Hypothalamic neuronal toll-like receptor 2 protects against age-induced obesity

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Toll-like receptors (TLRs) are traditionally associated with immune-mediated host defense. Here, we ascribe a novel extra-immune, hypothalamic-associated function to TLR2, a TLR-family member known to recognize lipid components, in the protection against obesity. We found that TLR2-deficient mice exhibited mature-onset obesity and susceptibility to high-fat diet (HFD)-induced weight gain, via modulation of food intake. Age-related obesity was still evident in chimeric mice, carrying comparable TLR2 immune cells, suggesting a non-hematopoietic-related involvement of this receptor. TLR2 was up-regulated with age or HFD in pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus, a brain area participating in central-metabolic regulation, possibly modulating the hypothalamic-anorexigenic peptide, α-melanocyte-stimulating hormone (α-MSH). Direct activation of TLR2 in a hypothalamic-neuronal cell-line via its known ligands, further supports its capacity to mediate non-immune related metabolic regulation. Thus, our findings identify TLR2 expressed by hypothalamic neurons as a potential novel regulator of age-related weight gain and energy expenditure.

Results

TLR2 deficient mice develop mature-onset obesity. Our initial serendipitous observation, that aged TLR2-deficient (TLR2D) mice had an increased body weight relative to age-matched wild type (WT) mice, led us to consider TLR2 as a possible regulator of metabolic functions. We therefore first monitored the weight gain and metabolic state of WT vs. TLR2D mice of different ages. The body weight of TLR2D mice began to deviate from that of WT at approximately 4 months of age. By 12 months of age, the TLR2D mice weighed 30% more, on average, than their WT counterparts (Fig. 1A,B), and exhibited a marked increase in fat mass, as measured by body composition MRI (Fig. 1C) and T2-weighted MRI images (Fig. 1D).

The increased weight gain observed in TLR2D mice prompted us to test whether this phenomenon was accompanied by metabolic alterations. We found elevated plasma levels of both cholesterol and triglycerides in TLR2D mice compared to the WT animals (Fig. 1E). Furthermore, the TLR2D mice showed decreased ability to handle a glucose load (assessed by glucose-tolerance test; GTT; Fig. 1F), and reduced insulin sensitivity (determined by insulin-tolerance test; ITT; Fig. 1G) as compared to their WT controls. Indirect calorimetry revealed that the respiratory exchange ratio (RER) of 12-month old TLR2D mice was higher throughout the day.
cycle, both in the dark (active) phase and the light phase, suggesting that TLR2D mice oxidized more carbohydrates and less fat as an energy source (Fig. 1H). Furthermore, while food consumption was similar in the two strains at 3 months of age, at 4 and 12 months, TLR2D mice consumed 17% and 23% more food than WT controls, respectively (Fig. 1I, J). Nevertheless, water intake, heat production, dark cycle locomotion, VO₂ (a measurement of oxygen consumption), and VCO₂ (a measurement of carbon dioxide production) did
not differ between strains (Fig. 1K). Together, these findings suggest that deficiency in TLR2 led to metabolic alterations that contributed to the development of mature onset obesity.

**TLR2 deficiency in adulthood exacerbates obesity induced by high fat diet.** Once we established that elderly TLR2D mice are more prone to develop obesity than their WT counterparts, we next tested the ability of adult TLR2D mice to cope with homeostatic challenge, such as high-fat diet (HFD). To this end, we maintained 3-month old TLR2D and WT mice, slightly before the emergence of mature onset obesity (3–4 months of age; Fig. 1B), on a HFD (45% kcal from fat) for 50 days. During their time on HFD, the response of TLR2D mice deviated from that of the WT mice, showing a greater rate of weight gain (Fig. 2A). Though TLR2D mice maintained on regular diet also exhibited increased body weight compared to their age matched WT controls, those maintained on HFD displayed even more pronounced weight gain compared to their HFD-maintained WT counterparts, suggesting that TLR2-deficiency resulted in a higher susceptibility to diet-induced obesity (DIO) (Fig. 2B). Furthermore, on HFD, TLR2D mice had higher food intake (expressed in grams, corrected for body weight, or not) compared to WT (Fig. 2C), their total fat mass was greater (Fig. 2D), and their percent lean mass of total body weight was lower, as measured by whole body composition MRI (Fig. 2E). Notably, comparison of age matched controls maintained on regular diet versus the HFD fed mice revealed that TLR2D mice maintained with HFD increased their total fat by 4.1 ± 0.05 fold, whereas the WT animals fed with HFD increased body fat by 3.1 ± 0.3 fold relative to those kept on regular chow (Fig. 2D). Moreover, more pronounced differences between TLR2D and their WT control in the % lean mass were noticed when the mice were kept on HFD (Fig. 2E). Taken together, these results suggest that in addition to its role in restricting age-induced obesity, TLR2 expression provides physiological protection at early adulthood against diet-induced obesity.

**The TLR2-mediated anorexic function extends beyond the hematopoietic system.** The TLRs are a family of receptors mainly known for their immunological roles. Therefore, their contribution to the development of obesity was mainly linked to their ability to induce inflammation in peripheral metabolic organs. As novel non-immune functions have recently been attributed to TLRs, we aimed here to determine whether TLR2-mediated regulation of age-related obesity extends beyond their role associated with hematopoietic cells. To test this, we created bone marrow chimeras, in which following total body irradiation, the bone marrow (BM) of the TLR2D mice was replaced with wild type BM cells that have normal TLR2 expression (termed (wt>TLR2D)). As a control for this manipulation, we similarly manipulated age matched WT mice, and replaced their BM with the same WT BM cells used for the TLR2 chimera preparation (these control chimeras are termed (wt>wt)). The identical wild type (TLR2-positive) BM cells used for the reconstitution of both chimera types allowed the attribution of any difference(s), if observed, to TLR2 deficiency by the non-hematopoietic, radio-resistant cells. We first verified that due to the manipulation, the (wt>TLR2D) chimeric mice gained TLR2 expression in their hematopoietic cells within the periphery, as demonstrated by the expression of TLR2 on myeloid cells (CD11b+) present in hematopoietic (blood, spleen, lymph-nodes, bone marrow) and metabolic organs (fat, liver and pancreas) (representative data from fat tissues is shown in Fig. 3A). In all tested organs, TLR2 expression levels on myeloid cells were comparable between TLR2D recipient mice that received BM from WT donors ((wt>TLR2D) chimeras) and WT mice that received WT BM ((wt>wt) chimeras) (Fig. 3A). Although displaying similar peripheral hematopoietic expression of TLR2, with maturity, the TLR2D mice that received the WT BM ((wt>TLR2D) chimeric) showed increased weight gain, similarly to the non-chimeric TLR2D mice, relative to their (wt>wt) control chimeras (Fig. 3B–D). Moreover, similar to the results observed in the whole-body TLR2D mice (Fig. 1F), (wt>TLR2D) chimeras demonstrated a robust decrease in glucose tolerance compared with the (wt>wt) chimeric controls (Fig. 3E). Our results thus demonstrated that the TLR2-mediated protective role in preventing age-related obesity extends beyond hematopoietic contribution, and is mediated, at least in part, via TLR2 expression by non-hematopoietic, radio-resistant cells.

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**Figure 2** | TLR2 protects against high-fat diet-induced obesity at adulthood. (A) TLR2D and WT mice (3 month old) maintained on HFD were monitored for weight change during 8 weeks on HFD (Repeated ANOVA; left F = 10.9, P < 0.0001; right F = 6.7, P = < 0.0001). (B) Weight and weight gain in [g] by TLR2D and WT mice, maintained either on regular chow or on HFD (ANOVA; left Pstrain = 0.001, Pchow < 0.0001; right Pstrain < 0.0001, Pchow < 0.0001). (C) Daily food intake of TLR2D and WT mice maintained on HFD (Student’s t-test; *P < 0.05). (D,E) Total fat mass (D; ANOVA, P < 0.0001) and percent lean mass (E; ANOVA, P < 0.0001) of TLR2D and WT mice, maintained either on regular chow or on HFD as measured by body composition MRI [gm]. Asterisks denote significant differences relative to control.
TLR2 is an intrinsic metabolic regulator in hypothalamic neurons. Food intake, energy balance and body weight are regulated in the CNS by metabolic neurons of the hypothalamus, which receive input from the periphery regarding the energetic state of the body, and relay this information to higher brain areas. The hypothalamic arcuate nucleus (ARC) has a key role in regulating feeding behavior and energy expenditure. The ARC contains two major neuronal populations with opposing effects on energy balance. One population co-expresses the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AGRP) and the other population expresses pro-opiomelanocortin (POMC) which is cleaved to produce the anorectic peptide α-melanocyte stimulating hormone (α-MSH), and co-express cocaine-and-amphetamine-regulating transcript (CART).

The observed involvement of TLR2 in the regulation of food intake (Fig. 1I), together with the finding of a non-hematopoietic associated role of TLR2 in the prevention of age-induced obesity (Fig. 3), encouraged us to test whether such protection is orchestrated by TLR2 activity in the metabolic region of the CNS. To address this question, we first analyzed the ARC, of middle-aged TLR2D mice and their age matched controls for the levels of α-MSH, one of the most potent anorectic peptides implicated in the central control of food intake. Coronal brain slices, which included the ARC, were chosen according to the Allen Brain Atlas, as detailed in the Methods. All slices included the hippocampal dentate gyrus and the third ventricle at the level of hypothalamus, as a mean to verify regional location; the adjacent Median Eminence (ME) is indicated. In the ARC, the levels of hypothalamic α-MSH immunoreactive fibers within the ARC were found in TLR2D mice (Fig. 4A,B). These results could be either an outcome of the obesity developed in the TLR2D mice, or a direct effect of TLR2 deficiency within the hypothalamus, indicating a role of TLR2 in the central regulation of food intake. Considering the latter option, we tested TLR2 expression in the brain of middle-aged wild type mice. TLR2 was found to be restricted to the hypothalamus, of TLR2 in the central regulation of food intake28. Coronal brain slices, which included the ARC, were prepared from wt and wt mice. TLR2 was found to be restricted to the hypothalamus, indicating a role of TLR2 in the prevention of age-induced obesity, through control of weight gain and food intake by TLR2. This receptor was found here to play a novel role that extends beyond its familiar inflammatory-related functions. This receptor was found to be expressed on the surface of neurons has some precedents. The TLR orthologue in Drosophila, Toll, was

Figure 3 | TLR2 mediated protection against age-related obesity extends beyond its expression by cells of the hematopoietic lineage. (A–D) [wt>TLR2] and [wt>TLR2D] chimeras were analyzed for TLR2 expression by flow cytometry (A; Representative analysis of fat tissue isolated from TLR2D as well as from wt mice) and [wt>TLR2] chimeras; histograms were pre-gated on myeloid cells (CD11b+); percentages of TLR2+ out of pre-gated cells are indicated, for weight gain (B,C,D), and for glucose sensitivity (E) (B-Repeated ANOVA, Pbetween groups < 0.0001; C- Student’s t-test, ***p < 0.0001; E- Repeated ANOVA, Pbetween groups = 0.0052). n = 11–14 mice per group.

In conclusion, TLR2 expression in the hypothalamus plays a role as a metabolic regulator in the CNS. Further studies of hypothalamic metabolic pathways are necessary to fully understand the role of TLR2 in the central regulation of food intake and energy balance.
shown, in parallel to its discovery in the context of fungal infection, to be a key player in the regulation of a non-immunological process, namely the establishment of dorsal-ventral axis polarity and synaptogenesis during embryogenesis. Research in the last few years reveals multi functions of TLRs in the CNS, including their diverse roles in neuronal-related processes in the adult, as manifested by their key functions in adult neurogenesis, neurosensory and neuronal death. Moreover, in addition to their roles in developmental processes, TLRs are involved in the regulation of neuronal-related processes in the adult, as manifested by their key functions in adult neurogenesis, neurosensory and neuronal death. The current study adds another piece to this growing puzzle by attributing a regulatory role to TLR2 in controlling metabolic processes within hypothalamic neurons. In line with our observation of age-related increase of TLR2 expression in metabolic neurons, TLR2/4 were shown to be induced in cerebral cortical neurons in response to ischemic injury, further supporting their dynamic expression in response to environmental challenges.

Our data suggest that hypothalamic TLR2 serves as an endogenous protective mechanism designed to maintain energy homeostasis and to compensate for metabolic alterations, which spontaneously develop in middle-age or following a metabolic challenge. This is in line with the recent study reporting increased levels of adipose tissue, higher weight gain, and a significant decrease in the rate of glucose uptake under high insulin stimulus in TLR2-deficient mice grown in a non-germ free facility. Notably, in our study, we were able to dissect the non-hematopoietic, neuronal anorexigenic contribution of TLR2 in protecting against obesity. According to our model, we suggest that TLR2-deficiency in the hypothalamic arcuate nucleus, participates in the inflammatory process of metabolic tissues that may progresses into obesity, while under conditions of homeostatic challenge (such as following increased microbial challenge, or with aging, or diet) it rather acts as an alternative universal protective mechanism against obesity. Additionally, the earlier studies were conducted in younger animals, in which the anorectic effect of TLR2 mediated by hypothalamic neurons may not be active yet. The apparent disagreement with previous reports showing an obesity-promoting role of TLR2, might thus reflect peripheral versus central effects, immune versus non-immune mechanisms, as well as the diet, housing, flora and age of the animals. In support of this complex picture, previous studies reported non-immune neural activities for TLRs, in which these neural TLR activities act in apparent opposition to their well-known immune-activating functions.

An intriguing question is the nature of the mechanism(s) that drive TLR2 into action. One possibility is that elevated plasma concentration of free fatty acids (FFAs), a major derangement of the extracellular milieu associated with obesity, induces hypothalamic TLR2 expression; TLR2 induction was previously shown to be affected by the presence of its ligands, which include, as discussed below, fat components. Alternatively, TLR2 induction can be regulated by pro-inflammatory cytokines, which accompany obesity, either HFD-induced or mature-onset, at the periphery and locally within the hypothalamus. Induction of TLR2 can also be regulated by NF-kappaB, mTOR, and c-Jun kinase, which are inter-crossed with metabolic pathways of insulin, and are shown to be activated in the hypothalamus in obese mice, even in neurons. In the context of neuronal TLRs, it was recently shown via in vitro studies that metabolic stress, in the form of glucose deprivation, regulates TLR levels.

Although a fundamental physiological role for TLR2 was established in this study, identification of the endogenous ligand(s) mediating this function necessitates further investigation. Interestingly, endogenous lipids such as FFA, facilitating many CNS processes, share common structural properties with some of the known exogenous lipid ligands of TLR2, and some lipids were shown to activate this receptor in ex vivo systems. Similarly, several laboratories have shown that long-chain saturated fatty acids (LCFAs), and in particular palmitic acid (PA), can activate TLR2. Furthermore, distinct fatty acids (saturated versus unsaturated) were shown to differentially affect the same TLR member, adding another level of complexity. Moreover, a crystallographic study recently showed that TLR2 heterodimerization discriminates between di- and
tri-acylated lipopeptides\textsuperscript{46}, suggesting this receptor as the ultimate molecule not only for rapidly responding to lipids in general, but for sensing the lipid saturation state of the body. The downstream mechanism operated by such TLR2-ligand interaction, which might be utilized in metabolic regulation requires further study. Interestingly, TLR2 challenge was previously shown to result in the rapid phosphorylation of STAT\textsuperscript{347}, a classical process in leptin activation, though not the classical downstream pathway of TLR2 ligation. This is in line with a previous suggestion that TLR activation in neurons drives a non-classical downstream signaling pathway\textsuperscript{17,20,37}.

Figure 5 | TLR2 is an intrinsic metabolic regulator acting in hypothalamic neurons. (A) Representative pictures for TLR2 staining in various coronal brain sections of middle-aged mice showing localization of this receptor to the arcuate nucleus (ARC). (B,C) Co-staining of TLR2 with astrocytes (GFAP\textsuperscript{1}; B) or microglia (Cx3cr1\textsuperscript{GFP/}; C) in the ARC of WT mice. (D, E) Co-localization of TLR2 to POMC neurons; staining for TLR2 in brain sections of mature Pomc\textsuperscript{RFP} mice (D) or co-staining with POMC (E) in WT mice. (F) Staining and quantification of TLR2 and TLR4 in the hypothalamus of adult and middle-aged WT mice (n = 3–4 mice per group; 3 depths). (G) Staining and quantification of TLR2 in ARC of mice fed with HFD or with regular chow (n = 3–4 mice per group; 3 depths; Student’s t-test ***P < 0.001). 3V- third ventricle; Arc- arcuate nucleus; ME- median eminence. Scale bars indicate 20 μm.

Figure 6 | Hypothalamic cell line expresses TLR2 and is affected by its ligands. (A–F) Analysis of TLR2 activation in the N42 hypothalamic cell line. TLR2 transcript levels in hypothalamic neurons (A), c-Fos activation (B,C) and changes in the transcript levels of the hypothalamic adipokines, resistin and fiaf (D), following TLR2 ligand administration, and in the presence of TLR2 neutralizing antibody (E,F). (B, Student’s t-test *P = 0.05; E, F ANOVA P < 0.0001). NC-negative control; MQ-microglia; PG-peptidoglycan; P3C- Pam3CysSK\textsubscript{4}. 
Moreover, the activities of STAT3 and NFkB, the classical TLR2 signaling, are closely intertwined, showing both transactivation and repression\(^4\). This suggests a potential platform at which TLR2 signaling, are closely intertwined, showing both transactivation and repression\(^4\). It is also possible that leptin-induced activation in TLR2 and leptin signaling may converge. It is also possible that leptin-induced activation in TLR2 and leptin signaling may converge. As the control of food intake is recently linked to obese individuals\(^5\). As the control of food intake is recently linked to obese individuals\(^5\). If so, this might assist in early diagnosis of susceptibility to obesity. Moreover, the activities of STAT3 and NFkB, the classical TLR2 signaling, are closely intertwined, showing both transactivation and repression48. This suggests a potential platform at which TLR2 signaling, are closely intertwined, showing both transactivation and repression\(^4\). Leptin signaling is also possible that leptin-induced activation in TLR2 and leptin signaling may converge. It is also possible that leptin-induced activation in TLR2 and leptin signaling may converge. Moreover, the activities of STAT3 and NFkB, the classical TLR2 signaling, are closely intertwined, showing both transactivation and repression48. This suggests a potential platform at which TLR2 signaling, are closely intertwined, showing both transactivation and repression\(^4\). As the control of food intake is recently linked to obese individuals\(^5\). As the control of food intake is recently linked to obese individuals\(^5\).

**Methods**

**Animals.** TLR2D mice were the generous gift of Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), C57BL/6 mice, a generous gift of Stefan Jantsch (Weizmann Institute, Israel) are heterozygous mice with GFP inserted in the C57BL/6 locus of one allele\(^1\). Mice were anesthetized with ketamine/xylazine and perfused transcardially with PBS. Brains were fixed, isolated, and sectioned, as previously described\(^15\). Primary antibodies were fixed, isolated, and sectioned, as previously described\(^15\). For all staining experiments, additional parts of the brains were routinely checked to verify staining specificity. TLR2D mice were used for the validation of TLR2-specific staining. The coronal brain slices, which included the ARC, were chosen according to the Allen Brain Atlas. To confirm their specific location, slices were verified to include hippocampal dentate gyrus and the third ventricle at the level of hypothalamus.

A Nikon fluorescence microscope (Nikon E800), equipped with a Nikon digital camera (DXM 1200F) and with either a 20 × (NA = 0.30) or 40 × (NA = 0.75) Plan Fluor objective lens (Nikon) was used for all fluorescence microscopy. The immunostaining intensity was determined automatically with Image-Pro Plus 4.5 software (Media Cybernetics). All measurements were performed by an observer blinded to the identity of the examined tissues.

**Fat and lean mass measurements.** Total fat [g] and lean mass [g] of TLR2D and WT mice were measured (MRI-MRI-100\(^TM\); EchoMRI Corporation, CA), a whole body composition MRI system. The EchoMRI-100\(^TM\) is a QNMR system that measures parameters of whole body composition including total body fat, lean mass, body fluids, and total water content in live laboratory animals.

**MRI studies.** Mice were anesthetized with ketamine/xylazine. MRI experiments were performed on a 9.4 Tesla Biomic Spectre Magnet 94/20 USR system (Bruker, Germany) equipped with gradient coil system capable of producing a pulse gradient of up to 40 gauss/cm in each of the three directions. The MRI protocol included axial T2-weighted images. The T2-weighted images were acquired using the spin-echo sequence with a TR of 1000 ms, TE of 10 ms, 256 × 128 matrix (interpolated to 256 × 256), and two averages, corresponding to an image acquisition time of 4 min 16 sec. In all experiments, 25 continuous slices with a thickness of 1.0 mm were acquired, with a field of view (FOV) of 4 cm × 4 cm. Spin-echo measurements were then repeated with application of fat suppression.

**Blood lipids.** For blood analysis, 250 μl of blood was collected through a small incision of the tail, and cholesterol and triglyceride levels were tested by a commercial lab (PathoVet Ltd., Kfar Bilu, Israel).

**Metabolic studies.** Indirect calorimetry, food and water intake as well as locomotor activity were measured using the LabMaster system (TSE-Systems, Bad Homburg, Germany). The LabMaster instrument consists of a combination of sensitive feeding and drinking sensors for automated online measurement. The calorimetry system is an open-circuit system that determines O₂ consumption, CO₂ production, and respiratory exchange ratio (RER). A photobeam-based activity monitoring system detects and records ambulatory movements, including rearing and climbing, in each cage. All parameters were measured simultaneously and continuously. Data were collected for 5 days after 48 hours of adaptation for singly housed mice.

**Glucose and insulin tolerance tests.** Glucose tolerance test was performed after 4 h of fasting. Glucose (2 g/kg of body weight) was injected i.p. and glucose concentration in whole venous blood was measured using an automatic glucometer (One Touch, LifeScan, CA) over 1.5 h. For insulin tolerance test, fasted mice were injected with insulin (0.75 units/kg of body weight, Sigma, St. Louis, MO), and blood glucose levels were measured over 1.5 h.

**Induction of obesity by high fat diet.** To induce obesity, 3 month old mice were maintained on an unlimited supply of high fat diet [Protein 20 kcal%, carbohydrates 35 kcal%, fat 45 kcal%; Research Diets, New Brunswick, NJ].

**Bone marrow chimera.** Recipient mice (2 months of age) were subjected to lethal whole-body irradiation (950 rad). On the following day, mice were reconstituted with TLR2-expressing 5 × 10⁹ BM cells (WT) isolated according to a previously described protocol\(^1\). For identification of donor derived cells, BM was isolated from C57B1/6 J mice, as termed in this article as Poms\(^1\).

**In-vitro studies of mouse hypothalamic neurons.** The N-42 hypothalamic cell-line (Cellulation Biosystems Inc.) was cultured in DMEM (Invitrogen Life Technologies), containing 10% FBS supplemented with 100 μg/ml of penicillin/streptomycin (Invitrogen Life Technologies). For activation assay, N-42 cells were plated in 24 well plates at 50% confluency. Cells were starved overnight in serum-free medium. Cells were cultured in the presence of synthetic Pam3CysSK4 (P3C; either 10 or 100 μg/ml EMIC Microcollections, Tuebingen, Germany) and peptidoglycan (PG 80–100 μg/ml, Fluka, Manila). The resulting antibody and cytokine release was quantified using a luminescence reader (ATCC). A Nikon fluorescence microscope (Nikon E800), equipped with a Nikon digital camera (DXM 1200F) and with either a 20 × (NA = 0.30) or 40 × (NA = 0.75) Plan Fluor objective lens (Nikon) was used for all fluorescence microscopy. The immunostaining intensity was determined automatically with Image-Pro Plus 4.5 software (Media Cybernetics). All measurements were performed by an observer blinded as to the identity of the examined tissues.

**RNA processing.** RNA was extracted using 5 PRIME PerfectPure RNA Cultured Cell kit (5 Prime GmbH). RNA preparations were reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.). The cDNA products were used as templates for Real-Time PCR analysis. Sense and antisense primers were selected from different exons to avoid false-positive results caused by DNA contamination.

**Real-time PCR reactions were carried out on a 7500 Real-Time PCR system using fluorescent SYBR Green technology (Applied Biosystems Inc.).** Reaction protocols were as follows: 10 min at 95°C for enzyme activation, followed by 45 cycles of 15 s at 94°C and 60 s at 60°C. The specificity of the amplification products was confirmed by melting curve analysis. All reactions contained the same amount of cDNA, 10 μl. Master Mix and 250 nM primers to a final volume of 20 μl.

**Statistical analysis.** The results were analyzed by Student’s t-test for comparison between two groups. Factorial ANOVA or repeated ANOVA, followed by Fisher’s exact test or Bonferroni post-tests, were used for comparison of multiple groups. Data are expressed as means ± S.E.M.

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Author contributions

R.S. and A.L. designed the experiments, analyzed the data, wrote the manuscript and created the figures. Y.K. planned, performed, and analyzed the in-vivo metabolic studies and the N42 in vitro studies. A.R. performed the in-vivo monitoring as well as the immunostaining studies, and assisted in the writing, data analysis, and figure creation. M.S. and A.C. supervised, guided and coordinated the project, the ideas, the experimental studies, and the manuscript writing. All the authors read and edited the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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