The hepatokine Tsukushi gates energy expenditure via brown fat sympathetic innervation

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Thermogenesis is an important contributor to whole-body energy expenditure and metabolic homeostasis. Although circulating factors that promote energy expenditure are known, endocrine molecules that suppress energy expenditure have remained largely elusive. Here we found that Tsukushi (TSK) is a liver-enriched secreted factor that is highly inducible in response to increased energy expenditure. Hepatic Tsk expression and plasma TSK levels were elevated in obesity. In mice, TSK deficiency increased sympathetic innervation and norepinephrine release in adipose tissue, leading to enhanced adrenergic signalling and thermogenesis, attenuation of brown fat whitening, and protection from diet-induced obesity. Our data reveal TSK as part of a negative feedback mechanism that gates thermogenic energy expenditure and highlights TSK as a potential target for therapeutic intervention in metabolic disease.

Endocrine hormones released by peripheral tissues, such as adipose tissue, skeletal muscle, and the liver, have an important role in metabolic cross-talk and homeostasis1–3. The adipose tissue hormone leptin acts on the central nervous system to suppress food intake and promote energy expenditure4,5. Defects in the neuroendocrine circuitry that mediates leptin action have been linked to obesity and metabolic disorders. Fibroblast growth factor 21 (FGF21) is a hepatokine that exerts pleiotropic metabolic effects, including the stimulation of brown and beige fat thermogenesis21–23. Genetic ablation of brown fat renders mice cold-sensitive and exacerbates diet-induced weight gain24, whereas activation of BAT thermogenesis has been linked to increased energy expenditure, reduced adiposity, and lower plasma lipid levels25–27. Brown fat thermogenesis is stimulated in response to cold exposure, through increased adrenergic signaling to the depot and local bioactive thyroid hormone production, leading to activation of the thermogenic gene programme and fuel oxidation25.

In this study, we performed liver secretome analysis and identified TSK as an inducible hepatokine that responds to increased energy expenditure. TSK acts as a hormonal checkpoint that suppresses adipose tissue sympathetic innervation, adrenergic action, and thermogenesis. Our work illustrates the existence of powerful endocrine hormones that dampen energy expenditure, and reveals an intriguing opportunity for targeting TSK to restore energy balance and improve metabolic parameters in obesity.

Results  
TSK is a hepatokine inducible in response to energy expenditure. We postulated that the liver provides a source of putative regulators of energy balance, for several reasons. The liver itself is highly responsive to nutritional, hormonal, and neural cues by integrating a wide array of nutrient and energy-sensing pathways. Hepatocytes undergo drastic reprogramming of the release of secreted factors, such as FGF21, under different physiological and stress conditions18. We took an unbiased bioinformatic approach to first identify secreted factors that exhibit enriched or restricted expression in the liver. We examined the mRNA expression profile of approximately 1,384 genes annotated to encode secreted proteins in a panel of 12 mouse tissues, including different brain regions and peripheral tissues (GSE54650). Clustering analysis revealed a highly restricted pattern of tissue distribution for this secretome gene set, illustrating the remarkable specificity and diversity of protein secretion by different tissues (Fig. 1a and Supplementary Table 1). As expected, leptin and neuregulin 4 were among the top secreted factor hits in liver. We identified a cluster of 129 genes exhibiting over fivefold enriched expression in mouse liver compared with the averaged expression values from other tissues (red box, Fig. 1a and Supplementary Table 2). As expected, many of these

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NATURE METABOLISM | VOL 1 | FEBRUARY 2019 | 251–260 | www.nature.com/natmetab

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liver-enriched secreted factors have known biological functions in lipoprotein metabolism, blood clotting, complement activation, and endocrine signaling. The latter included lipocalin 13 and fetuin B, two liver-derived endocrine factors that have been implicated in the regulation of glucose and lipid metabolism. As the tissue panel was obtained from healthy mice, it is possible that our analysis may not include secretome genes specifically induced under pathological conditions.

We hypothesized that the putative inhibitory regulators of energy expenditure may be induced in hypermetabolic states to restore energy balance by serving as a feedback signal. As such, we next examined regulation of the liver secretome genes in hypermetabolic states.

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**Fig. 1 | TSK is a hepatokine responsive to increased energy expenditure.**

- **a.** Heat map representation of secretome gene expression in chow-fed mouse tissues (left). The red box indicates a cluster of genes encoding liver-enriched secreted factors, four of which were induced by T3 treatment (right).
- **b.** Quantitative PCR (qPCR) analysis of Tsk expression in mouse tissues (n = 4) and in fractionated hepatocytes (Hep) and non-parenchymal cells (NPC) (n = 3). LacZ staining of tissues from mice containing a Tsk gene trap allele. Note the presence of strong β-galactosidase activity in the liver (blue asterisk).
- **d.** Immunoblots of total plasma proteins from WT and Tsk KO mice. Ponceau S staining is shown as loading control.
- **e.** TSK immunoblot of conditioned media collected from cultured WT and KO hepatocytes at indicated time points. qPCR analysis of hepatic Tsk expression (left) and immunoblots of plasma (right) from mice treated with T3 (500 μg kg⁻¹) (saline, n = 4; T3, n = 4), CL 316,243 (1 mg kg⁻¹) (saline, n = 4; CL, n = 4) or cold exposure for 4 h (room temperature (RT), n = 5; cold, n = 6).
- **f.** Rectal body temperature in WT (n = 7) and Tsk KO (n = 6) mice under ad libitum conditions and following 24 h of fasting.
- **h.** Body weight loss in WT (n = 7) and Tsk KO (n = 6) mice following 24 h of food deprivation. Two-tailed unpaired Student’s t test (b, f, h); two-way ANOVA (g); data are mean ± s.e.m. of biologically independent samples.
mice induced by chronic triiodothyronine (T3) treatment (GSE68867)\(^4\). This analysis identified a total of four genes exhibiting increased expression in T3-treated mouse livers, including the small leucine-rich-repeat-containing protein Tsk, fibronectin 1, apolipoprotein M (Apolm), and secreted phosphoprotein 2 (Spp2). Fibronectin and Spp2 are extracellular matrix proteins, whereas Apolm is a component of plasma lipoproteins. Tsk is a highly conserved secreted protein that exhibits the highest messenger RNA (mRNA) expression in mouse liver compared with other tissues (Fig. 1b and Supplementary Fig. 1). We confirmed this expression profile by performing LacZ staining on tissues from mice harboring a Tsk gene trap allele and observed strong β-galactosidase activity in the liver, but not in other tissues (Fig. 1c). Notably, Tsk mRNA was detected primarily in hepatocytes, but not in non-parenchymal cells (NPC), suggesting that Tsk encodes a bona fide hepatokine (Fig. 1b). To determine whether mature TSK is released into the circulation, we generated highly specific rabbit polyclonal antibodies against a C-terminal peptide of TSK. As shown in Figure 1d, TSK protein was readily detectable in mouse plasma; the specific band was completely absent in plasma samples from Tsk knockout (KO) mice. We observed robust secretion of endogenous TSK protein by cultured primary hepatocytes isolated from wild-type (WT), but not Tsk null, mice (Fig. 1e).

TSK was previously described as a regulator of commissure formation, although its expression in the brain was relatively low compared with the liver\(^6\)–\(^8\). Its role in metabolic signaling and energy balance remains unknown. To determine whether hepatic Tsk expression and plasma TSK levels are responsive to increased energy expenditure, we examined these parameters in mice treated with T3 or CL 316,243, a β3-selective adrenergic agonist, or subjected to cold exposure for 4 h. Similar to chronic treatments, a single injection of T3 resulted in robust induction of Tsk mRNA expression and elevated TSK levels in the circulation (Fig. 1f). Notably, a single dose of CL 316,243 treatment and acute cold exposure also stimulated hepatic Tsk mRNA expression, resulting in higher plasma TSK levels. These results strongly suggest that TSK is a hepatokine that is tightly linked to the activation of thermogenesis and energy expenditure. We next examined whether increased circulating TSK may provide a negative feedback signal to restrict energy expenditure in mice. In support of this, we observed that chow-fed Tsk null mice exhibited significantly higher core body temperatures than WT littermates under ad libitum conditions (Fig. 1g). This elevation of body temperature in KO mice persisted following overnight starvation. In addition, fasting-induced weight loss was higher by approximately 36% in mice lacking TSK than in controls (Fig. 1h), indicating that Tsk null mice are hypermetabolic and fail to suppress energy expenditure in response to food deprivation.

**TSK deficiency protects mice from high-fat-diet-induced obesity and metabolic disorders.** To determine whether TSK dysregulation might facilitate positive energy balance in obesity, we next examined hepatic Tsk expression in diet-induced and genetic obesity in mice. Compared with lean control, Tsk mRNA expression was significantly elevated in high-fat diet (HFD)-fed and leptin-receptor-deficient (db/db) obese mouse livers (Supplementary Fig. 2). Hepatic Tsk mRNA levels positively correlated with weight gain in a cohort of WT C57BL/6 mice following eight weeks of HFD feeding (Supplementary Fig. 2). Accordingly, plasma TSK levels were markedly elevated in HFD-fed and db/db mice (Fig. 2a), indicating that obesity is associated with abnormally high TSK levels in the circulation.

TSK-deficient mice appeared normal by gross examination, weighed slightly less than littermate controls and did not exhibit significant changes in their metabolic parameters when fed standard rodent chow (Supplementary Fig. 3). Following high-fat feeding, KO mice exhibited marked resistance to HFD-induced weight gain (Fig. 2b). WT mice gained an average of 66.3% body weight over a period of 14 weeks of high-fat feeding, whereas KO mice gained approximately 30.7%. Blood glucose levels were significantly lower in KO mice (Fig. 2c), whereas plasma total cholesterol and triglyceride (TAG) levels remained similar between the groups. Measurements of key circulating hormones indicated that, compared with control, plasma levels of insulin and leptin, but not FGF21, were markedly lower in Tsk null mice (Fig. 2d). Glucose tolerance test (GTT) and insulin tolerance test (ITT) indicated that Tsk null mice exhibited significantly improved insulin sensitivity and glucose tolerance (Fig. 2e).

Tsk null mice exhibited lower epididymal WAT (eWAT), BAT, and liver mass than controls following HFD feeding (Fig. 2f and Supplementary Fig. 4a). As expected, brown fat from HFD-fed WT mice appeared pale in color as a result of whitening of brown adipocytes. In marked contrast, brown fat from Tsk null mice remained dark red and appeared similar to brown fat obtained from cold-exposed mice (Fig. 2g). Histological analysis revealed that brown adipocytes in interscapular BAT from HFD-fed KO mice had reduced fat content and contained smaller lipid droplets (Fig. 2h). eWAT from Tsk KO mice contained smaller adipocytes and fewer crown-like structures, suggesting that obesity-associated adipose tissue inflammation is attenuated by TSK deficiency. In support of this, mRNA expression of genes associated with macrophage and adipose inflammation was significantly lower in eWAT from Tsk KO mice (Supplementary Fig. 4b). Tsk null mice had improved hepatic steatosis, as shown histologically and by measurement of liver TAG content (Fig. 2h–i). To establish how TSK deficiency influences energy balance, we performed metabolic cage studies in WT and KO mice fed HFD for 2 weeks to minimize confounding factors of prolonged HFD feeding. Compared with control, Tsk null mice exhibited significantly elevated VO₂ and energy expenditure rate (Fig. 3a and Supplementary Fig. 5). In contrast, food intake and total locomotor activity appeared comparable in the two groups. These results strongly suggest that TSK deficiency may abrogate a negative feedback brake on whole-body energy expenditure, resulting in resistance to diet-induced obesity.

**TSK deficiency promotes adipose thermogenesis and energy expenditure.** Brown fat thermogenesis is an important component of energy expenditure that contributes to defence against cold and obesity. We next examined whether Tsk inactivation results in stimulation of brown fat thermogenesis. Microarray analysis of brown fat gene expression identified a cluster of 202 genes that exhibited >1.4-fold increase in Tsk KO mice (Fig. 3b). Gene ontology analysis indicated that this cluster was highly enriched for pathways involved in mitochondria, lipid metabolism, nucleotide binding, and oxidoreductase. qPCR analysis confirmed that mRNA expression of key thermogenic markers, including Deiodinase 2 (Dio2) and Ucp1, was significantly higher in BAT from chow-fed Tsk null mice (Fig. 3c). UCP1 protein levels were also higher as a result of TSK deficiency (Fig. 3d). Notably, phosphorylation of hormone-sensitive lipase (HSL) and protein kinase A (PKA) substrates was markedly enhanced in brown fat obtained from Tsk KO mice. These results demonstrate that TSK deficiency augments adrenergic action and thermogenic stimulation in brown fat. Measurement of norepinephrine concentrations revealed that BAT, but not eWAT, exhibited significantly elevated norepinephrine levels as a result of TSK deficiency (Fig. 3e). In contrast, plasma norepinephrine levels were comparable between the groups, suggesting that TSK deficiency may enhance sympathetic outflow to brown fat to promote thermogenesis. Consistently, brown fat from HFD-fed Tsk null mice exhibited increased PKA substrate phosphorylation and UCP1 protein expression, characteristics of enhanced adrenergic activation (Fig. 3f). PKA substrate phosphorylation was also enhanced in inguinal WAT from HFD-fed Tsk null mice compared with control. Dio2 is...
responsible for the local activation of thyroid hormone, which acts in concert with adrenergic signaling to stimulate thermogenesis. We found that BAT from Tsk KO mice had slightly, but significantly, increased T3 levels (Fig. 3g).

The studies above illustrated that TSK serves an important role in energy balance and metabolic physiology. However, whole-body TSK deficiency may elicit unforeseen effects on developmental programmes. In addition, it remains unknown whether the liver provides a major source of plasma TSK in metabolic regulation. To address these issues, we employed a recently developed CRISPR/Cas9 method to knock out Tsk in the liver in adult mice. We generated a recombinant adenovirus-associated virus (AAV) expressing a pair of guide RNAs targeting the coding region of Tsk (gTsk). Tail vein injection of AAV-gTsk in Cas9 transgenic mice resulted in efficient deletion of Tsk in the liver and marked reduction of TSK in the circulation (Fig. 4a,b), illustrating the hepatic origin of circulating TSK. We subjected mice transduced with AAV-GFP and AAV-gTsk to HFD feeding and analysed their metabolic parameters. Compared with control, the latter group tended to gain less body weight and had lower blood glucose, yet the data did not reach statistical significance (Fig. 4c). In contrast, AAV-gTsk mice had significantly lower plasma insulin concentrations, improved insulin sensitivity (Fig. 4d), and reduced lipid content in brown adipocytes (Fig. 4e). Immunoblotting analysis indicated that hepatic Tsk inactivation resulted in increased tyrosine hydroxylase and UCP1 levels and enhanced adrenergic signaling (Fig. 4f), reminiscent of the

Fig. 2 | TSK facilitates diet-induced obesity and brown fat whitening. a, Immunobots of plasma from lean and obese mice. b, Body weight of male WT (n = 9) and Tsk KO (n = 12) mice during HFD feeding. c, Plasma glucose and lipid concentrations (WT, n = 8; KO, n = 10, except KO plasma TAG, n = 9). d, Plasma insulin (WT, n = 8; KO, n = 10), FGF21, and leptin (WT, n = 6; KO, n = 7) levels. e, GTT and ITT in HFD-fed WT (n = 9) and Tsk KO (n = 12) mice. f, Tissue weight of HFD-fed mice (WT, n = 7; KO, n = 11). g, Morphology of brown fat from HFD-fed mice. h, H&E staining of tissue sections (scale bar = 100 μm). i, Liver TAG content (WT, n = 7; KO, n = 11). Two-tailed unpaired Student’s t test (c, d, f, i); two-way ANOVA (b); data are mean ± s.e.m. of biologically independent samples.
Fig. 3 | TSK ablation relieved a brake on sympathetic action in adipose tissue. a, Oxygen consumption rate (top) and food intake (bottom) in male WT (n = 8) and Tsk KO (n = 8) mice following 2 weeks of high-fat feeding. Averaged oxygen consumption rate and food intake in dark and light phases are indicated on the right. b, Heat map of a cluster of genes induced by >1.4-fold in brown fat from Tsk KO mice. Enriched pathways in this cluster are indicated. c, qPCR analysis of Dio2 and Ucp1 mRNA expression in BAT (n = 7). d, Immunoblots of total brown fat lysates from chow-fed mice. Relative tyrosine hydroxylase (TH) band intensity was quantified using Image J (right) (n = 7). e, Plasma and adipose tissue norepinephrine concentrations from WT (n = 8) and Tsk KO (n = 8) mice. f, Immunoblots of total brown fat lysates from HFD-fed mice (WT, n = 6; KO, n = 7). g, Brown fat T3 content from WT (n = 6) and Tsk KO (n = 7) mice. Two-tailed unpaired Student’s t test (a, c–g); data are mean ± s.e.m. of biologically independent samples.

TSK is a negative regulator of sympathetic innervation in brown fat. Adipose tissue is densely innervated by sympathetic nerve fibres that control adipocyte lipolysis and thermogenesis. 1–3. Adrenergic stimulation as a result of sympathetic outflow provides a major catecholaminergic signal for adipose tissue that balances fat storage and utilization. 4–6. Low sympathetic activity and defective adrenergic signaling have been linked to reduced adipose tissue thermogenesis and brown fat whitening, leading to obesity and metabolic disorders. 7–9. The striking activation of adrenergic signaling in Tsk null adipose tissue raised the possibility that TSK may regulate sympathetic innervation in adipose tissue. In support of this, we observed elevated tyrosine hydroxylase protein levels in brown fat from Tsk KO mice (Fig. 3d). Tyrosine hydroxylase is a rate-limiting enzyme in catecholamine biosynthesis and serves as a marker for sympathetic nerve fibres. Notably, tyrosine hydroxylase protein levels were markedly higher in Tsk KO BAT and inguinal WAT than in littermate controls following HFD feeding (Fig. 3f). To directly visualize the effects of TSK deficiency on sympathetic innervation, we performed tyrosine hydroxylase immunofluorescence staining on brown fat sections. Confocal microscopy revealed that the abundance of tyrosine hydroxylase-positive punctae, which correspond to sympathetic fibres in cross-section, was markedly increased in brown fat from Tsk KO mice (Fig. 5a,b). These results suggest that TSK may serve as a checkpoint for sympathetic innervation of adipose tissue.

We next performed denervation studies to further analyse the significance of sympathetic innervation in mediating the effects of TSK on brown fat thermogenesis. We transected the nerve that targets brown fat on one side and performed a sham operation on the other side as control in the same animal. Similar to the above results, tyrosine hydroxylase levels and PKA substrate phosphorylation were strongly elevated in Tsk KO brown fat on the sham-operated side (Fig. 5c). In contrast, sympathetic denervation completely abolished tyrosine hydroxylase immunoreactivity and attenuated the increase in PKA substrate phosphorylation observed in KO brown fat. Histological analysis revealed that brown fat from Tsk null mice exhibited significant whitening following denervation (Fig. 5d). Furthermore, the induction of Ucp1 and Dio2 by TSK deficiency was also reversed by denervation of brown fat (Fig. 5e).

We took an alternative approach to suppressing sympathetic activity by thermoneutral housing. Although Tsk KO mice were protected from HFD-induced brown fat whitening when housed at ambient room temperature, this protective effect was largely abolished when mice were housed at thermoneutral temperature (Fig. 5f). Accordingly, protection from diet-induced obesity in Tsk KO mice was also partially lost when HFD feeding was performed at thermoneutral temperature (Fig. 5g). These results suggest that enhanced sympathetic innervation probably serves as a critical mediator of the stimulatory effects of TSK deficiency on brown fat thermogenesis and energy expenditure. In support of this,
recombinant TSK failed to exert significant effects on brown adipocyte differentiation and thermogenic activation (Supplementary Fig. 6). The expression of several neurotrophic factors, including neuregulins and nerve growth factor, was largely unaltered by TSK deficiency (Supplementary Fig. 7). In addition, recombinant TSK did not appear to affect nerve-growth-factor-induced differentiation and neurite outgrowth in PC-12 cells, a cell culture model commonly used for sympathetic neurons. To search for cellular targets of TSK action, we generated a fusion protein between TSK and secreted alkaline phosphatase (SEAP) and performed a hormone binding assay on frozen tissue sections. Both SEAP and TSK-SEAP exhibited similar background binding to the heart, lung, and liver (Fig. 5h). In contrast, strong binding of TSK-SEAP, but not SEAP, was observed on brown fat sections. Our results support a model where TSK acts on one or more unique cellular targets in brown fat to modulate sympathetic innervation.

**Discussion**

In this study, we identified TSK as a liver-derived endocrine hormone that gates energy expenditure by acting as a checkpoint for adipose sympathetic innervation and adrenergic signaling (Fig. 6). Several lines of evidence support the notion that TSK serves as a feedback hormonal signal that attenuates energy expenditure. Hepatic Tsk expression and its plasma levels were strongly induced by stimuli that increase thermogenesis and energy expenditure, such as T3, adrenergic agonist, and cold exposure. Mice lacking recombinant TSK exhibited elevated core body temperature and were unable adequately to suppress energy expenditure during starvation, leading to greater body weight loss. Tsk null mice were strongly resistant to diet-induced obesity, insulin resistance, and hepatic steatosis as a result of enhanced sympathetic activation and brown fat thermogenesis. In fact, TSK deficiency increased sympathetic innervation and nearly completely blocked HFD-induced whitening of brown fat. Together, these results support a critical role of TSK as a hepatokine in the regulation of adipose and systemic energy metabolism.

The metabolic effects of TSK inactivation are in marked contrast with leptin deficiency; the latter results in diminished sympathetic tone, thermogenesis and severe obesity in both mice and humans. As leptin serves as a fat-derived hormone that senses nutrient storage and excess, TSK appears to be highly responsive to thermogenesis and energy expenditure. We propose that a distributed endocrine signaling network involving multiple peripheral tissues and cell types acts together to ensure whole-body energy and nutrient homeostasis. The marked dichotomy of leptin and TSK action on energy balance raises the possibility that these two feedback pathways may converge at certain hitherto undefined biochemical and cellular targets in the body to drive the downstream metabolic effects. Whether TSK deficiency and/or polymorphisms in humans confer protection against weight gain and metabolic disorders remains an intriguing possibility for future exploration.

The sympathetic nervous system provides a powerful neural signal that controls adipose tissue fuel mobilization and thermogenesis.
Recent studies using three-dimensional imaging techniques revealed highly organized patterns and plasticity of sympathetic innervation in adipose tissue. TSK deficiency greatly increased tyrosine hydroxylase-positive fibre density in brown fat, suggesting that TSK signaling may serve an inhibitory role in adipose sympathetic innervation during development and postnatal growth. Recent work identified a unique population of adipose macrophages responsible for uptake and catabolism of catecholamines. However, mRNA expression of monoamine oxidase A, an enzyme responsible for catecholamine catabolism, appeared largely unaltered in TSK-deficient BAT (data not shown). These observations suggest that TSK modulates adipose tissue adrenergic signaling and thermogenesis primarily through its effects on sympathetic innervation. We observed TSK binding in brown fat; however, the identity...
of cellular targets for TSK and its receptor are currently unknown. Previous studies have demonstrated that TSK suppresses neurite outgrowth in cultured neurons. It is possible that local enrichment of TSK via binding to brown fat tissue may attenuate sympathetic nerve fibre outgrowth and/or branching. Alternatively, retrograde TSK signaling in postganglionic sympathetic neurons may suppress tyrosine hydroxylase expression and catecholamine synthesis. Our current study cannot rule out the possibility that TSK may act on the central nervous system to regulate sympathetic innervation and outflow. Nonetheless, our work delineates a negative feedback arm of energy expenditure that may serve as a target for therapeutic intervention in metabolic disease.

Methods

Mice. All animal studies were performed following procedures approved by the Institutional Animal Care and Use Committee at the University of Michigan. Unless otherwise stated, mice used in this study were male C57BL/6 mice maintained under 12:12 light/dark cycles with free access to food and water. To induce obesity, mice were fed standard chow (Teklad 5001) or a high-fat diet containing 60% of calories from fat (D12492, Research Diets) starting at 10–12 weeks of age. Tsk null mice were generated and maintained in a C57BL/6 background. Tsk null male mice at the University of Michigan Animal Phenotyping Core, as previously described. Energy expenditure, O2 consumption, total locomotor activity and fat innervation of cellular targets for TSK and its receptor are currently unknown. Previous studies have demonstrated that TSK suppresses neurite outgrowth in cultured neurons. It is possible that local enrichment of TSK via binding to brown fat tissue may attenuate sympathetic nerve fibre outgrowth and/or branching. Alternatively, retrograde TSK signaling in postganglionic sympathetic neurons may suppress tyrosine hydroxylase expression and catecholamine synthesis. Our current study cannot rule out the possibility that TSK may act on the central nervous system to regulate sympathetic innervation and outflow. Nonetheless, our work delineates a negative feedback arm of energy expenditure that may serve as a target for therapeutic intervention in metabolic disease.

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Cell culture. Primary brown adipocytes were immortalized and differentiated in culture as previously described. Briefly, confluent cultures were incubated with induction medium (DMEM containing 10% FBS, 0.5 μM isobutylmethylxanthine, 125 μM indomethacin, 1 μM dexamethasone, 1 nM T3 and 20 nM insulin). After 48 h, the medium was replaced with maintenance medium (DMEM supplemented with 10% FBS, 1 nM T3 and 20nM insulin). For TSK treatments, recombinant human TSK protein (no. 3940-TS R&D Systems) was added to the induction medium at different concentrations. PC-12 cells were cultured in RPMI 1630 medium containing 10% horse serum and 5% FBS. For PC-12 cell differentiation, 40–60% confluent cultures were exposed to RPMI 1640 containing 2% horse serum, 1% FBS and 100 mg/ml NGF (no. B5025, enVigo). Fresh differentiation medium was added every 2 d until day 6.

Gene expression and immunoblotting analyses. Total adipose tissue RNA was extracted using a commercial kit (no. 12183025, Invitrogen). Total RNA from other tissues was extracted using TRIzol (Invitrogen). Gene expression was analysed by qPCR using the primers shown in Supplementary Table 3. For microarray study of brown fat gene expression, total brown fat RNA from chow-fed WT and Tsk KO mice was analysed using Affymetrix Mouse Gene ST 2.1 array strips. Sample processing and data analyses were performed according to the manufacturer’s instructions. For immunoblotting, total lysates were prepared in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM Na2, 25 mM β-glycerol phosphate, 1 mM dithiothreitol and freshly added protease inhibitors. Protein lysates were separated by SDS–PAGE gels, transferred to polyvinylidene difluoride membrane and immunoblotted with the following primary antibodies: Rabbit polyclonal antibody to TSK was generated with mouse Tsk peptide (CR1RLYSSTKAYHRQPSSPK; Tsk amino acids 317–325) followed by affinity purification. Antibodies against phospho-PPA substrate (catalogue no. 9624), phospho-HSL (Ser660, catalogue no. 4126), and HSL (catalogue no. 4107) were purchased from Cell Signaling Technologies. Antibodies against phospho-perilipin (Ser497, catalogue no. 4855) and perilipin (catalogue no. 4854) were purchased from Vala Sciences. Antibody against UCP1 (UCP1-1-A) was purchased from Alpha Diagnosis. Antibody against tubulin (T6199) was purchased from Sigma-Aldrich. Antibody against HSP90 (sc-7947) was purchased from Santa Cruz. Antibody against tyrosine hydroxylase (ab112) was purchased from Abcam.

β-galactosidase staining. To determine the Tsk mRNA expression profile in mice, β-galactosidase staining was performed on tissues from WT and Tsk heterozygous mice. Briefly, tissues were dissected, cut into small pieces and fixed with cold formalin for 30 min. The tissues were washed three times with PBS and subsequently incubated with 1 mg/ml X-gal for 30 min.

Plasma and liver triglyceride measurement. Plasma concentrations of total cholesterol were measured using kits from Stanbio Laboratory (no. 1010–430). Plasma concentrations of triglycerides were measured using a kit from Sigma (no. Tr1000). Plasma concentrations of insulin and leptin were measured using commercial ELISA kits from Crystal Chem (nos. 90800 and 90900, respectively). The plasma FGE21 level was measured using the mFGF21 ELISA kit (no. MF2100, R&D Systems). Liver lipids were extracted and measured as previously described. Briefly, 100–150 mg liver tissue was homogenized in 2 ml lysis buffer containing 50 mM Tris (pH 7.5), 5 mM EDTA, 300 mM mannitol, and 1 mM PMSF. Homogenates (200 μl) were mixed with 5 μl KOH (10 M) and extracted with 800 μl chloroform/methanol (2:1). The organic phase was mixed with equal volumes of chloroform/methanol/ H2O (3:4:87) followed by vigorous vortexing and centrifuging at 10,000g for 10 min. The bottom layer was transferred to a new tube and dried in a fume hood overnight. The resulting lipids were resuspended with 50 μl triglyceride reuspension buffer (Butanol/[Trition-X114/methanol (2:1)]) (3:2). Triglyceride content was measured using a kit from Sigma (no. 337-B) and normalized to the liver weight.

GTT and ITT. GTT and ITT were performed as previously described. For the former, mice were fasted overnight (16 h) and injected intraperitoneally with a glucose solution at 1.0 g/kg body weight. Blood glucose concentrations were measured before and after 20, 45, 90, and 120 min after glucose injection. For ITT, mice were fasted for 4 h and then injected intraperitoneally with insulin at 0.7 U/kg body weight. Blood glucose concentrations were measured before and after 20, 45, 90, and 120 min after insulin injection.

Histology. Liver and WAT sections were embedded in paraffin and stained with H&E. For immunofluorescence, brown fat tissues were fixed with 4% paraformaldehyde overnight at 4 °C and incubated with 30% sucrose for 48 h before embedding in the optimal cutting temperature compound (OCT compound). Sections were blocked with 20% normal horse serum and incubated with the antibody against mouse tyrosine hydroxylase overnight at 4 °C, followed by incubation with a fluorophore-conjugated secondary antibody (Thermo Fisher Scientific).
Scientific) for 1 h. The sections were mounted with DAPI-containing mounting medium (VECTORSHIELD) for fluorescent microscopy.

Catecholamine measurement. Plasma and tissue norepinephrine levels were measured using the reverse-phase UltiMate 3000 HPLC system with electrochemical detection (Thermo Fisher). Briefly, individual BAT and eWAT samples were homogenized using perchloric acid (0.2 M) supplemented with ascorbic acid (1 mg ml–1) and dihydroxybenzylamide as an internal standard. Active aluminia was used to extract catecholamines from the homogenates, followed by elution with 200 µl of perchloric acid with ascorbic acid.

TSK binding assay. Hormone binding assay was performed as previously described[33]. Hepa-1 cells were transduced with retrovirus expressing SEAP or TSK-SEAP. Conditioned media from Hepa-1 cells were harvested and used in the binding assay. Briefly, frozen tissue sections were incubated with SEAP or TSK-SEAP CM for 45 min at room temperature. Slides were washed with PBS containing 0.1% Tween-20 and fixed in a solution containing 20 mM HEPES (pH 7.4), 60% acetone and 3% formaldehyde. After inactivation of endogenous alkaline phosphatase at 65°C for 30 min, enzymatic activity derived from the fusion protein was detected using NBT/BCIP substrate.

Statistics and reproducibility. Two-tailed Student’s t-test was used to analyse differences between the two groups. Two-way ANOVA with multiple comparisons was used for statistical analysis of GTT, ITT and body weight studies. A value of P < 0.05 was considered statistically significant. Statistical methods and corresponding P values for data in each panel are shown in the figure legends. All mouse experiments were independently replicated at least twice. The cell culture experiments were performed in triplicate and repeated at least three times. We did not exclude any data points or mice unless a technical issue or human error occurred.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary.

Data availability The microarray dataset described in the paper has been deposited in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE114361. All other data are available from the corresponding author on reasonable request.

Received: 7 May 2018; Accepted: 20 November 2018; Published online: 14 January 2019

References

1. Pedersen, B. K. & Febbraio, M. A. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat. Rev. Endocrinol. 8, 457–465 (2012).
2. Pothoff, M. J., Kliwer, S. A. & Mangelsdorf, D. J. Endocrine fibroblast growth factors 15/19 and 21: from feast to famine. J. Clin. Invest. 123, 3305–3345 (2013).
3. Cho, K. W., Zhou, Y., Sheng, L. & Ru, I. Lipocalin-13 regulates glucose metabolism by both insulin-dependent and insulin-independent mechanisms. Mol. Cell. Biol. 31, 450–457 (2011).
4. Meex, R. C. et al. Fetuin B is a secreted hepatocyte factor linking steatosis to impaired glucose metabolism. Cell. Metab. 22, 1078–1089 (2015).
5. Ohba, K. et al. Desensitization and inactivation of hyperglycemic target genes after chronic thyroid hormone treatment and withdrawal in male adult mice. Endocrinology 157, 1660–1672 (2016).
6. Hossain, M. et al. The combinatorial guidance activities of draxin and Tskukski are essential for forebrain commissure formation. Dev. Biol. 374, 58–70 (2013).
7. Ito, A. et al. Tsukushi is required for anterior commissure formation in mouse brain. Biochem. Biophys. Res. Commun. 402, 813–818 (2010).
8. Ohba, K. et al. Tskukski functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. Dev. Cell. 7, 347–358 (2004).
9. de Jesus, L. A. et al. The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J. Clin. Invest. 108, 1379–1385 (2001).
10. Xiao, Y. et al. Long noncoding RNA licensing of obesity-linked hepatic lipogenesis and NAFLD pathogenesis. Nat. Commun. 9, 2986 (2018).
11. Barton, T. J., Liu, Y., Shrestha, Y. B. & Ryu, V. Neural innervation of white adipose tissue and the control of lipolysis. Front. Neuroendocrinol. 35, 473–493 (2014).
12. Morrison, S. F. & Madden, C. J. Central nervous system regulation of brown adipose tissue. Compr. Physiol. 4, 1677–1713 (2014).
13. Zeng, W. et al. Sympathetic neuro-adipose connections mediate leptin-driven thermogenesis and obesity resistance. Science 297, 843–845 (2002).
14. Bray, G. A. & York, D. A. The MONA LISA hypothesis in the time of leptin. Cell 259, 24–26 (2017).
15. Roth, M. et al. Recruited brown adipose tissue as an antiobesity agent in humans. J. Clin. Invest. 123, 3404–3408 (2013).
16. Bartelt, A. et al. Brown adipose tissue activity controls triglyceride clearance. Nat. Med. 17, 200–205 (2011).
17. van der Lans, A. A. et al. Cold acclimation recruits human brown fat and increases energy expenditure. J. Clin. Invest. 132, 3395–3402 (2013).
18. Cho, K. W., Zhou, Y., Sheng, L. & Ru, I. Lipocalin-13 regulates glucose metabolism by both insulin-dependent and insulin-independent mechanisms. Mol. Cell. Biol. 31, 450–457 (2011).
19. Meex, R. C. et al. Fetuin B is a secreted hepatocyte factor linking steatosis to impaired glucose metabolism. Cell. Metab. 22, 1078–1089 (2015).
20. Ohba, K. et al. Desensitization and inactivation of hyperglycemic target genes after chronic thyroid hormone treatment and withdrawal in male adult mice. Endocrinology 157, 1660–1672 (2016).
21. Hossain, M. et al. The combinatorial guidance activities of draxin and Tskukski are essential for forebrain commissure formation. Dev. Biol. 374, 58–70 (2013).
22. Ito, A. et al. Tsukushi is required for anterior commissure formation in mouse brain. Biochem. Biophys. Res. Commun. 402, 813–818 (2010).
23. Ohba, K. et al. Tsukushi functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. Dev. Cell. 7, 347–358 (2004).
24. de Jesus, L. A. et al. The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J. Clin. Invest. 108, 1379–1385 (2001).
25. Xiao, Y. et al. Long noncoding RNA licensing of obesity-linked hepatic lipogenesis and NAFLD pathogenesis. Nat. Commun. 9, 2986 (2018).
26. Barton, T. J., Liu, Y., Shrestha, Y. B. & Ryu, V. Neural innervation of white adipose tissue and the control of lipolysis. Front. Neuroendocrinol. 35, 473–493 (2014).
27. Morrison, S. F. & Madden, C. J. Central nervous system regulation of brown adipose tissue. Compr. Physiol. 4, 1677–1713 (2014).
28. Zeng, W. et al. Sympathetic neuro-adipose connections mediate leptin-driven thermogenesis and obesity resistance. Science 297, 843–845 (2002).
29. Brav, G. A. & York, D. A. The MONA LISA hypothesis in the time of leptin. Cell 259, 226–236 e223 (2018).
30. Huang, D. et al. Inflammasome-driven catecholamine catabolism in macrophages blunts lipolysis during ageing. Nature 550, 119–123 (2017).
31. Pirigzka, R. et al. Metabolic syndrome-associated macrophages contribute to obesity by importing and metabolizing norepinephrine. Nat. Med. 23, 1309–1318 (2017).
32. Fan, R. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Methods 10, 226–236 (2013).
33. Zhao, X. Y., Li, S., Wang, G. X., Yu, Q. & Lin, J. D. A long noncoding RNA transcriptional regulatory circuit drives thermogenic adipocyte differentiation. Mol. Cell 55, 372–382 (2014).
34. Li, S. et al. Genome-wide coactivation analysis of PGC-1α-lipha identifies BAFO60a as a regulator of hepatic lipid metabolism. Cell. Metab. 8, 105–117 (2008).
35. Muller, H., Dai, G. & Soares, M. J. Placental lactogen-I (PL-I) target tissues identified with an alkaline phosphatase-PL-I fusion protein. J. Histochem. Cytochem. 46, 737–743 (1998).
50. Lin, J. & Linzer, D. I. Induction of megakaryocyte differentiation by a novel pregnancy-specific hormone. *J. Biol. Chem.* **274**, 21485–21489 (1999).

**Acknowledgements**

This work was supported by NIH grants (nos. DK102456 and AG055379 to J.D.L.; no. DK114220 to L.R.), the Michigan Diabetes Research Center (grant no. DK020572), and the Michigan Nutrition and Obesity Center (grant no. DK089503).

**Author contributions**

J.D.L. and Q.W. conceived the project and designed the research. Q.W., V.P.S., H.S., Y.X., Q.Z., X.X., I.G., H.S., S.L., L.R., and L.J. performed the experiments and analysed the data. K.O. provided the Tsk knockout mouse strain. J.D.L. and Q.W. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s42255-018-0020-9.

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Image J was used to measure the density of band in immunoblotting and quantify the positive area of Th in immunofluorescence staining assay. |
| Data analysis   | GraphPad Prism 8.0 was used to perform statistical analysis in the paper. |

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The microarray datasheet described in the paper has been deposited into Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with accession...
Full scans for western blots are provided in Supplementary Figure 8. Source Data for all mouse experiments have been provided. All other data are available from the corresponding author on reasonable request.

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**Life sciences study design**

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For animal studies, we did not predetermine the sample size but used group sizes typically for this type of work on basis of previous experiments using similar methodologies. To minimize any potential bias, we randomly assigned mice of same genotype to different treatments. For cell culture experiments, 3 or more biological replicates were used in the paper. |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the study. |
| Replication | All experimental findings were reproduced as stated in figure legends. All additional replication attempts were successful. |
| Randomization | Animals were assigned randomly to the experimental and control groups. |
| Blinding | For catecholamine measurements, samples were processed in random order and experimenters were blinded to experimental conditions. |

**Reporting for specific materials, systems and methods**

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|------------------------|
| [x] | Unique biological materials |
| [ ] | Antibodies |
| [x] | Eukaryotic cell lines |
| [ ] | Palaeontology |
| [x] | Animals and other organisms |
| [ ] | Human research participants |

**Methods**

| n/a | Involved in the study |
|-----|------------------------|
| [x] | ChIP-seq |
| [x] | Flow cytometry |
| [ ] | MRI-based neuroimaging |

**Antibodies**

| Antibodies used | Anti-phospho-PKA substrate (9624, Cell Signaling), Rabbit, 1:1,000 dilution; Anti-HSL (4107, Cell Signaling), Rabbit, 1:1,000 dilution; Anti-phospho-HSL (Ser660) (4126, Cell Signaling), Rabbit, 1:1,000 dilution; Anti-phospho-Perilipin (Ser497) (4855, Vala Sciences), mouse, 1:1,000 dilution; Anti-Perilipin (4854, Vala Sciences), mouse, 1:1,000 dilution; Anti-UCP1 (UCP11-A, Alpha Diagnostic), Rabbit, 1:20,000 dilution; Anti-Tubulin (T6199, Sigma-Aldrich), mouse, 1:2,000 dilution; Anti-HSP90 (sc-7947, Santa Cruz), Rabbit, 1:1,000 dilution; anti-tyrosine hydroxylase (ab112, Abcam), Rabbit, 1:1,000 dilution. Rabbit anti-TSK was generated with mouse TSK peptide. |

**Validation**

Except for anti-TSK, all antibodies were used in the paper according to the profile of manufacturer. The specificity of anti-TSK was evaluated using WT and TSK KO mice.

**Eukaryotic cell lines**

Policy information about cell lines

| Cell line source(s) | Primary brown adipocytes were immortalized by our lab. PC12 cells were kindly provide by Christin Carter-Su. |
|---------------------|--------------------------------------------------------------------------------------------------|
| Authentication | Primary brown adipocytes were induced to differentiation by adding a cocktail containing 0.5 μM IBMX, 125 μM indomethacin, 1 μM dexamethasone to DMEM medium containing 10% FBS, 20 nM insulin and 1 nM T3. Adipocyte gene expression was identified by qPCR. PC12 cell differentiation was induced by adding 100 ng/ml NGF to 1640 RPMI medium |
containing 2% horse serum and 1% FBS. Neurite outgrowth was observed at day 6, and differentiation marker gene expression was identified by qPCR.

**Mycoplasma contamination**
Mycoplasma contamination was not tested in the study.

**Commonly misidentified lines**
(See ICLAC register)
None of cell lines used in this study are listed by ICLAC.

### Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Unless otherwise stated, mice used were male C57BL/6J, and housed under 12/12 h light/dark cycles with free access to food and water. TSK KO mice were provided by Kunimasa Ohta, and maintained in C57BL/6 background. TSK null and littermate control male mice were used. The Cas9 transgenic mice (JAX #024858) were purchased from Jackson Laboratory. |
|--------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals       | N/A                                                                                                                                                                                                                                                                  |
| Field-collected samples | N/A                                                                                                                                                                                                 4

- **Animals and other organisms**

- **Policy information about studies involving animals:** ARRIVE guidelines recommended for reporting animal research

- **Laboratory animals:** Unless otherwise stated, mice used were male C57BL/6J, and housed under 12/12 h light/dark cycles with free access to food and water. TSK KO mice were provided by Kunimasa Ohta, and maintained in C57BL/6 background. TSK null and littermate control male mice were used. The Cas9 transgenic mice (JAX #024858) were purchased from Jackson Laboratory.

- **Wild animals:** N/A

- **Field-collected samples:** N/A