Original Article

MOLECULAR METABOLISM

Hypothalamic TTF-1 orchestrates the sensitivity of leptin

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ABSTRACT

Objective: Thyroid transcription factor-1 (TTF-1), a homeodomain-containing transcription factor, is predominantly expressed in discrete areas of the hypothalamus, which acts as the central unit for the regulation of whole-body energy homeostasis. Current study designed to identify the roles of TTF-1 on the responsiveness of the hypothalamic circuit activity to circulating leptin and the development of obesity linked to the insensitivity of leptin.

Methods: We generated conditional knock-out mice by crossing TTF-1$^{flox/flox}$ mice with leptin receptor (ObRb)^{Cre} or proopiomelanocortin (*POMC*)^{Cre} transgenic mice to interrogate the contributions of TTF-1 in leptin signaling and activity. Changes of food intake, body weight and energy expenditure were evaluated in standard or high fat diet-treated transgenic mice by using an indirect calorimetry instrument. Molecular mechanism was elucidated with immunohistochemistry, immunoblotting, quantitative PCR, and promoter assays.

Results: The selective deletion of *TTF-1* gene expression in cells expressing the ObRb or POMC enhanced the anorexigenic effects of leptin as well as the leptin-induced phosphorylation of STAT3. We further determined that TTF-1 inhibited the transcriptional activity of the ObRb gene. In line with these findings, the selective deletion of the *TTF-1* gene in ObRb-positive cells led to protective effects against diet-induced obesity via the amelioration of leptin resistance.

Conclusions: Collectively, these results suggest that hypothalamic TTF-1 participates in the development of obesity as a molecular component involved in the regulation of cellular leptin signaling and activity. Thus, TTF-1 may represent a therapeutic target for the treatment, prevention, and control of obesity.

Keywords Thyroid transcription factor-1; Leptin receptor; Leptin sensitivity; Leptin resistance; Obesity

1. INTRODUCTION

Obesity is a predisposing condition for several serious metabolic syndromes, including type 2 diabetes and various cardiovascular diseases, and is highly associated with complex abnormalities originating from disturbance of the delicate balance between caloric excess and body weight homeostasis [1]. The hypothalamus is the central unit that orchestrates homeostatic controls for feeding, physical activity, and energy expenditure by mediating the afferent inputs derived from metabolically active peripheral organs such as the adipose tissues, pancreas, and intestines [2]. In particular, leptin, an adipocyte-derived hormone, triggers catabolic responses by targeting hypothalamic neurons expressing leptin receptors [3]. Since the circulating level of leptin is proportional to the level of adiposity, leptin directly participates in the operation of a negative feedback loop to maintain energy balance in the hypothalamic neurons that control energy intake and expenditure [4]. Leptin action in the hypothalamic neurons mainly includes the regulation of two opposing neuronal populations: the inhibition of orexigenic agouti-related peptide (AgRP) neurons and the stimulation of anorexigenic proopiomelanocortin (POMC) neurons [5].

Over the last two decades, intensive studies have attempted to determine the usefulness of leptin in treating obesity. However, thus far, the application of leptin has not been effective in improving obesity. A major pathological event in the unsuccessful therapeutic usefulness of leptin is a perturbed responsiveness of the target cells to leptin,

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known as leptin resistance. A great deal of attention has been paid to unmasking the cellular and molecular causes in the development of leptin resistance. Nonetheless, strategies that have tried to mitigate leptin insensitivity have so far been unsuccessful.

Thyroid transcription factor-1 (TTF-1) is a transcription factor first found in the thyroid gland [6] and is also known as homeodomain-containing transcription factor Nkx2.1. TTF-1 plays a critical role in the development of the diencephalic brain regions [7]. Mice carrying the null mutation of the TTF-1 gene are stillborn due to lung failure, along with severe malformation or absence of some hypothalamic structures such as the ventral third ventricle and periventricular nuclei [8,9]. In line with this notion, we have found the presence of TTF-1 expression in several discrete hypothalamic nuclei, where it is involved in neural or neuroendocrine physiological mechanisms important in the control of body homeostasis [7,10,11]. Intriguingly, a previous study has reported that the brain-specific homeobox transcription factor (Bsx) was functionally linked to the whole-body energy metabolism [12]. In addition, our previous studies identified physiological roles of TTF-1 in the regulation of energy homeostasis [13,14]. Notably, TTF-1 was strongly expressed in the arcuate nucleus (ARC), where it stimulated the transcription of AgRP but inhibited the transcription of POMC [14]. The resulting intensive appetite-driving effect of TTF-1 opposes the well-known function of leptin. These previous results implicate the potential involvement of TTF-1 in leptin signaling pathways for the regulation of body energy homeostasis in the hypothalamic ARC.

In the present study, using transgenic mice harboring selective TTF-1 deletion in the cells expressing the long-form leptin receptor ObRb or in the POMC cells, we aim to provide experimental evidence suggesting that TTF-1 is a crucial molecular component involved in the leptin signaling pathway for the regulation of whole-body energy homeostasis and is involved in the responsiveness and resistance to leptin in obesity pathogenesis.

2. MATERIALS AND METHODS

2.1. Animals

All animals were housed individually in a temperature-controlled room with a 12-hour light—dark cycle and had ad libitum access to a standard chow diet (Feedlab, Gyeonggi-Do, Korea) and water unless otherwise stated. Transgenic mice (*POMC-EGFP* [stock no. 009593], *ObRb-Cre* [stock no. 008320], *POMC-Cre* [stock no. 005965] and *Ai14-Cre* [stock no. 007914]) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). *TTF-1*$^{f/f}$ mice [15] were crossbred with *ObRb-Cre* or *POMC-Cre* mice to generate TTF-1 conditional knockout mice specifically in the cells expressing ObRb or POMC. The resulting mouse lines that bore the TTF-1 deletion in the POMC cells (*POMC-TTF-1*$^{-/-}$) and in the cells expressing ObRb (*ObRb-TTF-1*$^{-/-}$) were first subjected to IHC observations to identify the specific deletion of TTF-1 in each cell (Supplementary Figures 2 and 3). All animals and procedures used were in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee at the University of Ulsan (permission numbers: BJL-14-050, BJL-14-052 and BJL-17-030).

2.2. Leptin administration and food intake assay

Animals were intraperitoneally injected with saline or recombinant mouse leptin (0.5, 1 or 3 mg/kg; Q544U0, R&D Systems, Minneapolis, MN, USA) after overnight fasting. Food intake of the individually caged animals was monitored for 2—24 h after injection. Their body weights were measured 24 h after administration of leptin.

2.3. Immunohistochemistry

Mice were first anesthetized with isoflurane, followed by transcardiac perfusion with 0.1 M phosphate buffer (PB, pH 7.4) and 3% paraformaldehyde in PB. Brains were dissected and incubated in 3% paraformaldehyde overnight at 4 °C. Coronal sections (thickness, 50 μm) were prepared using a vibratome (5100mz, Campden Instruments Ltd., Leicestershire, UK). Coronal sections containing the hypothalamic arcuate nucleus were preincubated with 0.2% Triton X-100 (T8787, Sigma-Aldrich, St. Louis, MO, USA) in PB for 30 min at room temperature (RT). After further washing with PB, the sections were incubated overnight at 4 °C with rabbit anti-TTF-1 (1:1000, SC-13040, Santa Cruz Biotechnology, Dallas, Texas, USA), sheep anti-α-MSH (1:10,000, AB5087, Millipore, Darmstadt, Germany), rabbit anti-POMC (1:1000, H-029-30, Phoenix Pharmaceuticals, Burlingame, CA, USA) and rabbit anti-c-Fos (1:1000, SC-52, Santa Cruz Biotechnology). For pSTAT3 staining, sections were incubated with 0.01 mol/L citrate buffer for 10 min at 80 °C and washed in PB at RT. The sections were then incubated with 2 N HCl for 30 min and incubated with 0.2% Triton X-100 in PB for 30 min at RT. Afterward, sections were incubated with rabbit anti-pSTAT3 (1:1000, #9148, Cell Signaling Technology, Danvers, MA, USA) overnight at RT. The next day, the sections were washed in PB for 30 min and incubated with the following secondary antibodies at RT: goat anti-rabbit Alexa Fluor 488 (1:500, A11008, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit Alexa Fluor 594 (1:500, A11012, Invitrogen), and donkey anti-sheep Alexa Fluor 594 (1:500, A21209, Invitrogen). For diaminobenzidine (DAB)-based IHC analysis to detect pSTAT3, the sections were washed thoroughly and incubated with biotinylated anti-rabbit secondary antibodies, Avidin—Biotin Complex (ABC) reagent (PK-6102, Vector Laboratories, Burlingame, CA, USA), and the DAB substrate (SK-4100, Vector Laboratories). The sections were then mounted onto glass slides and coverslips were then placed on these slides with a drop of mounting medium (Dako North America Inc., Carpinteria, CA, USA). The coverslips were sealed with nail polish to prevent the drying and movement of the samples under the microscope.

2.4. Microscopy and quantification

The stained brain sections were imaged using an Olympus FV-1200 laser scanning confocal microscope (Shinjuku, Tokyo, Japan) and a fluorescence microscope (Axioplan2 Imaging, Carl Zeiss Microimaging Inc., Thornwood, NY, USA). All parameters were kept constant for quantification purposes. Measurements of fluorescence-positive cell counts and density were performed using ImageJ software v. 1.47 (National Institute of Health, Bethesda, MD, USA).

2.5. Electrophysiology

Whole-cell patch-clamp recordings were conducted in the POMC-EGFP neurons of hypothalamic slices. The brains were removed and immediately submerged in ice-cold, carbogen-saturated (95% O_2, 5% CO_2) sucrose solution containing 75 mM sucrose, 2.5 mM KCl, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 25 mM glucose at pH 7.3. Hypothalamic coronal slices (300 μm) were made with a Leica VT1200 (Leica Biosystems, Wetzlar, Germany) vibratome in sucrose solution. The slices were then placed in a holding chamber filled with gassed artificial cerebrospinal fluid (ACSF) (95% O_2, 5% CO_2) that contained 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 25 mM glucose at pH 7.3. They were then incubated at RT for at least 1 h before recording. Slices were transferred to a recording chamber and bathed with continuously bubbled ACSF (30—32 °C) at a flow rate of ~3 ml/min. Whole-cell patch-clamp recordings were performed using pipettes

with 4–6 MΩ resistance after they were filled with internal solution. The recording pipettes were made of borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a PC-10 vertical puller (Narishige, Tokyo, Japan). The internal solution contained 135 mM potassium gluconate, 0.5 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM Na_2-GTP, and 10 mM phosphocreatine. POMC neurons in the arcuate nucleus were identified under fluorescence, and then, differential interference contrast was used to obtain a seal in these cells. After a gigaohm seal was obtained, a gentle negative pressure was applied to break through to the whole-cell configuration. Leptin (10 nM) was added to ACSF and perfused for 3–5 min into the recording chamber. All recordings were performed with a Multiclamp 700 B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were collected with pCLAMP software (Molecular Devices). Mean firing activity and membrane potential were calculated using Clampfit (Axon Instruments Inc., Union City, CA, USA) software.

2.6. Measurements of O_2 consumption, CO_2 production, and respiratory exchange ratio

Metabolic parameters of O_2 consumption (VO_2), CO_2 production (VCO_2), and respiratory exchange ratio (RER) were analyzed with a Promethion Metabolic Measurement System (Sable System, Las Vegas, NV, USA) using control *TTF-1*$^{f/f}$ and *ObRb-TTF-1*$^{-/-}$ mice. The mice were fed a high-fat diet containing 60% calories from lard and 20% calories from sugar (D12492; Research Diets, New Brunswick, NJ, USA) for 16 weeks. Food and water were provided ad libitum. Mice were acclimated to the metabolic cages for 48 h prior to data collection under 12 h light/dark cycle conditions. VO_2 and VCO_2 were measured in each mouse at 10 min intervals. The RER was calculated as the ratio of VCO_2 to VO_2. Data acquisition and instrument control were coordinated by the MetaScreen software (version 2.3.12, Sable Systems), and the obtained raw data were processed using Expe-Data (version 1.9.14, Sable Systems).

2.7. Transfection and western blotting

Mouse hypothalamic mHypoA cells purchased from CELLutions Biosystems (CELLutions Biosystems Inc., Toronto, Ontario, Canada) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Hyclone). The cells were maintained at 37 °C under 5% CO2 conditions. The mHypoA cells were seeded at 3 × 105 cells/well in 6-well plates and attached overnight. After starvation for 1 h, cells were transfected with a pLKO.1-Puro vector (Sigma-Aldrich) carrying shRNA that targets the 30th to 50th nucleotides (5′-GTT CTC AGT GTC TGA CAT CTT-3′) in the TTF-1 mRNA sequence [14] using Lipofectamine/PLUS (Invitrogen) for 4 h and were incubated in DMEM + 10% FBS for 24 h. Cells were then treated with leptin (500 ng/ml) and incubated for 30 min. Next, the cells were collected for total protein extraction. Extracted proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were probed with primary antibodies against pSTAT3 (1:1000, #9145, Cell Signaling) and β-actin (1:1000, A5441, Sigma-Aldrich).

2.8. Transfection and quantitative real-time PCR

mHypoA-POMC cells (CELLutions) had grown to 70% confluence and were transiently transfected for 24 h with expression vectors (pcDNA3.1 containing the TTF-1 coding region or control pcDNA3.1) using jetPRIME® reagent (Polyplus, New York, NY, USA). The cells were then serum-starved (6 h) and treated with leptin (100 ng/ml) or vehicle (Tris–HCl). Mediobasal hypothalamic samples were collected from mice, flash-frozen in liquid nitrogen, and stored at −80 °C until analysis. Total RNA was extracted from the hypothalami and cultured mHypoA-POMC cells using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized with 2 mg total RNA using a high-capacity cDNA reverse transcription kit (Intron Biotechnology, Seoul, Korea). Quantitative real-time PCR was performed using the SYBR Green Real-time PCR Master Mix Kit (TaKaRa Bio Inc., Foster, CA, USA), and mRNA expression levels were measured using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were normalized to the housekeeping gene *β-actin*. The primers used were as follows: *AgRP* forward: 5′-TGC AGA CCG AGC AGA AG-3′, reverse: 5′-ACT CGT GCA GCC TTA CAC AG-3′; *POMC* forward: 5′-TCC TAC TCC ATG GAG CAC TTC C-3′, reverse: 5′-ACT CGT TCT CAG CAA CGT TG-3′; *TTF-1* forward: 5′-GAT CTT GCT AGC GCC ATA GG-3′, reverse: 5′-AGC TGC AGG TGT TTG GAT CT-3′; *SOCS3* forward: 5′-GCT CCA AAA GCG AGT ACC AG-3′, reverse: 5′-TGA CGC TCA ACG TGA AGA AG-3′; *PTP1*B forward: 5′-GAC TCG TCA GTG CAG GAT CA-3′, reverse: 5′-CTG TCT TTC ATC CCC ACA GGT-3′; *ObRb* forward: 5′-GCA TGC AGA ATC AGT GAT ATT TGG-3′, reverse: 5′-CAA GCT GTA TCG ACA CTG ATT TCT TC-3′; *β-actin* forward: 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′, reverse: 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′. The *ObRb* primer was specifically designed to detect long-form leptin receptor [16].

2.9. Promoter assay

To determine whether TTF-1 regulates ObR transcription, mHypoA-POMC cells were co-transfected with an ObR promoter-luciferase reporter vector (500–1000 ng) and a TTF-1 expression vector (100–500 ng) using jetPRIME® (Polyplus). The 5′ flanking region of the mouse ObR gene (−1715 to +40 bp) that we used for this promoter analysis was cloned by PCR from mouse genomic DNA based on sequence information [17]. The 1755-nucleotide-long PCR product was inserted into a luciferase reporter plasmid (pGL4-promoter vector, Promega, Madison, WI, USA), and its sequence was confirmed by DNA sequencing. This 5′ flanking sequence of the mouse ObR gene includes 9 conserved core domains (CAAG) for the binding of TTF-1 [14]. Transfection efficiency was normalized by co-transfecting a Renilla reporter plasmid (pRL-SV40 vector; Promega) at 100 ng/well. Transfected cells were harvested 24 h after transfection, and luciferase activity was measured using a luciferase reporter assay system (Promega) according to the manufacturer's protocols.

2.10. ChIP assay

mHypoA-POMC cells were transiently transfected using jetPRIME® reagent (Polyplus) with expression vector pcDNA3.1 containing the TTF-1 coding region or control pcDNA3.1. To determine the effect of leptin on TTF-1 binding to the ObR 5′ flanking region the cells were treated with leptin (100 ng/ml) for 24 h after serum starvation for 6 h. For cross-linking of the DNA and protein binding, cells were treated with formaldehyde to a final concentration of 1% and the cross-link reaction was stopped by adding glycine to a final concentration of 125 mM. The cells were lysed with cell lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP40, and protease inhibitors) and nuclei were extracted and resuspended with nuclear lysis buffer (50 mM Tris, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication; then, the mixture was diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, 134 mM NaCl and protease inhibitors). The reactions were incubated with 5 μg of rabbit anti-TTF-1 antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology) at 4 °C

for two days. Immune complexes were collected by reaction with 60 μl of protein G agarose Resin 4 B (Lugen Sci Inc., Gyeonggi-do, Korea) and then washed with washing buffer containing different concentrations of salts (150 mM—500 mM) and 0.25 M LiCl. DNA from the protein—DNA cross-links was extracted by incubating the reactions with extraction solution (1% SDS and 0.1 M NaHCO3), and formaldehyde cross-links were reversed by adding NaCl to a final concentration of 0.3 M and incubation at 65°C for 5 h. The samples were precipitated by treatment with 100% ethanol and incubated overnight at −20°C. DNA was purified using spin columns (Geneall Biotechnology, Seoul, Korea) and was eluted with 10 mM Tris. PCR amplification was performed using 35 cycles of 94 °C for 30 s, 57—63 °C for 30 s, and 72 °C for 30 s, preceded by 94 °C for 30 s and followed by 72 °C for 10 min using the following primer sets: TTF-1 at −1567, forward, 5′-CCA GCC ATT TTT CTC TGC C-3′; TTF-1 at −1567, reverse, 5′-GAT CCA TCT CCC CCA TAC C-3′; TTF-1 at −1357, forward, 5′-CCT AGA ACC AGT GAA AAG CTG G-3′; TTF-1 at −1357, reverse, 5′-GGC CAA TGG ACA TCA AGG AC-3′; TTF-1 at −1238, forward, 5′-GTC CTT GAT GTC CAT TGG C-3′; TTF-1 at −1238, reverse, 5′-CGT GGT TGG TGT CTT TCC-3′; TTF-1 at −1181/-1201, forward, 5′-GGA AAG ACA CCA ACC ACG-3′; TTF-1 at −1181/-1201, reverse, 5′-ACC TGC TAG TGG ATG TGT AC-3′; TTF-1 at −807, forward, 5′-CCT CAA CAG ATT GGC CTG-3′; TTF-1 at −807, reverse, 5′-GCA GGA ACC TTA CCA GC-3′; TTF-1 at −728, forward, 5′-GTG CTG TAA GAG AGC TGG-3′; TTF-1 at −728, reverse, 5′-GGG ACC AAA GGG TTT ATC TC-3′; TTF-1 at −669, forward, 5′-GAT GAT GGA TCA ATC ATT GTG TGG-3′; TTF-1 at −669, reverse, 5′-GTC TGT GTG TGT GTG TGT G-3′.

2.11. Statistical analysis

The number of mice in each experimental group is indicated in the figure legends. All values are expressed as the mean ± standard error of the mean (SEM), and a two-tailed Student's *t*-test was used for pairwise comparisons. One-way and two-way analysis of variance (ANOVA) tests, followed by the Bonferroni post hoc test, were used to compare the data from more than two groups. Statistical evaluation was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Hypothalamic TTF-1 responds to leptin

Leptin, an adipose-derived hormone, regulates whole-body energy homeostasis via governing of the hypothalamic neurons expressing *ObRb* to reduce appetite and enhance energy expenditure [18—20]. To investigate whether TTF-1 is involved in leptin action on energy metabolism, we determined the presence of TTF-1 in ObRb-positive cells by performing immunohistochemistry (IHC) with an antibody against the TTF-1 protein in brain sections from a transgenic mouse line retaining tomato reporter signals in ObRb-positive cells (*ObRb* cre:Ai14-tdTomato). Double positive signals of TTF-1 and ObRb were found in the ARC, dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic area (LH), but not in the ventromedial hypothalamic nucleus (VMH) (Figure 1A). To verify whether hypothalamic TTF-1 responds to leptin, we next tested the number of TTF-1-positive cells in the hypothalamus of mice systemically administrated with leptin by performing IHC with an antibody against TTF-1 protein. Administration

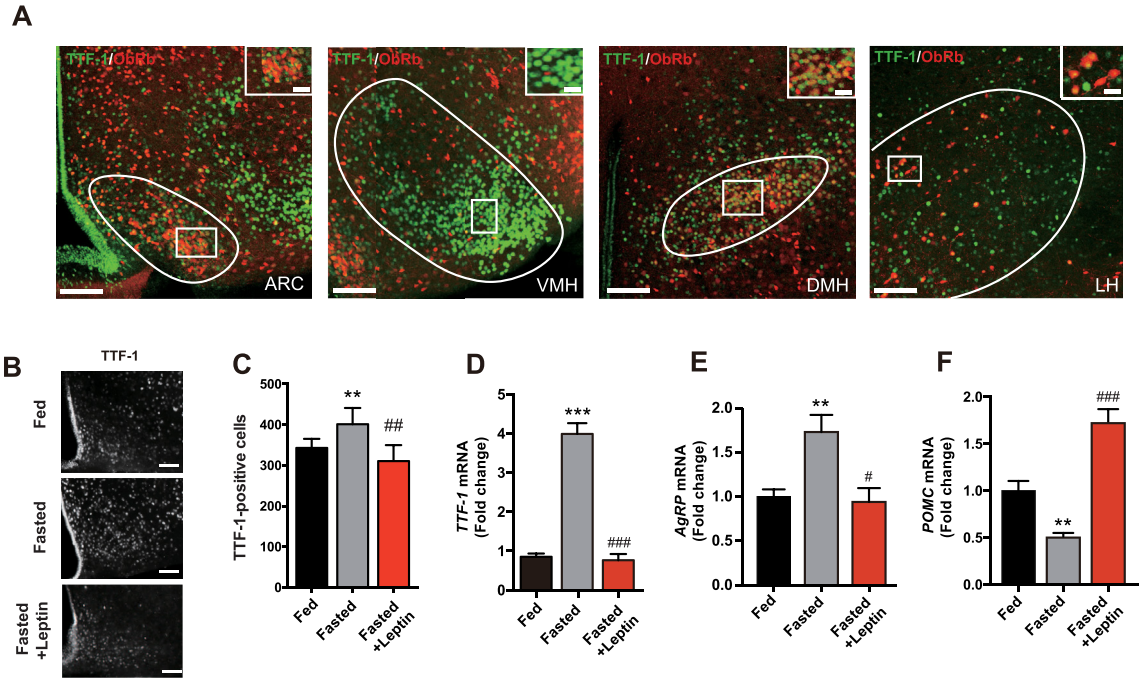

Figure 1: Leptin regulates the hypothalamic expression of TTF-1. (A) Representative images show TTF-1 protein (green) in the ObRb-positive cells (red) in hypothalamic regions including the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic nucleus (LH), but not in the ventromedial hypothalamic nucleus (VMH) of transgenic mice expressing td-Tomato signals selectively in the ObRb-positive cells. The insets show the signals at higher magnifications. (B, C) Representative images of (B) immunohistochemistry and (C) calculated data revealed that TTF-1 synthesis was increased after overnight fasting, and this increase was reversed by the i.p. injection of leptin (3 mg/kg), observed at 90 min after the injection (fed, n = 8 sections/4 mice; fasted, n = 7 sections/4 mice; fasted + leptin, n = 8 sections/4 mice). (D—F) Quantitative real-time PCR results revealed that systemic administration of leptin (3 mg/kg) suppressed the overnight fasting-induced increases in the (D) *TTF-1* and (E) *AgRP* mRNA levels and decrease in the (F) *POMC* mRNA level in the mediobasal hypothalamic area (n = 6/group). Data are presented as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. fed group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. fasted group. Scale bar = 50 μm (20 μm for higher magnification view in the insets). *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-test.

of leptin effectively suppressed the elevation of TTF-1 immunosignals induced by overnight food deprivation in the ARC (Figure 1B and C). Similarly, elevated TTF-1 immunosignals in response to fasting were almost completely rescued by leptin treatment in the DMH. However, no alteration of TTF-1 immunosignals was observed in the paraventricular nucleus (PVN) in response to the fasting and leptin treatment (Supplementary Figure 1). Additionally, we found that leptin suppressed the overnight fasting-induced increase of *TTF*-1 mRNA levels in the mediobasal hypothalamus (Figure 1D). The effectiveness of systemic administration of leptin was confirmed by changes in hypothalamic *AgRP* and *POMC* mRNA levels (Figure 1E and F). These findings suggest that TTF-1 is negatively controlled by leptin in energy homeostasis.

3.2. Deletion of *TTF-1* in ObRb-positive cells leads to enhanced sensitivity to leptin with regard to feeding behavior

Hypothalamic TTF-1 expression was altered according to the whole-body energy state and was under the control of circulating leptin. Thus, we next observed the participation of TTF-1 in regulating leptin control of energy homeostasis using a mouse model (*ObRb-TTF-1*$^{-/-}$) bearing a selective deficiency of *TTF-1* expression in ObRb-positive cells. The model was generated by crossing a *TTF-1* floxed mouse with a mouse expressing Cre recombinase in ObRb-positive cells. Specific knock-down of *TTF-1* expression was verified by IHC in the ARCs of *ObRb-TTF-1*$^{-/-}$ mice (Supplementary Figure 2). The body weights of control and *ObRb-TTF-1*$^{-/-}$ mice were similar before leptin treatment (29.38 g $\pm$ 0.75 for control mice versus 28.53 g $\pm$ 0.67 for *ObRb-TTF-1*$^{-/-}$ mice). Reduced food intake and weight loss induced by the systemic administration of leptin (1 mg/kg) were significantly enhanced in the *ObRb-TTF-1*$^{-/-}$ mice compared to the control mice (Figure 2A and B). Intriguingly, *ObRb-TTF-1*$^{-/-}$ mice displayed significantly decreased food intake in response to a low concentration of leptin (0.5 mg/kg) that normally did not induce a reduction of food intake in the control group (Figure 2C). To confirm the enhanced responsiveness to leptin seen in the *ObRb-TTF-1*$^{-/-}$ mice, we determined the food intake of individual mice in both groups of experimental animals before and after systemic administration of leptin (3 mg/kg). The leptin-induced reduction of food intake seen in each individual mouse was much greater in the *ObRb-TTF-1*$^{-/-}$ mice than in the control mice (Figure 2D). Similarly, significantly elevated immunosignals of c-Fos, a molecular marker for neuronal activity, induced by leptin treatment (3 mg/kg) was observed in the ARCs of *ObRb-TTF-1*$^{-/-}$ mice compared to the control mice (Figure 2E and F). In addition, leptin-induced c-Fos activation was sustained until 6 h after treatment in the *ObRb-TTF-1*$^{-/-}$ mice, while it disappeared after 3 h in the control mice (Figure 2F).

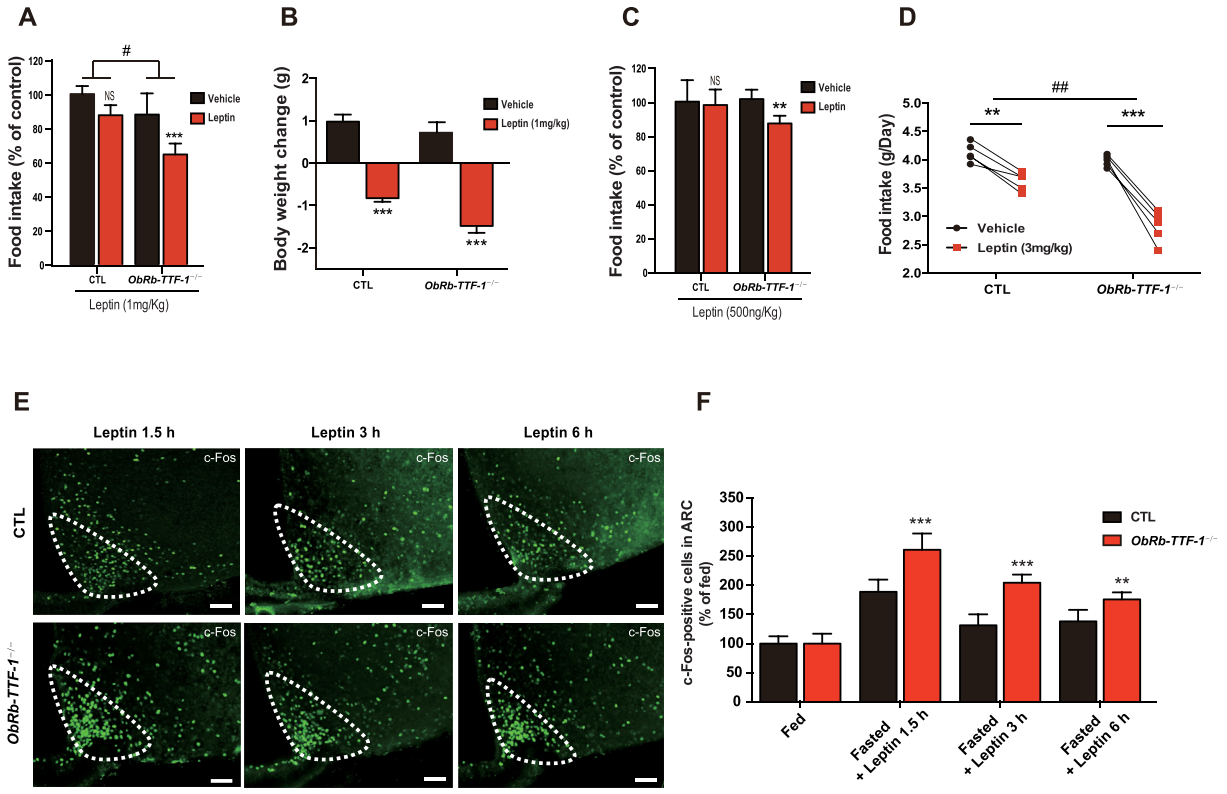

Figure 2: Selective deletion of TTF-1 in ObRb-positive cells enhances sensitivity to leptin. (A) Reduced cumulative food intake for 24 h was greater in *ObRb-TTF-1*$^{-/-}$ mice than in the control mice after the i.p. injection of leptin (1 mg/kg) (n = 5/group). (B) Weight loss for 24 h in response to i.p. leptin injection (1 mg/kg) was significantly greater in *ObRb-TTF-1*$^{-/-}$ mice than in control mice (n = 5/group). (C) I.p. injection with a low dose of leptin (0.5 mg/kg) was associated with a decrease in food intake for 24 h in the *ObRb-TTF-1*$^{-/-}$ mice but not in the control mice (CTL, n = 5; *ObRb-TTF-1*$^{-/-}$, n = 7). (D) Anorexigenic effects of leptin were significantly more prominent in the individual *ObRb-TTF-1*$^{-/-}$ mouse than in the control mouse, based on the measurement of food intake for 24 h after the i.p. administration of leptin (3 mg/kg; CTL, n = 4; *ObRb-TTF-1*$^{-/-}$, n = 5). (E, F) Leptin-induced activation of neurons was determined by assessing the change of c-Fos immunoreactivity in the ARCs of mice that were treated with i.p. leptin (1 mg/kg) 90 min before sacrifice. (E) Representative photographs and (F) calculated graphs show an elevation of leptin-induced c-Fos activity in the ARCs of *ObRb-TTF-1*$^{-/-}$ mice compared to that observed in case of the control mice (n = 4–9 sections/3–5 mice/group). Data are presented as the mean $\pm$ SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle-treated control group (A–D) or control wild mice (F). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. change in food intake by control wild mice. NS, not significant. *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.

Thus, the selective blockade of *TTF-1* expression in ObRb-positive cells may result in the increased responsiveness of these neurons to leptin.

3.3. TTF-1 regulates responsiveness to leptin in POMC neurons

Since TTF-1 is a negative regulator for the expression of POMC [14], we further analyzed the contribution of TTF-1 to the leptin responsiveness of POMC neurons. We generated a mouse model that bears the POMC neuron-specific deletion of *TTF-1* (*POMC-TTF-1*$^{-/-}$) and analyzed TTF-1's function in mediating leptin's action on POMC neurons. The selective knock-down of *TTF-1* expression in *POMC-TTF-1*$^{-/-}$ mice was verified in POMC cells expressing green fluorescent protein (GFP) (Supplementary Figure 3). The body weights of control and *POMC-TTF-1*$^{-/-}$ mice were similar before leptin treatment (29.03 g ± 0.52 for control mice versus 28.86 g ± 0.76 for *POMC-TTF-1*$^{-/-}$ mice). Although systemic administration of leptin at a low concentration (1 mg/kg) did not induce a significant decrease in food intake in the control mice, the *POMC-TTF-1*$^{-/-}$ mice responded to this low dose of leptin treatment with significantly reduced food intake (Figure 3A). In addition, this elevated leptin sensitivity seen in the *POMC-TTF-1*$^{-/-}$ mice was observed until 24 h after the leptin injection, indicating a prolonged responsiveness to leptin. Accordingly, this low dose of leptin induced a much greater decrease in body weight in

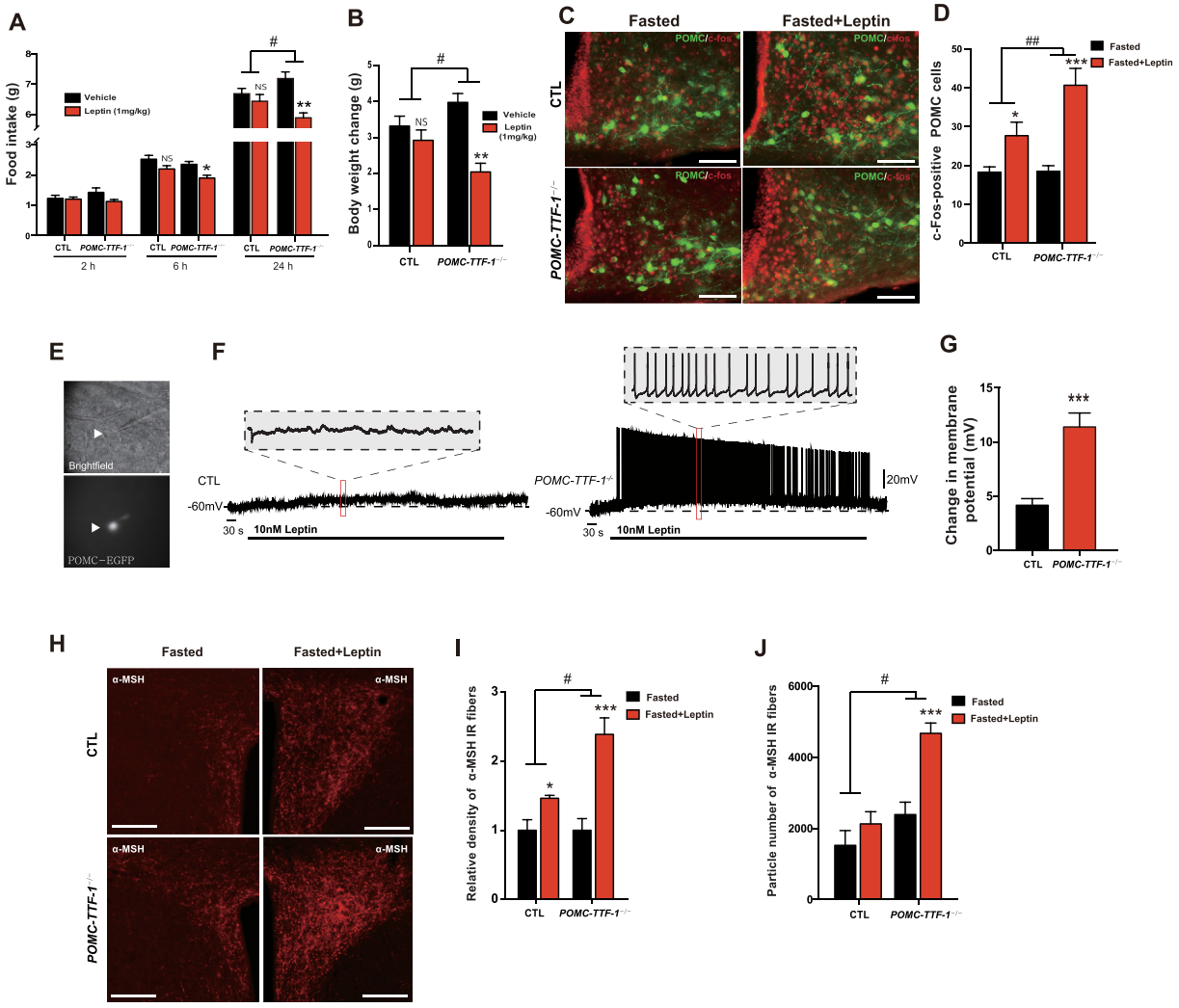

Figure 3: POMC neuron-specific deletion of TTF-1 leads to increased sensitivity to leptin. (A, B) Leptin (i.p., 1 mg/kg) induced a significant reduction of (A) food intake for 6 h and 24 h and (B) body weight for 24 h in the *POMC-TTF-1*$^{-/-}$ mice, but not in the control mice (n = 4–5/group). (C, D) Leptin-induced activation of POMC neurons was determined by measuring the change in c-Fos immunoreactivity (red) in the POMC-GFP cells (green) of overnight-fasted control and *POMC-TTF-1*$^{-/-}$ mice that were treated with i.p. leptin (1 mg/kg) 90 min before sacrifice. (C) Representative photographs and (D) calculated data show a greater increase in leptin-induced c-Fos activation in the POMC cells of *POMC-TTF-1*$^{-/-}$ mice than in those of the control mice (n = 4 sections/4 mice/group). (E–G) To identify changes in the membrane potential of the POMC neurons of *POMC-TTF-1*$^{-/-}$ mice, the electrical property was measured by whole cell patch-clamp recoding. (E) Bright-field and fluorescent illumination of POMC-GFP neurons attached to a whole-cell recording pipette (the arrowhead indicates the target cell). (F) Treatment with leptin (10 nM) produced high-frequency spikes in the POMC neurons of the *POMC-TTF-1*$^{-/-}$ mice, but not in those of the control (*POMC-TTF-1*$^{+/+}$) mice. (G) Leptin depolarized the POMC neurons of the *POMC-TTF-1*$^{-/-}$ mice to a greater extent than that observed in case of the control mice (n = 7 cells/4 mice/group). (H–J) Changes in α-MSH innervation were determined in the PVN of *POMC-TTF-1*$^{-/-}$ mice. (H) Representative images show α-MSH immunosignals in the PVN. Leptin (1 mg/kg)-induced increases in (I) relative density and (J) particle numbers of α-MSH fiber signals were significantly higher in *POMC-TTF-1*$^{-/-}$ mice than those observed in case of the control mice (n = 4 sections/4 mice/group). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$ vs. vehicle-treated control group (A, B, G) or fasted group of each genotype (D, I, J). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. change in control mice. NS, not significant. Scale bar = 50 μm. *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.

the *POMC-TTF-1*$^{-/-}$ mice than in the control mice (Figure 3B). As stronger neuronal activity was induced by leptin in the ARCs of the *ObRb-TTF-1*$^{-/-}$ mice than in the control mice, we observed that there were more c-Fos-positive POMC cells following leptin administration (1 mg/kg) in *POMC-TTF-1*$^{-/-}$ mice compared with the control mice (Figure 3C and D). To verify whether the specific ablation of *TTF-1* in the POMC neurons caused enhanced activation of POMC neurons in response to leptin, the electrical activity of POMC^{GFP} neurons was measured by whole-cell recording (Figure 3E). The POMC neurons of the *POMC-TTF-1*$^{-/-}$ mice were strongly depolarized in response to a low dose of leptin (10 nM), whereas those of the control mice were not (Figure 3F). The resting membrane potential was more depolarized in the POMC neurons of *POMC-TTF-1*$^{-/-}$ mice than in control mice following leptin administration (Figure 3G). Since α-melanocyte stimulating hormone (α-MSH) is produced by a proteolytic cleavage process from a *POMC* gene product and targets the PVN, where it triggers satiety signals, we next evaluated the pattern of α-MSH innervation into the PVN after intraperitoneal (i.p.) injection of leptin (1 mg/kg). Leptin induced much greater increases in the density and number of α-MSH fibers into the PVN of *POMC-TTF-1*$^{-/-}$ mice than those observed in the control mice (Figure 3H–J). Thus, TTF-1 is a critical molecular orchestrator for leptin responsiveness in POMC neurons, thus affecting leptin-induced melanocortin inputs to the PVN neurons.

3.4. TTF-1 controls cellular sensitivity to leptin by regulation of phosphorylation of STAT3

Leptin receptor signaling in target cells induces a series of tyrosine phosphorylations: the binding of leptin induces phosphorylation of the receptor itself and of Janus kinase, which results in STAT3 phosphorylation. Thus, phosphorylated STAT3 (pSTAT3) can be used as a molecular indicator to evaluate the activity of the leptin signaling pathway [21]. We examined pSTAT3 in hypothalamic sections from control and *ObRb-TTF-1*$^{-/-}$ mice at 1.5 h, 3 h, and 6 h after systemic injection of leptin (1 mg/kg). Levels of pSTAT3 were below that of a detectable level in both control and *ObRb-TTF-1*$^{-/-}$ mice when they were fed normally or fasted overnight (Figure 4B). However, pSTAT3 signals were dramatically elevated by leptin treatment after overnight fasting (Figure 4A and B). Leptin-induced levels of pSTAT3 were significantly higher in the *ObRb-TTF-1*$^{-/-}$ mice than in the control mice at every observation time. We next found that increased pSTAT3 levels in response to leptin were also observed in the *POMC-TTF-1*$^{-/-}$ mice compared to the control mice (Figure 4C and D). Small hairpin RNA (shRNA)-mediated blockade of TTF-1 expression consistently enhanced leptin-induced pSTAT3 levels in the mouse hypothalamic mHypoA cell line (Figure 4E).

In parallel with these findings, we observed a significant increase in the expression of *ObRb*, a long-form leptin receptor, in the hypothalamus of *ObRb-TTF-1*$^{-/-}$ mice (Figure 4F), suggesting that the increased *ObRb* mRNA expression could be linked to the enhanced STAT3 phosphorylation and increased sensitivity to leptin in *ObRb-TTF-1*$^{-/-}$ mice. Interestingly, we observed a marked increase in the mRNA levels of molecular switches for the intracellular feedback loop of leptin signaling, such as *suppressor of cytokine signaling 3* (*SOCS3*) and *protein-tyrosine phosphatase 1B* (*PTP1B*), in *ObRb-TTF-1*$^{-/-}$ mice (Figure 4G and H). These paradoxical results likely reflect that a sustained increase in cellular leptin sensitivity may cause activation of the

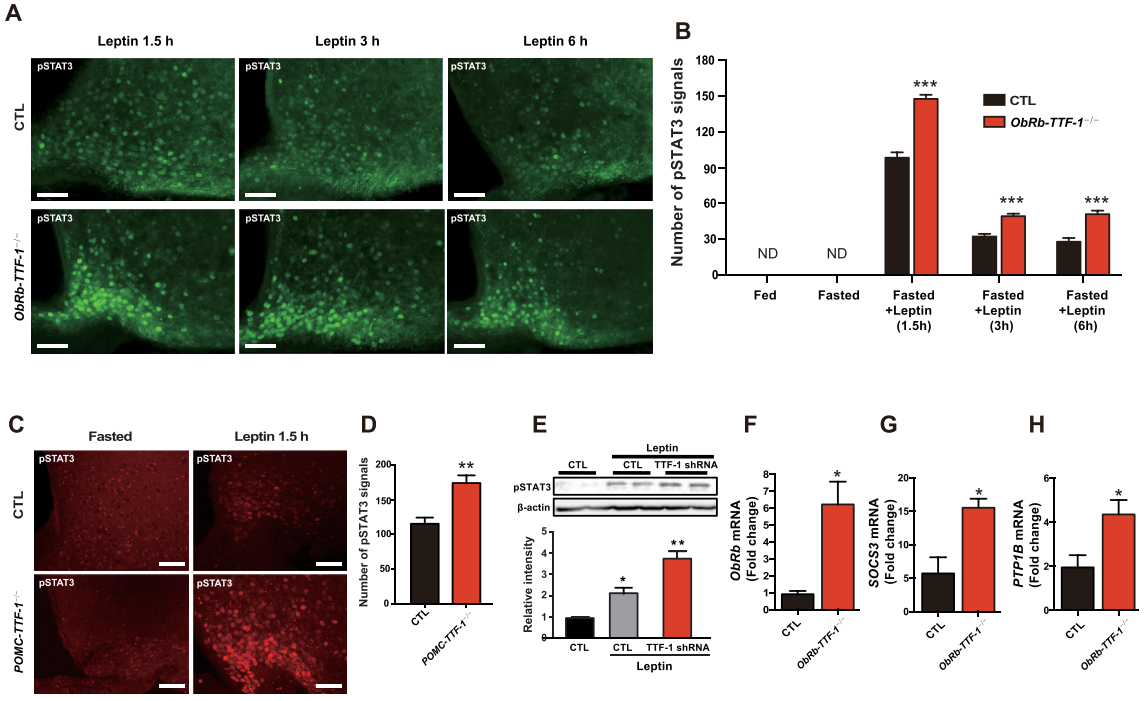

Figure 4: TTF-1 is involved in STAT3 phosphorylation and *ObRb* expression. (A, B) Leptin-induced phosphorylation of STAT3 was observed in the hypothalami of *ObRb-TTF-1*$^{-/-}$ mice fasted overnight. (A) Representative images and (B) calculated data show a greater increase in the intensity of pSTAT3 signals after leptin treatment in the ARCs of *ObRb-TTF-1*$^{-/-}$ mice than that observed in case of the control mice at the indicated time points (n = 10–14 sections/5–7 mice/group). (C, D) The hypothalamic ARCs of *POMC-TTF-1*$^{-/-}$ mice also showed a significantly higher level of pSTAT3 at 1.5 h after i.p. leptin (1 mg/kg) treatment than that observed in case of the control mice (n = 5 sections/5 mice/group). (E) Immunoblot analysis revealed that the shRNA-mediated blockade of TTF-1 synthesis resulted in an increase in the pSTAT3 level in the mHypoA cells in response to leptin (500 ng/ml per well, n = 4/group). (F–H) Quantitative real-time PCR data revealed that (F) *ObRb*, (G) *SOCS3*, and (H) *PTP1B* mRNA levels showed higher expression in the hypothalami of ObRb-TTF-1−/− mice than in those of the control mice. (n = 3/group). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. leptin-treated control mice (B, D, F–H) or vehicle-treated control cells (E). Scale bar = 50 μm. *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-test.

negative feedback loop of leptin receptor signaling. Thus, TTF-1 may affect cellular sensitivity to leptin, at least in part, by regulating *ObRb* expression and STAT3 phosphorylation.

3.5. TTF-1 controls the transcription of the ObRb gene

To better understand the enhanced leptin sensitivity seen in TTF-1-deficient mice, we investigated the transcriptional regulation of the ObRb gene by TTF-1 in an adult mouse hypothalamic cell line (mHypoA-POMC) originally generated and immortalized from the hypothalamic cells that express POMC and secrete α-MSH. We first confirmed the responsiveness of these cells to leptin with an observation of change in pSTAT3 levels after leptin treatment (Supplementary Figure 4). In support of findings showing enhanced leptin sensitivity in *ObRb-TTF-1*$^{-/-}$ and *POMC-TTF-1*$^{-/-}$ mice, overexpression of TTF-1 led to a decrease in *ObRb* mRNA levels, and moreover almost completely blunted the increased *ObRb* mRNA expression that was induced by leptin treatment in the mHypoA-POMC cells (Figure 5A and B). We next performed promoter assays using a luciferase construct containing the 5′-flanking region of the mouse ObR gene (−1755 to +40) in mHypoA-POMC cells. The ObR promoter was active over a wide range of concentrations, indicating that this promoter construct is transcriptionally active in mHypoA-POMC cells (Figure 5C). When the ObR promoter was co-transfected with a TTF-1 overexpression vector, the promoter activity of ObR was significantly reduced in a dose-dependent manner in mHypoA-POMC cells (Figure 5D). To identify the functionally active TTF-1 binding sites in the ObR promoter region, ChIP assays were performed using TTF-1 antibodies and PCR primer sets specific to the promoter regions recognized by TTF-1. Promoter sequences at −669, −728, −1181, −1201, −1238, −1357, and −1567 were detected by PCR and their band intensities were markedly decreased in DNA samples isolated from the leptin-treated cells (Figure 5E). No detectable band was observed in the control IgG precipitations and the PCR performed using the primer sets for the −807 and −1317 binding sites (Figure 5E). These results suggest a molecular mechanism that supports the inhibitory function of TTF-1 on the actions of leptin through regulation of ObRb expression.

3.6. Deletion of TTF-1 in ObRb-positive cells protects against diet-induced obesity by improving leptin resistance

To determine whether the enhanced leptin sensitivity of *ObRb-TTF-1*$^{-/-}$ mice affects leptin resistance and development of obesity, we fed mice a high-fat diet (HFD) for 16 weeks to generate a diet-induced obesity (DIO) model and analyzed their metabolic phenotypes. *ObRb-TTF-1*$^{-/-}$ mice gained significantly less body weight than the control mice during HFD feeding (Figure 6A and B). To confirm whether alteration of energy expenditure was responsible for the lower weight gain observed in *ObRb-TTF-1*$^{-/-}$ mice, we assessed carbon dioxide generation (VCO_2) and oxygen consumption (VO_2), determined by indirect calorimetry, during HFD feeding. VCO_2 and VO_2 were

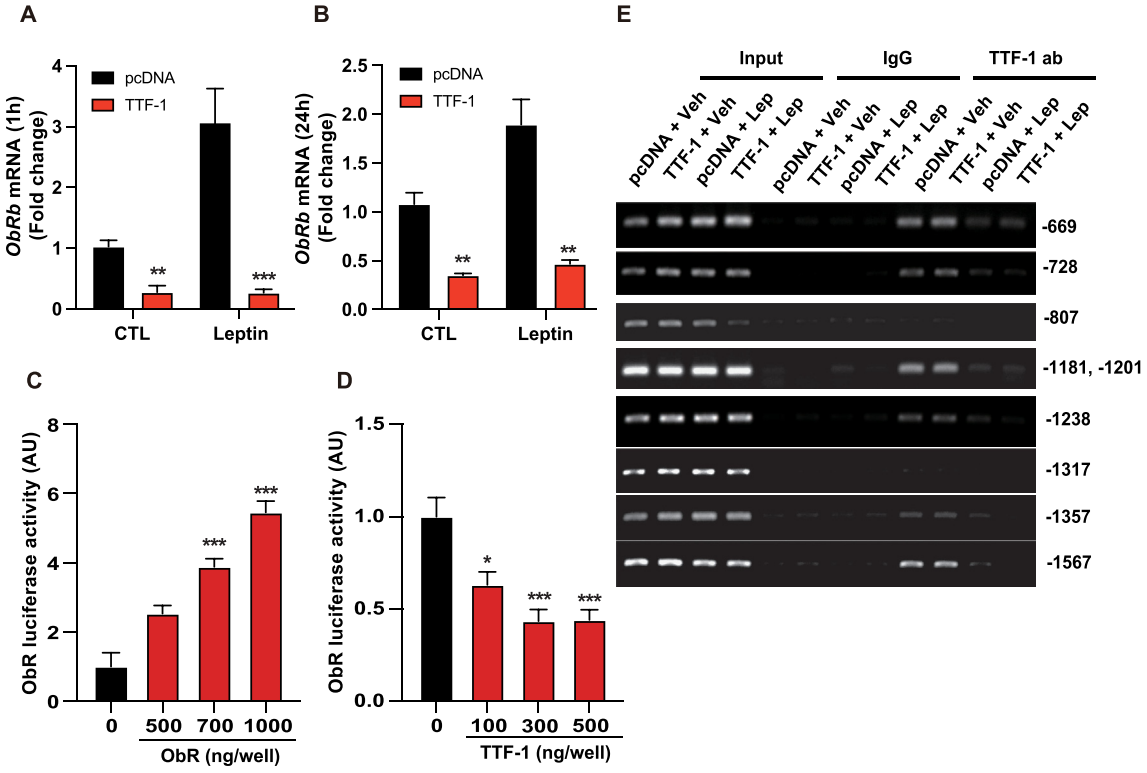

Figure 5: TTF-1 participates in the control of leptin receptor transcription. (A, B) mHypoA-POMC cells were transfected with pcDNA control vector or TTF-1 expression vectors and treated with leptin (100 ng/ml) or the vehicle control for 1 h and 24 h. Quantitative real-time PCR data revealed that the leptin-induced increase in ObRb expression was suppressed by TTF-1 overexpression (n = 5/group). (C, D) Luciferase reporter constructs (pGL4) containing the 5′-flanking region of the mouse ObR gene were transfected into cells and the transcriptional activity of ObR gene after the overexpression of TTF-1 was evaluated. Promoter activities of ObR were positively correlated with the increasing concentrations of the transfected promoter constructs (n = 4/group) and were significantly inhibited by the overexpression of TTF-1 (n = 6/group). (E) ChIP assays were performed using an anti-TTF-1 antibody and PCR primer sets targeting the mouse ObR promoter sequences that encompass putative TTF-1 binding sites under TTF-1 overexpression and leptin-treated conditions (n = 3/group). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group. *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-test.

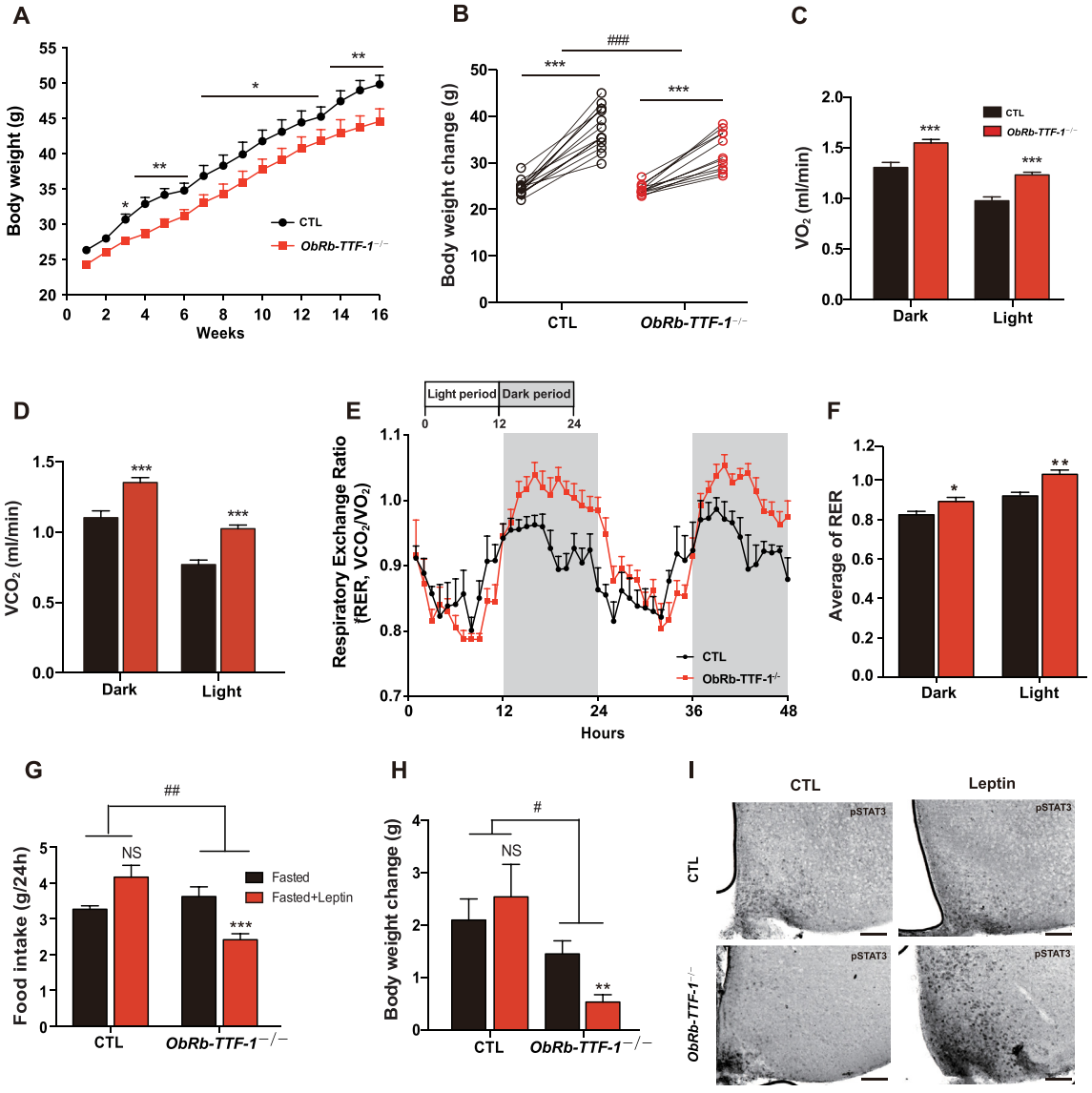

Figure 6: Deletion of TTF-1 in ObRb-positive cells protects against diet-induced obesity by improving leptin resistance. Mice were fed HFD for 16 weeks and underwent measurement of metabolic phenotypes. (A, B) Weight gain during HFD feeding for 16 weeks was significantly lower in the *ObRb-TTF-1*$^{-/-}$ mice than in the control mice (CTL, n = 14; *ObRb-TTF-1*$^{-/-}$, n = 12). (C, D) *ObRb-TTF-1*$^{-/-}$ mice showed higher (C) oxygen consumption (VO_2) and (D) carbon dioxide generation (VCO_2) than the control mice during the dark and light periods (n = 4/group). (E, F) *ObRb-TTF-1*$^{-/-}$ mice showed increased RER compared to the control mice during the dark and light periods (n = 4/group). (G, H) *ObRb-TTF-1*$^{-/-}$ mice showed a significant reduction in (G) food intake and (H) body weight for 24 h in response to systemic administration of leptin (3 mg/kg, i.p.), while the control HFD-fed mice were unresponsive to leptin with respect to anorexia and weight loss (n = 5–6/group). (I) Hypothalamic sections from the *ObRb-TTF-1*$^{-/-}$ mice, but not those from the control mice, showed increased pSTAT3 levels in response to the i.p. injection of leptin (3 mg/kg). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ vs. control group (A-D, F) or fasted group of each genotype (G, H). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. change in the control mice. Scale bar = 50 μm. *P*-values for unpaired comparisons were analyzed by two-tailed Student's *t*-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.

significantly higher in the *ObRb-TTF-1*$^{-/-}$ mice than in the control mice (Figure 6C and D). The respiratory exchange ratio (RER), calculated based on the collected VO_2 and VCO_2, was also higher in the *ObRb-TTF-1*$^{-/-}$ mice during both dark and light periods (Figure 6E and F). We next determined whether selective deletion of the *TTF-1* gene in leptin receptor-positive cells enhanced the leptin sensitivity of the DIO mice. Systemic administration of leptin (3 mg/kg) was unable to induce anorexia and weight loss in the control mice fed the HFD for 16 weeks (Figure 6G and H), indicating the development of leptin resistance. However, the same treatment with leptin effectively decreased the food intake and body weight of the *ObRb-TTF-1*$^{-/-}$ mice fed the HFD for 16 weeks (Figure 6G and H). In support of the metabolic phenotypes showing improved leptin resistance, the *ObRb-TTF-1*$^{-/-}$ mice displayed markedly increased pSTAT3 levels in the ARC in response to leptin treatment after 16 weeks of HFD feeding, while the control DIO mice failed to show a leptin-induced increase in pSTAT3 levels (Figure 6I). Thus, the selective deletion of the *TTF-1* gene in *ObRb*-positive cells improves leptin responsiveness and thus, protects against obesity development in association with leptin resistance induced by over-nutrition.

4. DISCUSSION

The present study highlights the contribution of TTF-1 to the leptin signaling pathway in the hypothalamic neuronal circuit and its resulting impact on the development of obesity in association with leptin resistance. The current observations suggest that TTF-1 is an important molecular cue in the leptin signaling pathway during development of leptin resistance.

Whole body energy metabolism is intimately related to the communication between the peripheral organs that propagate signals reflecting the energy state and central compartments involved in signal integration for the triggering of proper behavioral outputs. In particular, it has long been recognized that the hypothalamic neuronal circuit is essential for interpreting afferent chemical messengers such as adiposity signals and gut-derived hormones [22].

Since leptin is one of the most effective hormones for controlling energy balance, and establishment of multiple leptin secretion- or receptor signaling-impaired genetic lines have led to the development of obesity and metabolic disorders, leptin has been referred to as an attractive therapeutic target for anti-obesity drugs [23,24]. Despite its strong effects on appetite reduction and enhanced energy expenditure, using leptin therapeutically has not been feasible thus far as it has not been shown to be effective for the severely obese [1]. Indeed, the disruption of leptin signaling in hypothalamic cells gives rise to insensitivity to leptin's actions, or so-called leptin resistance, and this could be a critical pathogenic element in the development of obesity [25,26]. In line with these findings, numerous studies have explored the primary cellular events and molecular mediators for the actions of leptin in order to interpret the development of leptin resistance. Until now, the most acceptable explanation has been that the development of leptin resistance induced by over-nutrition is closely linked to a variety of cellular stresses such as endoplasmic reticulum stress, oxidative stress, and inflammation [27–29].

Transcriptional regulation is a crucial molecular action in the mechanism of cellular stress-induced leptin resistance. STAT3 is a pivotal transcriptional modulator in the leptin signaling pathway. Phosphorylation of STAT3 gives rise to an increase in *POMC* transcription and a decrease in *AgRP* transcription via its specific binding motif, known as the leptin response element [30]. A mouse line in which STAT3 is specifically impaired in neural cells displayed obesity accompanied by hyperphagia [31]. TTF-1 has recently been considered a key component in the hypothalamic complex of regulatory systems for energy homeostasis as it has been found that alteration of TTF-1 expression triggers a marked change in metabolic phenotypes including food intake [14] and thermogenesis [32] through the transcriptional regulation of several downstream targets such as the *POMC* and *AgRP* genes. These previous studies have raised further questions as to whether TTF-1 is responsive to hormonal inputs for the triggering of feeding or satiety. At the beginning of this study, we studied localization of TTF-1 in the entire brain and found that *TTF-1* is predominantly expressed in leptin receptor-expressing cells and the hypothalamic areas that are critical in the control of energy metabolism. These histological findings and previous reports together led us to design the current study exploring the molecular contribution of TTF-1 to the leptin signaling pathway.

We found that mice with selective deletion of *TTF-1* in ObRb- or POMC-positive cells responded to leptin with more sensitivity, which promoted the development of anorexia and body weight loss. This suggests that these mice may display a lower leptin-responsiveness threshold. These genetically modified mice also displayed prolonged responsiveness to leptin, suggesting that TTF-1 is an important molecular modulator for the action of leptin in the control of energy balance. Since TTF-1 actively participates in the development of the neural tube ventral midline and the diencephalic brain region [7,33], we interrogated whether altered number of POMC cells could be a cellular event for the enhanced leptin sensitivity seen in *ObRb-TTF-1*$^{-/-}$ or *POMC-TTF-1*$^{-/-}$ mice. However, no difference in number of POMC cells was observed between control and TTF-1-deficient mice (Supplementary Figure 5), suggesting that enhanced leptin sensitivity induced by TTF-1 ablation is coupled to the activity of POMC neurons rather than the density of POMC cells in hypothalamic ARC. Moreover, these mice displayed changes in the intracellular molecules involved in leptin signaling in the hypothalamus: for example, the mRNA levels of *ObRb* were increased in the hypothalmi of mutant mice, and phosphorylation of STAT3 was greatly increased by leptin treatment in the mutant mice compared to the control mice. These molecular findings suggest a potential explanation as to how TTF-1 is involved in cellular responsiveness to leptin. In this way, the TTF-1 knockout-induced increase in *ObRb* expression may cause an increase in the phosphorylation of STAT3, and the increased ObRb and pSTAT3 together, in turn, render the cells more sensitive to leptin [34,35]. Intriguingly, *ObRb-TTF-1*$^{-/-}$ mice showed higher expression levels of both *SOCS3* and *PTP1B*, both of which are involved in the intracellular feedback loop of leptin receptor signaling. These increases in negative feedback activity might be due to the homeostatic cellular response to compensate for the enhanced activation of leptin receptor signaling in the mutant cells. We verified the transcriptional regulation of the ObR gene by TTF-1 in cultured hypothalamic cells. These observations further confirmed that enhanced leptin sensitivity is associated with increased activity of leptin signaling triggered by TTF-1 deficiency.

Although deficiency in leptin can lead to obesity, a high level of circulating leptin in diet-induced obesity can neither reduce food intake nor activate energy expenditure [36]. Thus, the majority of studies have been conducted to understand the mechanism of leptin resistance and to manipulate the sensitivity of leptin action. It has been well established that mice with long-term exposure to an HFD develop leptin resistance that accounts for the development of obesity and its related pathogenesis [1,37]. In accordance with the aforementioned cellular and molecular changes, *ObRb-TTF-1*$^{-/-}$ mice also displayed metabolic phenotypes indicating higher sensitivity to leptin after a long-term HFD-fed condition that caused leptin resistance in the control mice.

In terms of molecular event, leptin resistance was effectively reversed by modulating the molecules involved in leptin signaling pathway such as SOCS3 and pSTAT3 [21]. Moreover, an elevation of ObRb expression also improved the leptin resistance and obesity pathogenesis [38]. From these evidences, we thought that increased ObRb expression could be a molecular clue to interpret the enhanced leptin sensitivity seen in *TTF-1*- deficient mice models. However, further studies are required to better understand the contributions of TTF-1 in the regulation of obesity pathogenesis coupled to the leptin resistance. Particularly, it is valuable to uncover detailed molecular mechanisms of TTF-1 functions in the leptin signaling pathway, especially its role with respect to molecules such as SOCS3 and PTP1B in the negative regulation of cellular leptin action. It is also important to clarify whether TTF-1 affects intracellular factors in other neuronal populations beyond POMC neurons in the hypothalamus. In this study, we identified the changes in number of TTF-1-positive cells in response to fasting and leptin treatment in hypothalamic DMH where ObRb is also existed. These data suggest that enhanced leptin sensitivity seen in *TTF-1*-deficient mice models used in this study might be also linked to the functions of TTF-1 in ObRb-positive cells in hypothalamic DMH. Although we highlighted the functions of TTF-1 in hypothalamic ARC,

further studies in other hypothalamic nuclei are required for better understating the physiological and pathological functions of TTF-1 in the regulation of leptin sensitivity.
In summary, the present study identified TTF-1 as a key regulator in the leptin signaling pathway and highlighted its contribution to the leptin sensitivity of the hypothalamic neuronal circuit controlling energy homeostasis. Thus, TTF-1 may serve as a novel therapeutic target for the treatment of obesity.

AUTHOR CONTRIBUTIONS

Byong Seo Park: Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Writing - original draft; Dasol Kang: Formal analysis, Investigation, Methodology, Writing - original draft; Kwang Kon Kim: Investigation, Methodology; Bora Jeong: Investigation, Methodology; Tae Hwan Lee: Investigation, Methodology; Jeong Woo Park: Formal analysis, Investigation; Shioko Kimura: Resources; Jung-Yong Yeh: Investigation; Gu Seob Roh: Investigation; Chang-Joong Lee: Writing - review and editing; Sungchil Yang: Writing - review and editing; Sunggu Yang: Formal analysis, Writing - review and editing; Jae Geun Kim: Conceptualization, Formal analysis, Supervision, Writing - original draft, Writing - review and editing; Byung Ju Lee, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing.

DATA AVAILABILITY

Data will be made available on request.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101636.

REFERENCES

[1] Myers, M.G., Leibel, R.L., Seeley, R.J., Schwartz, M.W., 2010. Obesity and leptin resistance: distinguishing cause from effect. Trends in Endocrinology and Metabolism: Trends in Endocrinology and Metabolism 21(11):643–651. https://doi.org/10.1016/j.tem.2010.08.002.

[2] Saper, C.B., Lowell, B.B., 2014. The hypothalamus. Current Biology: CB 24(23):R1111–R1116. https://doi.org/10.1016/j.cub.2014.10.023.

[3] van de Wall, E., Leshan, R., Xu, A.W., Balthasar, N., Coppari, R., Liu, S.M., et al., 2008. Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion. Endocrinology 149(4):1773–1785. https://doi.org/10.1210/en.2007-1132.

[4] Friedman, J.M., Halaas, J.L., 1998. Leptin and the regulation of body weight in mammals. Nature 395(6704):763–770. https://doi.org/10.1038/27376.

[5] Varela, L., Horvath, T.L., 2012. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. EMBO Reports 13(12):1079–1086. https://doi.org/10.1038/embor.2012.174.

[6] Civitareale, D., Lonigro, R., Sinclair, A.J., Di Lauro, R., 1989. A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. The EMBO Journal 8(9):2537–2542.

[7] Lee, B.J., Cho, G.J., Norgren, R.B., Junier, M.P., Hill, D.F., Tapia, V., et al., 2001. TTF-1, a homeodomain gene required for diencephalic morphogenesis, is postnatally expressed in the neuroendocrine brain in a developmentally regulated and cell-specific fashion. Molecular and Cellular Neuroscience 17(1):107–126. https://doi.org/10.1006/mcne.2000.0933.

[8] Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C.H., Ward, J.M., et al., 1996. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes & Development 10(1):60–69.

[9] Marín, O., Baker, J., Puelles, L., Rubenstein, J.L.R., 2002. Patterning of the basal telencephalon and hypothalamus is essential for guidance of cortical projections. Development (Cambridge, United Kingdom) 129(3):761–773.

[10] Kim, M.S., Hur, M.K., Son, Y.J., Park, J.-I., Chun, S.Y., D'Elia, A.V., et al., 2002. Regulation of pituitary adenylate cyclase-activating polypeptide gene transcription by TTF-1, a homeodomain-containing transcription factor. Journal of Biological Chemistry 277(39):36863–36871. https://doi.org/10.1074/jbc.M206443200.

[11] Kim, J.G., Son, Y.J., Yun, C.H., Kim, Y.I., Nam-Goong, I.S., Park, J.H., et al., 2007. Thyroid transcription factor-1 facilitates cerebrospinal fluid formation by regulating aquaporin-1 synthesis in the brain. Journal of Biological Chemistry 282(20):14923–14931. https://doi.org/10.1074/jbc.M701411200.

[12] Kaji, T., Nonogaki, K., 2013. Role of homeobox genes in the hypothalamic development and energy balance. Frontiers in Bioscience 18(2):740–747. https://doi.org/10.2741/4136.

[13] Kim, J.G., Nam-Goong, I.S., Yun, C.H., Jeong, J.K., Kim, E.S., Park, J.J., et al., 2006. TTF-1, a homeodomain-containing transcription factor, regulates feeding behavior in the rat hypothalamus. Biochemical and Biophysical Research Communications 349(3):969–975. https://doi.org/10.1016/j.bbrc.2006.08.147.

[14] Kim, J.G., Park, B.S., Yun, C.H., Kim, H.J., Kang, S.S., D'Elia, A.V., et al., 2011. Thyroid transcription factor-1 regulates feeding behavior via melanocortin pathway in the hypothalamus. Diabetes 60(3):710–719. https://doi.org/10.2337/db10-0183.

[15] Kusakabe, T., Kawaguchi, A., Hoshi, N., Kawaguchi, R., Hoshi, S., Kimura, S., 2006. Thyroid-specific enhancer-binding protein/NKX2.1 is required for the maintenance of ordered architecture and function of the differentiated thyroid. Molecular Endocrinology (Baltimore, Md 20(8):1796–1809. https://doi.org/10.1210/me.2005-0327.

[16] Pan, W., Hsuchou, H., Tu, H., Kastin, A.J., 2008. Developmental changes of leptin receptors in cerebral microvessels: unexpected relation to leptin transport. Endocrinology 149(3):877–885. https://doi.org/10.1210/en.2007-0893.

[17] Tsuchiya, H., Ikeda, Y., Ebata, Y., Kojima, C., Katsuma, R., Tsuruyama, T., et al., 2012. Retinoids ameliorate insulin resistance in a leptin-dependent manner in mice. Hepatology (Baltimore, MD 56(4):1319–1330. https://doi.org/10.1002/hep.25798.

[18] Myers, M.G., Olson, D.P., 2012. Central nervous system control of metabolism. Nature 491(7424):357–363. https://doi.org/10.1038/nature11705.

[19] Laque, A., Zhang, Y., Gettys, S., Nguyen, T.-A., Bui, K., Morrison, C.D., et al., 2013. Leptin receptor neurons in the mouse hypothalamus are colocalized with the neuropeptide galanin and mediate anorexigenic leptin action. American Journal of Physiology. Endocrinology and Metabolism 304(9):E999–E1011. https://doi.org/10.1152/ajpendo.00643.2012.

[20] Leinninger, G.M., Jo, Y.-H., Leshan, R.L., Louis, G.W., Yang, H., Barrera, J.G., et al., 2009. Leptin acts via leptin receptor-expressing lateral hypothalamic

neurons to modulate the mesolimbic dopamine system and suppress feeding. Cell Metabolism 10(2):89–98. https://doi.org/10.1016/j.cmet.2009.06.011.

[21] Myers, M.G., Cowley, M.A., Münzberg, H., 2008. Mechanisms of leptin action and leptin resistance. Annual Review of Physiology 70(1):537–556. https://doi.org/10.1146/annurev.physiol.70.113006.100707.

[22] Zhang, Z.-Y., Dodd, G.T., Tiganis, T., 2015. Protein tyrosine phosphatases in hypothalamic insulin and leptin signaling. Trends in Pharmacological Sciences 36(10):661–674. https://doi.org/10.1016/j.tips.2015.07.003.

[23] Bates, S.H., Stearns, W.H., Dundon, T.A., Schubert, M., Tso, A.W.K., Wang, Y., et al., 2003. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature 421(6925):856–859. https://doi.org/10.1038/nature01388.

[24] Kwon, O., Kim, K.W., Kim, M.-S., 2016. Leptin signalling pathways in hypothalamic neurons. Cellular and Molecular Life Sciences 73(7):1457–1477. https://doi.org/10.1007/s00018-016-2133-1.

[25] Lin, S., Thomas, T.C., Storlien, L.H., Huang, X.F., 2000. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. International Journal of Obesity 24(5):639–646. https://doi.org/10.1038/sj.ijo.0801209.

[26] Scarpace, P.J., Zhang, Y., 2009. Leptin resistance: a prediposing factor for diet-induced obesity. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 296(3):R493–R500. https://doi.org/10.1152/ajpregu.90669.2008.

[27] Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., Cai, D., 2008. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell 135(1):61–73. https://doi.org/10.1016/j.cell.2008.07.043.

[28] Ropelle, E.R., Flores, M.B., Cintra, D.E., Rocha, G.Z., Pauli, J.R., Morari, J., et al., 2010. IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKbeta and ER stress inhibition. PLoS Biology 8(8):e1000465. https://doi.org/10.1371/journal.pbio.1000465.

[29] Ozcan, L., Ergin, A.S., Lu, A., Chung, J., Sarkar, S., Nie, D., et al., 2009. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metabolism 9(1):35–51. https://doi.org/10.1016/j.cmet.2008.12.004.

[30] Banks, A.S., Davis, S.M., Bates, S.H., Myers, M.G., 2000. Activation of downstream signals by the long form of the leptin receptor. Journal of Biological Chemistry 275(19):14563–14572.

[31] Gao, Q., Wolfgang, M.J., Neschen, S., Morino, K., Horvath, T.L., Shulman, G.I., et al., 2004. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proceedings of the National Academy of Sciences of the United States of America 101(13):4661–4666. https://doi.org/10.1073/pnas.0303992101.

[32] Yun, C.H., Kim, J.G., Park, B.S., Lee, H.M., Kim, D.H., Kim, E.O., et al., 2011. TTF-1 action on the transcriptional regulation of cyclooxygenase-2 gene in the rat brain. PLoS One 6(12):e28959. https://doi.org/10.1371/journal.pone.0028959.

[33] Toda, C., Santoro, A., Kim, J.D., Diano, S., 2017. POMC neurons: from birth to death. Annual Review of Physiology 79:209–236. https://doi.org/10.1146/annurev-physiol-022516-034110.

[34] Zhang, Y., Wu, X., He, Y., Kastin, A.J., Hsuchou, H., Rosenblum, C.I., et al., 2009. Melanocortin potentiates leptin-induced STAT3 signaling via MAPK pathway. Journal of Neurochemistry 110(1):390–399. https://doi.org/10.1111/j.1471-4159.2009.06144.x.

[35] Mainardi, M., Scabia, G., Vottari, T., Santini, F., Pinchera, A., Maffei, L., et al., 2010. A sensitive period for environmental regulation of eating behavior and leptin sensitivity. Proceedings of the National Academy of Sciences of the United States of America 107(38):16673–16678. https://doi.org/10.1073/pnas.0911832107.

[36] Breslow, M.J., Min-Lee, K., Brown, D.R., Chacko, V.P., Palmer, D., Berkowitz, D.E., 1999. Effect of leptin deficiency on metabolic rate in ob/ob mice. American Journal of Physiology 276(3 Pt 1):E443–E449.

[37] Meng, Q., Cai, D., 2011. Defective hypothalamic autophagy directs the central pathogenesis of obesity via the IkappaB kinase beta (IKKbeta)/NF-kappaB pathway. Journal of Biological Chemistry 286(37):32324–32332. https://doi.org/10.1074/jbc.M111.254417.

[38] Andreoli, M.F., Donato, J., Cakir, I., Perello, M., 2019. Leptin resensitization: a reversion of leptin resistant states. Journal of Endocrinology 1(aop):R81–R96. https://doi.org/10.1530/joe-18-0606.

