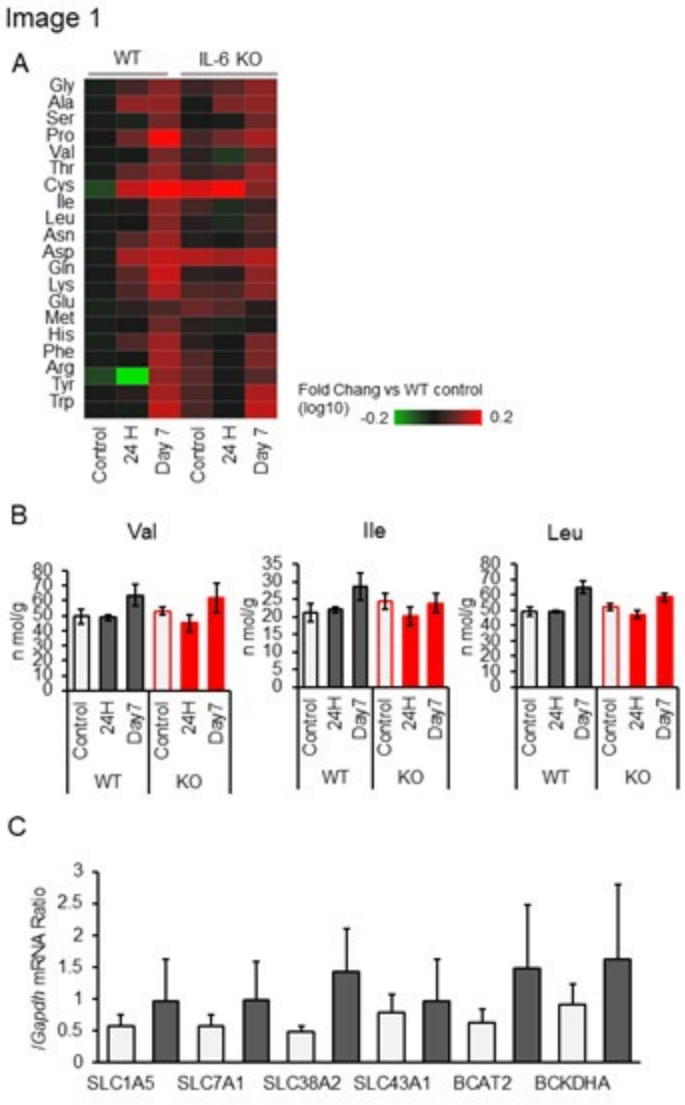
> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In this study, the authors identified a previously unrecognized organ interaction where limb immobilization induces thermogenesis in BAT. They showed that limb immobilization by cast fixation enhances the expression of UCP1 as well as amino acid transporters in BAT, and amino acids are supplied from skeletal muscle to BAT during this process, likely contributing to increased thermogenesis in BAT. Furthermore, the experiments with IL-6 knockout mice and IL-6 administration to these mice suggest that this cytokine is likely involved in the supply of amino acids from skeletal muscle to BAT during limb immobilization.*
>
> *Strengths:*
>
> *The function of BAT plays a crucial role in the regulation of an individual's energy and body weight. Therefore, identifying new interventions that can control BAT function is not only scientifically significant but also holds substantial promise for medical applications. The authors have thoroughly and comprehensively examined the changes in skeletal muscle and BAT under these conditions, convincingly demonstrating the significance of this organ interaction.*
>
> *Weaknesses:*
>
> *Through considerable effort, the authors have demonstrated that limb-immobilized mice exhibit changes in thermogenesis and energy metabolism dynamics at their steady state. However, The impact of immobilization on the function of skeletal muscle and BAT during cold exposure has not been thoroughly analyzed.*
>
> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, the authors show that impairment of hind limb muscle contraction by cast immobilization suppresses skeletal muscle thermogenesis and activates thermogenesis in brown fat. They also propose that free BCAAs derived from skeletal muscle are used for BAT thermogenesis, and identify IL-6 as a potential regulator.*
>
> *Strengths:*
>
> *The data support the conclusions for the most part.*
>
> *Weaknesses: The data provided in this manuscript are largely descriptive. It is therefore difficult to assess the potential significance of the work. Moreover, many of the described effects are modest in magnitude, questioning the overall functional relevance of this pathway. There are no experiments that directly test whether BCAAs derived from adipose tissue are used for thermogenesis, which would require more robust tracing experiments. In addition, the rigor of the work should be improved. It is also recommended to put the current work in the context of the literature.*

We appreciate the reviewer's valuable feedback. As the reviewer pointed out, many of the effects described in this study are modest in magnitude. This reflects a limitation of our study, which used skeletal muscle immobilization as a model. To clarify the overall functional relevance of this pathway, we therefore plan to use alternative models in which BAT thermogenesis and systemic cachectic effect are more strongly induced. We have added this point to the 'Conclusion' section on page 18.

In addition, previous findings reported that mitochondrial BCAA catabolism in brown adipocytes promotes systemic BCAA clearance, suggesting that BCAAs may be supplied to BAT from other organs during BAT thermogenesis.[5] However, as the reviewer rightly pointed out, the current study did not directly investigate whether BCAAs derived from adipose tissue contribute to thermogenic processes. In light of this, we have revised the manuscript to include a statement in the limitations section on page 20 that addresses this point.

Metabolomic analysis of white adipose tissue (WAT) following skeletal muscle immobilization revealed alterations in amino acid concentrations in WAT in response to cast immobilization (Author response image 1A). Notably, levels of BCAAs in WAT remained largely unchanged at 24 hours after cast immobilization, but increased significantly by day 7 (Author response image 1B). At the 24-hour time point, when BAT thermogenesis is known to be activated, WAT weights was found to be reduced (Fig. 2H). Gene expression analysis of amino acid metabolism-related genes in WAT at this time point revealed a modest upregulation of several genes (Author response image 1C). Furthermore, a slight increase in the uptake of [^{3}H] leucine into WAT was observed following immobilization (Fig. 3C). Collectively, these findings suggest that BCAAs within WAT may be primarily metabolized locally rather than being mobilized and supplied to BAT. In addition, given the relatively low levels of BCAAs per tissue mass and the limited capacity for BCAA uptake in WAT compared to other tissues, we consider it unlikely that WAT serves as a major reservoir of BCAAs.

Author response image 1.



(A) Amino acids in epididymal white adipose tissue (eWAT) of IL-6 KO (–/–) and WT (+/+) mice without (control) or with bilateral cast immobilization for the indicated times. Results are presented as heat maps of the log10 value of the fold change relative to control WT mice and are means of four mice in each group. (B) BCAA concentrations in eWAT of IL-6 KO and WT mice without (control) or with bilateral cast immobilization for 1 or 7 days. (n = 4 per group) (C) RT and real-time PCR analysis of the expression of SLC1A5, SLC7A1, SLC38A2, SLC43A1, BCAT2 and BCKDHA genes in eWAT of mice without (control) or with bilateral cast immobilization for 24 h. (n = 6 per group). All data other than in (A) are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by Dunnett's test (B) or by the unpaired t test (C).

> ***Reviewer #1 (Recommendations for the authors):***
>
> *• Gypsum is an irrelevant label. Label consistently, with a procedure acronym, like CI or Imm.*

We apologize for any confusion that our notation may have caused. We corrected all labels relating to the skeletal muscle immobilization model in mice to 'Imm'.

> *There are many grammatical errors and typos. Search for an example on Fudure1. The sense of some sentences is enough to obscure their meaning.*

We appreciate the reviewer's points. We have checked the article for grammatical and typographical errors, correcting them where necessary.

> *• Figures 6E and F need to be re-annotated in the legend and on figures.*

Following the peer reviewer's advice, we have re-annotated the Figure legends of this result.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) It is difficult to understand how the data presented in Supplemental Table 1 were obtained. This appears to be data showing that the skeletal muscle weight of the hind limbs in mice accounts for 40 to 50% of the total skeletal muscle weight. How did the authors calculate the muscle weight? Specifically, how did they measure the weight of muscles that are neither in the hind limbs nor in the forelimbs ("Other")? Was this estimated from whole-body CT or MRI data?*
>
> *In the legend, it mentions "the posterior cervical region," but what exactly was measured in the posterior cervical region? The methods for this data should be clearly described.*

We appreciate the reviewers' comments. We apologize for any confusion caused by inadequate explanation of this data. This data was obtained by removing skeletal muscle from the posterior cervical region and measuring the weight of the wet tissue. We have taken care to remove most of the skeletal muscle, but some will remain. However, we do not believe that these errors are significant enough to alter the interpretation of the results. This has now been added to the 'Methods' section on page 21.

> *(2) Through considerable effort, the authors have demonstrated that limb-immobilized mice exhibit changes in thermogenesis and energy metabolism dynamics at their steady state. However, it remains unclear why limb-immobilized mice have reduced tolerance to cold exposure. Was there any change in the abundance of energy metabolism-related genes during cold exposure between the immobilized and control mice? For example, if the gene expression of UCP1 and UCP2, which are typically upregulated in brown adipose tissue (BAT) and skeletal muscle during cold exposure, was suppressed in the immobilized mice, it might explain their reduced cold tolerance. Thus, the changes in the response of skeletal muscle and BAT to cold exposure between immobilized and control mice should also be analyzed.*

We thank the reviewer for the constructive comments. We consider the main weakness of this study to be the fact that we were unable to measure the temperature and electromyography (EMG) of the skeletal muscles of the cast-immobilized mice. Following the reviewers' advice, we analyzed the expression levels of several genes related to heat production or energy metabolism (Ucp1, Ucp2, Ucp3, Sln and Ppargc1a) in BAT and skeletal muscle of cast-immobilized mice after acute cold exposure (Figure1_figure supplement 1G-1K). The results showed that the expression of several genes that are usually increased in BAT and skeletal muscle during cold exposure was repressed in cast-immobilized mice. Notably, cast immobilization did not induce the UCP2 and PGC-1α genes at room temperature, and their upregulation during cold exposure was also suppressed in cast-immobilized mice. UCP2 is known to alter its expression in relation to energy metabolism, but it is unclear whether it

regulates energy metabolism.[2] Additionally, UCP2 is understood to play a non-role in thermogenesis, and the function of the UCP2 in skeletal muscle remains unclear.[3] On the other hands, PGC-1α is widely recognized as a transcriptional coactivator that regulates various metabolic processes, including thermogenesis.[4] In our study, we found that the amounts of metabolites in the TCA cycle and the expression of the PGC-1α gene were decreased rapidly in immobilized skeletal muscle. This suggests that the metabolic rate is reduced in immobilized skeletal muscle (Figure 1_figure supplement 2A and 2F). In endothermic animals, energy expenditure in skeletal muscle plays a significant role in maintaining body temperature during both activity and rest. Hence, it is assumed that the reduced metabolic rate in skeletal muscle significantly impacts the maintenance of body temperature in cold conditions. Further investigation is required into the function of these genes in skeletal muscle thermogenesis, but we expect that the additional data suggest that the loss of muscle function due to immobilization affects the maintenance of body temperature under cold temperature. These results were discussed further on page 15.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *There are also more specific concerns related to the data supporting the claims.*
>
> *(1) The relevance of increasing thermogenesis in BAT after cast immobilization is unclear, as adult humans have very little BAT. Thermogenesis gene and protein expression should be measured in white adipose tissue.*

We would like to thank the reviewers for highlighting this important issue. We agree with the reviewer's comments. We did not observe significant changes in UCP1 expression in the subcutaneous adipose tissue of the inguinal region following skeletal muscle immobilization. We suspect that this is because skeletal muscle immobilization in mice did not exert a strong enough effect to induce browning of white adipose tissue. The ability of immobilizing skeletal muscle to activate thermogenesis in brown or beige adipocytes in adults remains unclear. We have therefore noted this limitation in our study in line 6.

Additionally, in this study, we aimed to clarify the role of skeletal muscle as an amino acid reservoir under metabolic stress conditions that increase BAT thermogenesis. To this end, we employed models of skeletal muscle immobilization, acute cold exposure, and restraint stress. We also intend to analyze the metabolic interactions between beige adipose tissue and skeletal muscle in more detail using models that induce browning, such as exercise or cold acclimation.

> *(2) In Figures 1E-G, there is no significant difference in UCP1 levels relative to the control, but body temperature is lowered from day 2 to day 7. How do the authors explain this?*

This is an important point. We consider the decrease in body temperature of mice following cast immobilization at room temperature to be the result of a reduction in systemic locomotor activity.

> *(3) The small induction of PGC1α seen at 10 hours goes away after day 3. Why is this?*

This is an important point. Our investigation showed that the norepinephrine concentration in BAT and blood of cast-immobilized mice tends to increase, peaking at 24 hours of immobilization (Fig. 1H and Figure 2_figure supplement 2D), and then gradually returns to baseline. We speculate that this transient activation of the sympathetic nervous system may affect the expression of PGC1α in BAT. Additionally, although thermogenesis in BAT temporarily increases after skeletal muscle immobilization, studies from other research groups suggest that long-term skeletal muscle immobilization (two weeks) may increase non-shivering thermogenesis in skeletal muscle via high expression SLN.[6] Therefore, we

hypothesize that other thermogenic mechanisms besides BAT might be involved during prolonged cast immobilization. We have added a discussion of these topics on page 16.

> *(4) The metabolic cage data are marked in multiple places as significant, but the effect size is extremely small. Please describe how significance was calculated (Figure 5 supplement 1B, E, F).*

This is a valid point. This data was statistically analyzed using daily averages, with the results then being compiled. However, the figure was amended because it was not appropriate to use the original to demonstrate significant differences.

> *(5) How does IL-6 increase BCAA levels in muscle?*

This is an important point. We are also investigating this issue with great interest. In future, we will use RNA-seq profiling to investigate the mechanism by which IL-6 regulates amino acid metabolism in skeletal muscle. This point was added as a

limitation of the study on page 19.

> *(6) What is the mechanism behind the elevated il6 levels after cast immobilization?*

We appreciate the reviewer's points. Since IL-6 gene expression in skeletal muscle increases in response to acute cold exposure and acute stress, we hypothesize that IL-6 is regulated by β-adrenergic effectors. In our preliminary experiments, stimulation with norepinephrine or with clenbuterol, a β2-adrenergic receptor agonist, suggests an increase in IL-6 gene expression and the intracellular free BCAA concentration in cultured mouse muscle cells (Author response image 2A-2D). Going forward, our plans include conducting further studies using a mouse model in which the sympathetic nervous system is activated by administering LPS intracerebroventricularly, as well as using muscle-specific β2-adrenergic receptor knockout mice.

Reference:

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(3) C Y Zhang, et al., Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes., Cell. 2001 Jun 15;105(6):745-55. doi: 10.1016/s0092-8674(01)00378-6.

(4) Christophe Handschin and Bruce M Spiegelman, Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism., Endocr Rev. 2006 Dec;27(7):728-35. doi: 10.1210/er.2006-0037.

(5) Yoneshiro, et al., BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature. 2019 572(7771): 614-619 doi: 10.1038/s41586-019-1503-x.

(6) Shigeto Tomiya, et al., Cast immobilization of hindlimb upregulates sarcolipin expression in atrophied skeletal muscles and increases thermogenesis in C57BL/6J mice., Am J Physiol Regul Integr Comp Physiol. 2019 Nov1;317(5):R649-R661.doi:10.1152/ajpregu.00118.2019.

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