Innervation of thermogenic adipose tissue via a calsyntenin 3β–S100b axis

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The sympathetic nervous system drives brown and beige adipocyte thermogenesis through the release of noradrenaline from local axons. However, the molecular basis of higher levels of sympathetic innervation of thermogenic fat, compared to white fat, has remained unknown. Here we show that thermogenic adipocytes express a previously unknown, mammal–specific protein of the endoplasmic reticulum that we term calsyntenin 3β. Genetic loss or gain of expression of calsyntenin 3β in adipocytes reduces or enhances functional sympathetic innervation, respectively, in adipose tissue. Ablation of calsyntenin 3β predisposes mice on a high–fat diet to obesity. Mechanistically, calsyntenin 3β promotes endoplasmic-reticulum localization and secretion of S100b—a protein that lacks a signal peptide—from brown adipocytes. S100b stimulates neurite outgrowth from sympathetic neurons in vitro. A deficiency of S100b phenocopies deficiency of calsyntenin 3β, and forced expression of S100b in brown adipocytes rescues the defective sympathetic innervation that is caused by ablation of calsyntenin 3β. Our data reveal a mammal–specific mechanism of communication between thermogenic adipocytes and sympathetic neurons.

A hallmark of mammalian evolution is the emergence of brown adipose tissue (BAT), an organ that is specialized for performing non-shivering thermogenesis.1 The presence of BAT confers an evolutionary advantage to mammals by enhancing their adaptability to cold stress and the survival of newborns. The powerful ability of thermogenic fat to oxidize substrates and increase energy expenditure has drawn growing interest as a therapeutically approach to address obesity and associated metabolic disorders2.

BAT thermogenesis requires innervation by the sympathetic nervous system. Sympathetic nerves release the neurotransmitter noradrenaline from local axons,3,4 which activates the β-adrenergic receptor–cAMP–PKA pathway in adipocytes to drive lipolysis and thermogenic respiration.3,5,6 Consistent with the thermogenic function of BAT, this tissue is much more richly innervated by sympathetic nerves than white adipose tissue (WAT).7 Nevertheless, the molecular basis of this selective recruitment of sympathetic innervation has remained largely unexplored.

PRDM16 has recently been identified as an important transcriptional regulator that drives the thermogenic program in beige adipocytes8,9, which are a distinct type of inducible thermogenic adipocytes that mainly reside in subcutaneous WAT.10 Forced expression of PRDM16 leads to increased sympathetic innervation of beige adipocytes11; and adipose-specific ablation of PRDM16 has the opposite effect12. These observations strongly suggest that adipocyte-derived factors can influence the extent of sympathetic innervation of the adipose tissue. Here we show that a previously unknown, mammal–specific membrane protein, which we have named calsyntenin 3β (CLSTN3β), promotes the sympathetic innervation of both brown and beige adipocytes. CLSTN3β binds to—and enhances the protein expression and secretion of—S100b, a protein that is unconventionally secreted without a signal peptide. S100b, in turn, acts as a neurotrophic factor to stimulate sympathetic axon growth.

Clstn3b is an adipose–specific gene
Adipose-specific ablation of lysine-specific demethylase 1 (Lsd1, also known as Kdm1a) has previously been found to cause severe BAT dysfunction13. Expression of calsyntenin 3 (Clstn3) is strongly down-regulated in LSD1-deficient BAT (Extended Data Fig. 1a). Clstn3 is a plasma–membrane protein that promotes synaptogenesis in the central nervous system14. In contrast to the known form of Clstn3, the completely distinct and unannotated form of Clstn3b is expressed in BAT (Fig. 1a). Clstn3b appears to contain three exons: the first exon, which is large and unique, lies within an intron of Clstn3, and the final two exons are shared with Clstn3 (Fig. 1a). Chromatin immunoprecipitation with sequencing (ChIP–seq) analyses for histone markers and transcription regulators suggest that a promoter and enhancer of Clstn3b that are distinct from those of Clstn3 exist in BAT (Extended Data Fig. 1b). Taken together, these observations suggest that Clstn3b is a previously unknown gene that is expressed in BAT, rather than a splicing variant of Clstn3.

Clstn3b mRNA expression is highly restricted to adipose tissue: the level of expression in the interscapular BAT is sixfold higher than in either the inguinal subcutaneous WAT or the perigonadal visceral WAT (Fig. 1b). Clstn3b expression is strongly induced in the inguinal subcutaneous WAT upon exposure to cold (Extended Data Fig. 1c), which suggests that Clstn3b is also highly expressed in beige adipocytes.

We next attempted to clone the Clstn3b cDNA. We successfully amplified a 1,074-bp open reading frame that was predicted to encode a protein of 357 amino acids in length, from a cDNA library of mouse BAT (Fig. 1c); this confirmed the existence of Clstn3b at the transcript level. Notably, the N-terminal extracellular portion of CLSTN3—which is essential for interaction between CLSTN3 and α-neurexins, and for the synaptogenic activity of this protein—is completely missing in CLSTN3β, which strongly suggests that the two proteins have distinct functions.
To detect CLSTN3β at the protein level, we developed a rabbit polyclonal antibody against a C-terminal peptide of CLSTN3β (amino acids shown in green in Fig. 1c). The antibody detected a doublet band of the expected size for the full-length protein in BAT (Extended Data Fig. 1d). We immunoprecipitated endogenous CLSTN3β with this antibody, and mass spectrometry analysis identified two peptides that were consistent with the predicted sequence; one derived from the region shared with Clstn3 and the other derived from the unique exon that was not included in the mouse protein database (corresponding to the underlined amino acids in Fig. 1c, Extended Data Fig. 1d), which thus provided definitive evidence at the peptide level for the existence of CLSTN3β.

We next examined the evolution of Clstn3b. Unequivocal homologies can be identified only in mammals that give birth to live progeny (Extended Data Fig. 1e). A fragment with limited homology, but which is unproductive in terms of protein coding, exists in an intron of Clstn1 from the Chinese softshell turtle (Extended Data Fig. 1f), which suggests Clstn3b evolved in mammals after their divergence from reptiles. By contrast, Clstn3 is conserved throughout the entire vertebrate family.

CLSTN3β localizes to the endoplasmic reticulum

We first studied the function of CLSTN3β by determining its subcellular localization. Ectopically expressed CLSTN3β in primary brown adipocytes was found to co-localize with KDEL, which is a marker for the endoplasmic reticulum; this suggests that CLSTN3β localizes to the endoplasmic reticulum (Fig. 2a). Endogenous CLSTN3β was detected as more-weakly stained puncta that were also positive for KDEL (Fig. 2b). To visualize the subcellular localization of CLSTN3β at the ultrastructural level, we expressed an APEX2 reporter fused to the C terminus of CLSTN3β in brown adipocytes; APEX2 localization based on 3,3′-diaminobenzidine tetrahydrochloride reactivity was then examined by electron microscopy. Consistent with the immunofluorescence data, APEX2 strongly labelled the endoplasmic reticulum (Fig. 2c). By contrast, we observed no APEX2 labelling of other membranous organelles (Extended Data Fig. 2a, b). The endoplasmic reticulum localization of CLSTN3β was further supported by BAT fractionation and western blot analysis (Extended Data Fig. 2c).

CLSTN3β promotes adipose thermogenesis

To study the function of CLSTN3β in adipose thermogenesis and whole-body energy metabolism, we generated a global Clstn3b-knockout mouse strain. Deletion of Clstn3b was verified by DNA sequencing, quantitative PCR, western blot and immunofluorescence (Extended Data Fig. 3a–d). Expression and splicing of Clstn3 is unaffected in Clstn3b-knockout mice (Extended Data Fig. 3e). On a chow diet, the body weights of Clstn3b-knockout mice were not significantly different from wild-type mice, but Clstn3b-knockout mice had significantly higher body-fat mass (Extended Data Fig. 3f, g). Histological analysis and triglyceride quantification showed that CLSTN3β-deficient mice exhibited a significant increase in BAT size, as well as in the absolute and relative amount of brown adipocytes (Fig. 3).

**Fig. 1 | Clstn3b encodes an adipocyte-specific protein.** a, Distribution of RNA sequencing reads at the Clstn3 locus, from BAT and cerebral cortex. The arrow denotes the exon that is unique to Clstn3b. b, Tissue specificity of Clstn3b expression as determined by quantitative PCR (n = 3 mice). eWAT, epididymal WAT; iWAT, inguinal WAT. c, Cloning of Clstn3b from a BAT cDNA library. Red denotes nucleotides and amino acids derived from the unique exon of Clstn3b. The underlined region denotes peptides that were detected by mass spectrometry. Green denotes the peptide used expressed (a) or endogenous (b) Clstn3b in primary brown adipocytes. Green, KDEL; red, Clstn3β. Arrows denote puncta specific for CLSTN3β. In b, the region inside the white box is enlarged for clearer view. c, Electron microscopy analysis of the localization of CLSTN3β–APEX2. Arrows denote endoplasmic reticulum that is positive or negative for APEX2 in infected or uninfected cells, respectively. Scale bars, 10 μm (a, b), 500 nm (c).
BAT has significantly more lipid deposition than wild-type BAT; this parameter often reflects BAT dysfunction (Fig. 3a). An indirect calorimetry study showed that Clstn3b-knockout mice had lower rates of O₂ consumption and CO₂ production than wild-type mice (Fig. 3b, Extended Data Fig. 3b), although their food intake and physical activity were unaltered (Extended Data Fig. 3i, j). The acute respiratory response to an injection with the β3-adrenergic agonist CL-316,243 showed altered cold sensitivity—coupled with their normal responses to the β3-adrenergic agonist—suggested that CLSTN3 may affect the BAT response to pharmacological β3-adrenergic stimulation.

Together, these findings suggest that the Clstn3b-knockout mice have defective adaptive thermogenesis compared to wild-type mice, which is consistent with these knockout mice displaying BAT dysfunction. The transgenic mice displayed a phenotype that was opposite to that of the knockout mice in all of the assays (Fig. 4a–h, Extended Data Fig. 4a–h). These data provide strong support for a pro-thermogenic function of CLSTN3/3.

CLSTN3/3 enhances sympathetic innervation

The observation that both Clstn3b-knockout and -transgenic mice showed altered cold sensitivity—coupled with their normal responses to the β3-adrenergic agonist—suggested that CLSTN3/3 may affect sympathetic innervation of adipose tissue. Indeed, we observed no significant difference in noradrenaline-induced respiration between wild-type or knockout brown adipocytes (Fig. 5a), which suggests that the phenotype at the organismal level cannot be explained by alterations in the intrinsic thermogenic capacity of brown adipocytes.

To assess BAT sympathetic innervation, we performed tyrosine hydroxylase immunostaining. Notably, tyrosine hydroxylase immunoreactivity of the knockout BAT was substantially reduced relative to the wild type (Fig. 5b). Immunostaining of TUBB3, a neural-specific tubulin, yielded similar results (Fig. 5b). These results indicate that knockout BAT has reduced growth of sympathetic nerves, relative to wild type. Decreased expression of several thermogenesis-related genes in knockout BAT was observed upon acute cold exposure following pre-acclimatization at thermoneutrality, which is consistent with.
transgenic mice (\textit{Clstn3b} knockout) BAT, we chemogenetically activated sympathetic pre-motor neurons, and assessed the downstream BAT response. Previous studies have identified medullary raphe neurons that express vesicular glutamate transporter 3 (VGLUT3), which are proposed to activate BAT thermogenesis via direct projections to preganglionic sympathetic neurons in the spinal cord\textsuperscript{15}. We crossed the \textit{Slc17a8-ires-cre} (\textit{Slc17a8} is also known as \textit{Vglut3}) mouse line\textsuperscript{17} to \textit{Clstn3b}-knockout and \textit{-transgenic} lines, and stereotaxically injected Cre-dependent AAV-hM3Dq-mCherry or AAV-mCherry into the medullary raphe region of the brain stem to drive stable expression of the transgene specifically in the neurons that express VGLUT3. Injection of clozapine-N-oxide\textsuperscript{10} (CNO; a ligand of hM3Dq), but not of saline, into mice that received AAV-hM3Dq-mCherry induced FOS expression in the medullary raphe region, as well as an increase of 0.9 °C in interscapular temperature (Fig. 5g). CNO injection into mice that received AAV-mCherry produced neither FOS expression nor a temperature response (Fig. 5g), which confirmed that CNO specifically activates sympathetic premotor neurons in the medullary raphe region to trigger the thermogenic response.

We next examined the response of \textit{Clstn3b}-knockout and \textit{-transgenic} mice to CNO. The response was reduced from 0.9 °C in wild-type mice to 0.3 °C in knockout mice, but increased from 0.6 °C in control mice to 1.2 °C in transgenic mice (Fig. 5h–i). Taken together, these findings indicate that ablation of \textit{Clstn3b} impairs the functional sympathetic innervation of thermogenic adipose tissue, whereas transgenic expression has the opposite effect.

\textbf{CLSTN3\textsuperscript{3} promotes the secretion of SI100b}

Our findings raised the critical question of how an intracellular membrane protein might regulate sympathetic innervation of thermogenic adipocytes. To gain insight into this question, we performed quantitative
CLSTN3 β promotes functional sympathetic innervation of the adipose tissues. a, Respiration rates of wild-type or Clstn3b-knockout brown adipocytes (n = 4 biologically independent samples). NA, noradrenaline. b, c, Immunostaining of cryo-section of wild-type and Clstn3b-knockout BAT (b), and wild-type and Clstn3b-transgenic BAT (c) (n = 5 mice). TH, tyrosine hydroxylase. d-f, Histology and triglyceride quantification (d), tyrosine hydroxylase staining (e) and cold tolerance test (f) of Clstn3b-knockout ± Adipoq-cre mice, receiving AAV-DIO-CLSTN3b (n = 4 mice). g, FOS immunoreactivity and thermogenic response to CNO administration (n = 8 mice for hM3Dq + CNO, n = 3 mice for hM3Dq + saline; and mCherry + CNO). h, i, Thermogenic response to CNO administration in wild-type and Clstn3b-knockout mice (n = 8 mice) (h), and wild-type and Clstn3b-transgenic mice (n = 4 mice) (i). Scale bars, 100 μm. Data are mean ± s.e.m. and analysed by unpaired Student’s two-sided t-test.

Whole-tissue proteomic analysis of wild-type and Clstn3b-knockout BAT (Fig. 6a, Supplementary Table 1). The most strongly downregulated (47% down) protein in the knockout BAT was S100b. Previous studies have established that S100b is a protein that is highly expressed by astrocytes in the central nervous system, and that it has neurotrophic activity. S100b expression is much higher in BAT than in WAT, and is strongly induced in the inguinal subcutaneous WAT upon exposure to cold (Extended Data Fig. 6a, b). Notably, S100b transcription is positively regulated by PRDM16 (Extended Data Fig. 6c-e). We therefore examined the hypothesis that S100b might be a critical adipocyte-derived neurotrophic factor that promotes sympathetic innervation of adipose tissue.

We first asked whether S100b possesses neurotrophic activity for sympathetic neurons. S100b significantly induced neurite growth from primary sympathetic neurons (Fig. 6b), thus demonstrating that S100b can act as a neurotrophic factor for sympathetic neurons.

We next tested whether forced expression of S100b in brown adipocytes could alleviate the Clstn3b-knockout defects, by injecting Cre-dependent AAV-S100b into the BAT of Clstn3b-knockout mice with or without Adipoq-cre. We observed a significantly reduced triglyceride content and increased sympathetic innervation only in the presence of Adipoq-cre (Fig. 6c, d). Moreover, Cre + mice showed significantly increased cold tolerance and energy expenditure, compared with Cre − mice (Fig. 6e, Extended Data Fig. 6f), which indicates that forced S100b expression is sufficient to reverse the innervation and thermogenic defects caused by CLSTN3 β deficiency.

We determined whether S100b-deficient BAT phenocopies CLSTN3 β-deficient BAT by analysing BAT sympathetic innervation of a global S100b-knockout mouse line. S100b-deficient BAT displayed a notable decrease in the level of sympathetic innervation (Fig. 6f), which suggests that S100b could be a target of CLSTN3 β that mediates sympathetic-adipose communication. We observed no difference between sympathetic innervation of the salivary gland of wild-type and S100b-knockout mice (Extended Data Fig. 6g). Moreover, ablation of S100b completely obliterates the ability of Clstn3b-transgenesis to enhance BAT sympathetic innervation (Fig. 6f), which suggests that S100b works downstream of CLSTN3 β. Finally, we mechanistically examined how CLSTN3 β deficiency results in a reduction in S100b protein level in BAT. We observed no difference in S100b mRNA level between wild-type and Clstn3b-knockout BAT (Extended Data Fig. 6b), which suggests that S100b is post-transcriptionally regulated by CLSTN3 β. We then cultured primary Clstn3b-knockout brown adipocytes, and ectopically expressed S100b with or without CLSTN3 β. In these experiments, we observed that CLSTN3 β led to a modest increase in cellular level of the S100b protein (Extended Data Fig. 6i). Similar results were obtained when using HEK293T cells (Extended Data Fig. 6j). CLSTN3 β did not have such an effect (Extended Data Fig. 6j). Co-expression of CLSTN3 β significantly promotes co-localization of S100b with the endoplasmic reticulum marker KDEL in brown adipocytes (Fig. 6g, h), which suggests that CLSTN3 β promotes the association of S100b with the endoplasmic reticulum. The amount of S100b secreted into the medium increased strongly in the presence of CLSTN3 β (Fig. 6i), thus indicating that CLSTN3 β promotes secretion of S100b. S100b is highly unusual in that it is a secreted protein with no signal peptide; the pathway of its secretion has therefore been unclear. CLSTN3 β did not promote secretion of complement factor D (also known as adipasin)
**Fig. 6** CLSTN3β promotes secretion of S100b, an adipocyte-derived neurotrophic factor. **a,** Proteomics of wild-type and Clstn3b-knockout BAT. **b,** TUBB3 immunostaining of sympathetic neurons ± S100b (n = 25 cells). AU, arbitrary unit. **c–e,** Histology, and triglyceride quantification (c), tyrosine hydroxylase staining (d) and cold tolerance test (e) of Clstn3b-knockout ± Adipoq-cre mice, receiving AAV-DIO-S100b (n = 4 mice). **f,** Tyrosine hydroxylase and TUBB3 immunostaining of sympathetic neurons. The secretion pathway of S100b is involved in the innervation of thermogenic fat. Genetic ablation of Clstn3b leads to a deficiency in adipose innervation and thermogenic function. Human adipose tissue also expresses CLSTN3 and CLSTN3β. Scale bars, 100 μm. Data are mean ± s.e.m. and analysed by unpaired Student’s two-sided t-test.

(Fig. 6i, Extended Data Fig. 6k), a secreted protein with a signal peptide that is abundantly expressed in adipocytes.

The observation that CLSTN3β promotes the endoplasmic reticulum association of S100b suggests that CLSTN3β may directly bind S100b. We found that CLSTN3β bound S100b much more strongly than did CLSTN3 (Fig. 6i). Our data thus suggest that CLSTN3β—but not CLSTN3—may enhance secretion of S100b via direct physical interaction at the endoplasmic reticulum.

**Discussion**

Innervation by the sympathetic nervous system is important for both the development and activation of brown and—potentially beige—fat.21–24. Previously, very little has been known about the identity of adipocyte-derived factors that promote the innervation of thermogenic fat. We show here that the newly identified protein CLSTN3β, which is selectively and abundantly expressed in thermogenic adipocytes, is involved in the innervation of thermogenic fat. Genetic ablation of Clstn3b leads to a deficiency in adipose innervation and thermogenic function. Human adipose tissue also expresses Clstn3b (Extended Data Fig. 7), which suggests CLSTN3β may have a similar role of promoting adipose innervation in humans.

We have shown that CLSTN3β resides mainly in the endoplasmic reticulum but is also involved in innervation. It appears to do so—at least in part—by driving the secretion of S100b, a protein with trophic activity on sympathetic neurons. The secretion pathway of S100b is unclear, as this protein does not have a signal peptide.25. Our findings...
support a chaperone-like activity of CLSTN3β on S100β, which explains why the ablation of Clstn3 in mice results in a reduction in the level of S100β protein in BAT. Understanding the detailed mechanism of non-canonical secretion requires more analysis. However, the notion that S100β is used as a physiological neurotrophic factor is supported by both gain- and loss-of-function evidence. In addition to being regulated at the protein level by CLSTN3β, S100β transcription is positively regulated by PRDM16; this provides an explanation for the altered levels of adipose sympathetic innervation observed in PRDM16 gain- and loss-of-function mouse models.\(^{11,12}\). CLSTN3β expression is not as sensitive as S100β to changes in PRDM16 levels.

Previous studies have suggested that nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) are factors that potentially promote the sympathetic innervation of thermogenic fat.\(^{26,27}\). Nevertheless, ribosome profiling data driven by adipose-specific Cre revealed extremely low-to-undetectable levels of Ngf and Bdnf mRNA, which indicates that these factors may not be produced by adipocytes at a physiologically relevant level.\(^{28,29}\). By contrast, CLSTN3β and S100β are abundantly and selectively expressed in thermogenic adipocytes, which provides strong support for their roles as adipoctye-derived neurotrophic factors. Our data do not exclude the possibility that NGF or BDNF also contribute to adipose innervation.

Our findings should help to advance understandings of the basic biology of thermogenic fat and its innervation. Moreover, the identification of a soluble protein with neurotrophic actions on the sympathetic nervous system may also provide a therapeutic opportunity for promoting thermogenic fat activity in the treatment of obesity and associated metabolic disorders.

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1156-9.

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### Author contributions

X.Z. conceived the project and designed experiments. X.Z., M.Y. and B.H. performed imaging experiments and data analysis. X.Z. and B.H. performed metabolic assays. J.M.R. performed stereotaxical surgeries, X.Z., M.Y. and B.H. performed imaging experiments and data analysis. X.Z. and B.M.S. wrote the manuscript with discussion and contributions from all authors.

### Competing interests

The authors declare no competing interests.

### Additional information

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METHODS

No statistical methods were used to predetermine sample size. Except imaging data analysis, investigators were not blinded to allocation during experiments and outcome assessment.

**Mouse strains.** *Clstn3b*-knockout mice were generated using the CRISPR–Cas9 technique. Two guide RNAs (CCCCGACGAGAACTGAACTCGT, ATGG CCCCCGACGAGAACACCCGAG) flanking the unique exon of *Clstn3b* were synthesized by PNA-BIO. They were microinjected with Cas9 enzyme (CP02, PNA-BIO) into fertilized eggs of mice of the C57BL/6 background at the transgenic core of Beth Israel Deaconess Medical Center. F1 progeny were genotyped with the following primers: CTGCCAGGGAACGACGGGTCCAG, GACGAGTTCTCATAACAGTTGCC and CACCGAGAAGAACAGGAGCTTC. The wild-type allele yielded a band of 368 bp and the knockout allele yielded a band of approximately 480 bp. F1 progeny carrying the knockout allele were crossed with C57BL/6 mice purchased from Jackson Laboratory to screen for germline transmission. F2 *Clstn3b*−/− mice were then crossed with *Clstn3b*+/+ to yield *Clstn3b*−/− and *Clstn3b*−/+ mice for analyses. To generate CLSTN3−/−adipose-specific mice, the mouse *Clstn3b* cDNA was cloned into a construct for integration into the Rosa26 locus as previously described. The construct was linearized with KpnI and microinjected into C57BL/6 embryonic stem cells. Successful integration of the transgene was validated using long-range PCR (5′-forward: CTTAAAGAAGGACGCTGTTCTGGG, 5′-reverse: TGGGCTTAGAACTATGACCCGG; 3′-forward: TTGGCAGAAGATCTCTCCCAACCTGGG; 3′-reverse: CAGTTAAGGGCATACACAGTGTGG) before crossing into the Rosa26 background (Jackson Laboratory). Tyrosine hydroxylase antibody was generated with a C-terminal peptide DSPSSDERRIIESPPHRY with an N-terminal cysteine for conjugation to the carrier protein keyhole limpet haemocyanin, and affinity-purified with the antigenic peptide (New England Peptide; Covance). The C-terminal peptide DSPSSDERRIIESPPHRY was expressed and purified. Adipoq-cre and Vglut3-ires-cre lines were maintained at the animal facility of Beth Israel Deaconess Medical Center. The *Stro2−/−* knockout line was recovered from cryopreservation at RIKEN Institute, and genotyping was performed following the protocol in the original publication. All mice were maintained under a 12 h light/12 h dark cycle at constant temperature (23 °C), unless otherwise specified, with free access to food and water. All animal studies were approved by and in full compliance with the ethical regulation of the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Male mice of 8–12 weeks of age were used for experiments. Sample size was chosen based on the literature and results of pilot experiments to ensure statistical significance could be reached. Randomization was not performed because mice were grouped based on genotype.

**CLSTN3-3′ antisense.** Rabbit polyclonal antibody to CLSTN3 was generated with a C-terminal peptide DSPSSDERRIIESPPHRY for conjugation to the carrier protein keyhole limpet haemocyanin, and affinity-purified with the antigenic peptide (New England Peptide; Covance).

**Cryo-section immunostaining.** Tissue was collected immediately after euthanizing the mice, and fixed with 4% paraformaldehyde overnight. Tissue was then washed with PBS 5 times, for 10 min each time and incubated in PBS with 30% sucrose for 8 h, and then frozen in Tissue-Tek OCT Compound (Sakura Finetek). Sections were then washed with saline, and transcardially perfused first with 0.1 M phosphate buffered saline (PBS) and then with 4% paraformaldehyde. Brains were extracted and post-fixed with 1% osmium tetroxide, dehydrated and embedded in EPON resin. Forty-eight hours later, coverslips were removed and areas containing cells were randomly selected and mounted.

**Histology.** Mice were terminally anaesthetized with 7% chloral hydrate (500 mg kg−1; Sigma, Aldrich) diluted in saline, and transcardially perfused first with 0.1 M phosphate-buffered saline (PBS) and then with 10% neutral-buffered formalin solution (NBF) (Thermo Fisher Scientific). Brains were extracted and post-fixed overnight at 4 °C in NBF. The next day, brains were switched to PBS containing 20% sucrose for cryoprotection. Finally, brains were sectioned coronally at 30 μm on a freezing microtome (Leica Biosystems), and stored in cryoprotectant solution at −20 °C until used for immunofluorescence. For immunofluorescence, brain tissue sections were washed 3× in PBS before a blocking step containing 3% normal donkey serum and 0.4% Triton X-100 in PBS for 1 h at room temperature. Primary antibody was prepared in the same blocking solution and incubated overnight at 4°C in NBF. The next day, brains were switched to PBS containing 20% sucrose for cryoprotection. Finally, brains were sectioned coronally at 30 μm in a freezing microtome (Leica Biosystems), and stored in cryoprotectant solution at −20 °C until used for immunofluorescence. For immunofluorescence, brain tissue sections were washed 3× in PBS before a blocking step containing 3% normal donkey serum and 0.4% Triton X-100 in PBS for 1 h at room temperature. Primary antibody was prepared in the same blocking solution and incubated until the following concentrations: rat anti-mCherry (Life Technologies, M12117) 1:3,000, rabbit anti-FOS (EMD Millipore, ABE457) 1:3,000. The next day sections were washed 5× in PBS, then incubated for 2 h at room temperature in Alexa Fluor fluorescent secondary antibody (Life Technologies, 1:1,000) prepared in PBS containing 2% normal donkey serum and 0.4% Triton X-100. Sections were then washed 3× in PBS, mounted on gelatin-coated slides and coveredslips with Vectashield mounting medium containing DAPI (Vector Labs). Fluorescent images were captured using an Olympus VS120 slide-scanning microscope.
Chemogenetic activation of BAT assay. To monitor BAT response in real time, temperature probes (Bio Medic Data Systems, IPTT-300) were implanted into the interscapular region of mice and temperature was read with a transponder (Bio Medic Data Systems, DAS-7007R). To activate sympathetic neurons, mice were intraperitoneally injected with saline or CNO (Sigma, C0832) at 1 mg/kg.

B­AT fractionation and CLSTN3β isolation. BAT fractionation was performed based on a previously described procedure. In brief, whole BAT was dissected and homogenized in a dounce homogenizer in 10 ml of 20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4 (HES buffer). The homogenate was filtered with two layers of cheesecloth and centrifuged at 16,000 g for 15 min. The fat layer was removed and the supernatant (S1) was saved. The pellet (P1) was resuspended in 5 ml HES and applied on top of a 1.12 M sucrose cushion that contained 20 mM HEPES, 1 mM EDTA, pH 7.4, and was centrifuged at 101,000 g for 70 min to yield a pellet (P2) and fluffy material at the interface (plasma-membrane fraction). P2 was resuspended in 5 ml HES, and centrifuged at 700 g to yield the nuclei pellet; the supernatant was centrifuged again at 8,000 g to yield the mitochondria pellet. S1 was centrifuged at 212,000 g for 70 min to yield the microsome pellet, and the supernatant was the cytosol fraction. To isolate endogenous CLSTN3β, the microsome pellet was solubilized in IP buffer (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 5% glycerol, pH 7.4) and incubated with Dynabeads protein G, pre-loaded with CLSTN3β antibody at 4 °C for 2 h. The beads were washed with IP buffer 3 times, for 5 min each time and incubated with IP buffer containing 100 μg/ml antigenic peptide at 4 °C for 30 min.

AAV injection. Six-week-old male mice were anaesthetized with isoflurane and an incision was made above the interscapular area to expose the underlying adipose tissue. About 3 × 1010 AAV particles were injected into each BAT lobe and the incision was closed with suture. Mice received one injection of meloxicam (2 mg/kg) 24 h before surgery, and another injection immediately after surgery. Mice were allowed to recover for three weeks before analysis. AAV8-EF1a-DIO-Clstn3b or AAV8-EF1a-DIO-S100b were packaged at the Boston Children’s Hospital Viral Vector Core (Addgene plasmid 27056; donating investigator, K. Deisseroth).

Cold tolerance assay. Mice were pre-acclimatized at thermoneutrality (28–30°C) for two weeks and then shifted to 4°C. Body temperature was measured with a rectal probe (Physitemp, RET3) and a reader (Physitemp, BAT-12).

S100b assays. To detect binding between S100b and CLSTN3β or CLSTN3, HEK293T cells transfected with pcDNA, pcDNA-Clstn3b-Flag or pcDNA-Clstn3-Flag were homogenized with IP buffer (25 mM HEPES 150 mM NaCl, 1 mM CaCl2, 1% lauryl maltose–neopentyl glycol, 5% glycerol, pH 7.4) and centrifuged at 16,000 g for 10 min. The supernatant was incubated with anti-Flag M2 resin (Sigma, A2220) at 4°C for 1 h, and the resin was washed with binding buffer (same as IP buffer except lauryl maltose–neopentyl glycol concentration was reduced to 0.02%) 3 times, for 5 min each time. The resin was then incubated with 10 μg/ml recombinant S100b (R&D Systems, 1820-SB-050) in binding buffer at 4°C for 30 min and then washed with binding buffer 3 times, for 5 min each time. Bound proteins were eluted with 150 μg/ml 3 × Flag peptide (Sigma, F4799) in binding buffer at 4°C for 30 min. To assess S100b secretion, medium was collected from cells that express S100b and centrifuged at 16,000 g for 20 min. The supernatant was subjected to S100b quantification with an enzyme-linked immunosorbent assay kit (Millipore, EZHS100B-33K). S100b antibody (Abcam, ab51642) was used for immunofluorescence and western blot analysis. The HEK293T cell line used in these experiments was purchased from ATCC and maintained in the Spiegelman laboratory. This cell line has been routinely tested negative for mycoplasma. Authentication of this cell line was not performed for this study.

Quantitative PCR. The following primers were used for quantitative PCR analysis of gene expression. Clstn3b fwd, CTCGCAGGAACACAGCAGCC; rev, AGGATAACCATAACGCACCCAG; S100b fwd, TGTTGGCCTCATGATGTCT; rev, CCCATGCCATCTTGCTCC. Primers for other genes have previously been described.

ChIP–seq and metabolic assays. All ChIP and metabolic experiments were performed as previously described.

Whole-mount immunofluorescence. The whole-mount immunostaining of subcutaneous inguinal WAT was performed as previously described. The inguinal, rather than the dorsolumbar, region was selected for imaging.

Mass spectrometry analysis. Quantitative whole tissue proteomics analysis was performed as previously described.

Statistics and reproducibility. All experiments have been successfully repeated with similar results for at least three times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
Histone modification marker and transcription factor ChIP–seq datasets generated in this study are available at NIH Sequence Read Archive under the accession code PRJNA526243. Any other relevant data are available from the corresponding author upon reasonable request.

30. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods 12, 51–54 (2015).

31. Simpson, I. A. et al. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim. Biophys. Acta 763, 393–407 (1983).
Extended Data Fig. 1 | Clstn3b encodes an adipocyte-specific protein.

a, Quantitative PCR analysis of Clstn3b expression in wild-type and Lsd1-knockout BAT \( (n = 3 \text{ mice}) \). b, Histone marker and transcription regulator ChIP–seq at the Clstn3 locus from BAT. c, Quantitative PCR analysis of Clstn3b expression in inguinal subcutaneous WAT from mice acclimatized to room temperature or 4 °C \( (n = 4 \text{ mice}) \). d, Mass spectrometry identification of CLSTN3β peptides. e, Conservation of CLSTN3β within the mammalian class. The red cross and green ticks indicates the absence and presence, respectively, of homologues of CLSTN3β in mammalian subclasses. f, Sequence alignment between the unique exon of Clstn3b from human, and a fragment, in an intron upstream of the penultimate exon of Clstn1, in the genome of Chinese softshell turtle. Note how the position of this fragment corresponds to the β-selective exon in Clstn3. All data are mean ± s.e.m. Statistical significance was calculated by unpaired Student's two-sided t-test.
Extended Data Fig. 2 | CLSTN3/3 localizes to the endoplasmic reticulum. a, b. Electron microscopy analysis of primary brown adipocytes that express CLSTN3β–APEX2. In a, arrows denote the Golgi apparatus. In b, arrows denote peroxisomes. Scale bars, 100 nm. c, Western blot analysis of the fractionation pattern CLSTN3. Asterisk denotes a nonspecific band. For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 3 | Ablation of Clstn3b impairs adipose thermogenesis. a–d, Sanger sequencing (a), western blot (b), quantitative PCR (c) (n = 4 mice) and immunofluorescence (d) confirmation of CRISPR–Cas9 deletion of Clstn3b. Scale bars, 10 μm. e, Quantitative PCR analysis of Clstn3 expression in a panel of wild-type mouse tissues, and wild-type and Clstn3b-knockout brain (n = 2 mice for surveying tissue specificity in wild-type mouse; n = 3 mice for wild type and knockout). The primers target the junction between the third and the penultimate exons. f, g, Body weight curve (f) and body composition (g) of wild-type and Clstn3b-knockout mice on chow diet (n = 8 mice). h, Rates of CO₂ production from indirect calorimetry analysis of wild-type and Clstn3b-knockout mice (n = 6 mice). i, j, Movement (i) and daily food intake (j) of wild-type and Clstn3b-knockout mice in metabolic chambers (n = 6 mice). k, Oxygen consumption response to acute β3 agonist injection, of wild-type and Clstn3b-knockout mice (n = 6 mice). All data are mean ± s.e.m. Statistical significance was calculated by unpaired Student’s two-sided t-test.
Extended Data Fig. 4 | Transgenic expression of Clstn3b increases adipose thermogenesis. a, b, Western blot (a) and quantitative PCR (b) confirmation of transgenic overexpression of CLSTN3β in BAT (n = 5 mice). c, d, Body-weight curve (c) and body composition (d) of wild-type and Clstn3b-transgenic mice on chow diet (n = 6 mice). e, Rates of CO2 production from indirect calorimetry analysis of wild-type and Clstn3b-transgenic mice (n = 4 mice). f, g, Movement (f) and daily food intake (g) of wild-type and Clstn3b-transgenic mice in metabolic chambers (n = 4 mice). h, Oxygen consumption response to acute β3 agonist injection of wild-type and Clstn3b-transgenic mice (n = 4 mice). All data are mean ± s.e.m. Statistical significance was calculated by unpaired Student’s two-sided t-test.
Extended Data Fig. 5 | CLSTN3/β increases sympathetic innervation of thermogenic adipose tissue. a, Gene expression analysis of wild-type and Clstn3b-knockout BAT upon 5 h of acute cold exposure, following mice being pre-acclimatized to thermoneutrality (n = 4 mice). Blue, wild-type; orange, knockout. b, Indirect calorimetry analysis of Clstn3b-knockout mice with or without Adipoq-cre, receiving AAV-DIO-Clstn3b injection (n = 4 mice). c, Whole-mount tyrosine hydroxylase staining of the inguinal region of the posterior subcutaneous WAT from wild-type and Clstn3b-knockout mice, acclimatized at 4 °C for 1 week. Scale bars, 50 μm. All data are mean ± s.e.m. Statistical significance was calculated by unpaired Student’s two-sided t-test.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | CLSTN3β promotes secretion of S100b, an adipocyte-derived neurotrophic factor. a, b, Quantitative PCR analysis of S100b expression in various fat depots (a) and in inguinal subcutaneous WAT (b), from mice acclimatized to room temperature or 4 °C (n = 4 mice). c, Quantitative PCR analysis of S100b expression in control or Prdm16-transgenic inguinal subcutaneous WAT (n = 4 mice). d, Quantitative PCR analysis of S100b expression in control or Prdm16-knockout inguinal subcutaneous WAT (n = 4 mice). e, PRDM16 ChIP–seq showing binding at the S100b locus. f, Indirect calorimetry analysis of Clstn3b-knockout mice with or without Adipoq-cre, receiving AAV-DIO-S100b injection (n = 4 mice). g, Tyrosine hydroxylase immunostaining of salivary gland from wild-type and S100b-knockout mice. h, Quantitative PCR analysis of S100b expression in wild-type and Clstn3b-knockout BAT from mice housed at room temperature (n = 4 mice). Note that this is a different housing condition from that used for experiments in Extended Data Fig. 5a. i, Western blot analysis of intracellular level of S100b in Clstn3b-knockout brown adipocytes that express S100b alone, or co-expressing S100b with CLSTN3β. j, Western blot analysis of S100b protein level in HEK293T cells transfected with various constructs as indicated. k, Western blot analysis of S100b and complement factor D secretion from HEK293T cells co-transfected with or without CLSTN3β. All data are mean ± s.e.m. Statistical significance was calculated by unpaired Student’s two-sided t-test.
Extended Data Fig. 7 | Clstn3b is specifically expressed in human adipose tissue. RNA sequencing in human tissues that shows adipose-specific expression of Clstn3b. RNA sequencing of 13 human tissue types was analysed for reads that uniquely map to the Clstn3b-specific exon.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- **n/a**
- **Confirmed**

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- 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

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

**Data collection**
- Oxymax 5.0 for CLAMS; NIS element for confocal imaging; LAS-3000 Imaging System from Fuji for Western blot; Applied Biosystems 7500 for qPCR.

**Data analysis**
- Imaris 8.4 and Image J 1.52 for imaging analysis; Excel 2016 for statistical analysis. Integrated genome viewer for sequencing data

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data will be available in publicly available datasets or upon request to the Spiegelman laboratory after publication.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences
Life sciences study design

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

| Sample size | No sample size pre-determination was performed. Samples size was chosen based on previous experience that would be sufficient to achieve statistical significance. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded.                                                                                                                                                                                |
| Replication | All experiments have been successfully repeated with similar results for at least three times.                                                                                                           |
| Randomization | Mice were grouped based on genotype and therefore no randomization was required for this study.                                                                                                |
| Blinding | Imaging investigator was blinded during data analysis.                                                                                                                                                  |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | Antibodies |
| ☐ | ☒ Eukaryotic cell lines |
| ☐ | Palaeontology |
| ☒ | Animals and other organisms |
| ☐ | Human research participants |
| ☒ | Clinical data |
| n/a | Involved in the study |
| ☒ | ChIP-seq |
| ☐ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Antibodies

| Antibodies used | Clstn3β antibody was generated at Covance. S100b antibody, Abcam, ab51642. TH antibody, EMD Millipore, AB1542. TUBB3 antibody, Abcam, ab52623. KDEL antibody, Abcam, ab50601. Ucp1 antibody, Abcam, ab10983. Cav1 antibody, Abcam, ab192869. GAPDH, Abcam, ab9485. FLAG antibody, Sigma-Aldrich, F1804. Cfd antibody, Abcam, ab213177. mCherry antibody, ThermoFisher M11217; cfos antibody, EMD Millipore, ABE457. Donkey anti sheep Alexa Fluor 488 antibody, ThermoFisher, A-11015; Goat anti rabbit Alexa Fluor 617 antibody, ThermoFisher, A-27040; Goat anti rat Alexa Fluor 488 antibody, ThermoFisher, A-11006; Donkey anti rat Alexa Fluor 594 antibody, ThermoFisher, A21209; Donkey anti rabbit Alexa Fluor 488 antibody, ThermoFisher, A21206. |
| Validation | Clstn3β antibody was validated by both KO and transgenic tissue samples. All commercially available antibodies were validated for detecting mouse proteins by immunostaining or Western blot by the manufacturers. |

Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HEK293T from ATCC. |
| Authentication | This cell line was maintained in the Spiegelman and not specifically authenticated for this study. |
| Mycoplasma contamination | The cell line was tested negative for mycoplasma. |
| Commonly misidentified lines | No commonly misidentified lines were used. |

Animals and other organisms

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

| Laboratory animals | Mus musculus. Clstn3β KO, Clstn3β transgenic, adiponectin-cre, and S100b KO mice were all on the C57Bl/6 background. Vlug3-ires-cre line had a mixed background. Male mice at age 8-12 weeks were used for experiments. |
Wild animals | This study did not involve wild animals.
---|---
Field-collected samples | This study did not involve field-collected samples.
Ethics oversight | Beth Israel Deaconess Medical Center IACUC.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### ChIP-seq

**Data deposition**

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

**Data access links**

- BAT H3K4me3 and H3K27Ac are from ENCODE database. Accession code: ENCSR000CFB for H3K4me3 and ENCSR000CEZ for H3K27Ac.
- All other datasets are available at NIH SRA under the accession code PRJNA526243.

**Files in database submission**

- BAT_H3K4me1_rep1.bam
- BAT_H3K4me1_rep2.bam
- BAT_H3K4me2_rep1.bam
- BAT_H3K4me2_rep2.bam
- BAT_H3K79me3_rep1.bam
- BAT_H3K79me3_rep2.bam
- BAT_LSD1.bam
- BAT_PPARG.bam

**Genome browser session**

- N/A.

**Methodology**

- **Replicates**
  - Two replicates for each sample for Histone markers. One replicate for LSD1 and PPARG.
- **Sequencing depth**
  - 30-50 million reads per sample
- **Antibodies**
  - H3K4me1, EMD Millipore 07-436; H3K4me2, EMD Millipore 07-030; H3K79me3, Abcam ab2621; PPARG, SantaCruz, H-100; LSD1, Abcam, ab17721
- **Peak calling parameters**
  - This study does not involve peak calling but only concerns reads distribution at a specific locus.
- **Data quality**
  - Not relevant for this study.
- **Software**
  - CLC Genomics Workbench 11.