

Autocrine TGFB1 suppresses β 3-adrenergic stimulation-induced adipose browning in obesity via its EMI domain and RGD motif

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Abstract

Background: Transforming growth factor- β -induced protein (TGFB1) is a secreted extracellular matrix protein induced by TGF- β signaling. Obesity is associated with reduced β 3-adrenergic receptor (β 3-AR) expression and impaired adipose browning. To date, the autocrine role of adipocyte-derived TGFB1 and its involvement in β 3-AR signaling remains unclear. This study investigated the autocrine role and underlying mechanism of adipocyte-derived TGFB1 in β 3-adrenergic stimulation-mediated adipose browning.

Methods: Analyses of human and mouse adipose tissue datasets identified TGFB1 as a potential therapeutic target for enhancing β 3-adrenergic stimulation-induced adipose browning in obesity. *In vivo* using TGFB1-knockout mice, *in vitro* using adipocytes with TGFB1 knockdown or overexpression, and *ex vivo* using adipocytes differentiated from bone marrow-derived mesenchymal stem cells were established to investigate the role of TGFB1. Western blotting, quantitative polymerase chain reaction, co-immunoprecipitation, Notch signaling modulation, and TGFB1 domain-deletion mutants were used to elucidate the underlying molecular mechanisms.

Results: β 3-Adrenergic stimulation reduced TGFB1 secretion and TGFB1–Notch1 signaling. Adipocyte-derived TGFB1 suppressed β 3-AR expression through Notch/HES1-mediated downregulation of C/EBP α . Increased TGFB1 expression suppressed β 3-adrenergic stimulation-induced adipose browning, whereas TGFB1 deficiency enhanced it. TGFB1 expression was inversely associated with β 3-AR expression in adipose tissues from humans and mice with obesity. The EMI domain and Arg–Gly–Asp (RGD) motif of TGFB1 were identified as the principal regions regulating adipose browning. The FAS1 domain showed no involvement.

Conclusion: These findings identify the EMI domain and RGD motif of adipocyte-derived TGFB1 as functional regions that regulate β 3-adrenergic stimulation-mediated adipose browning and suggest TGFB1 as a potential therapeutic target for obesity.

Background

Obesity results from an imbalance between energy intake and expenditure, disrupting body mass homeostasis (Webber, 2003). In adipose tissue, sympathetic catecholamine signaling promotes the conversion of white adipocytes to a beige, thermogenic phenotype, thereby increasing energy expenditure, a process known as adipose browning. Adipose browning is impaired in obesity, suggesting reduced responsiveness of obese adipose tissue to physiological stimulation (Valentine et al., 2022).

Cold exposure or exercise physiologically induces adipose browning by activating the sympathetic nervous system (Gado et al., 2023) and promoting norepinephrine release from peripheral nerve terminals (Young et al., 1982), which in turn activates β -adrenergic receptors on adipocytes (Jiang et al., 2017). β -adrenergic receptors (AR) are classified into β 1-AR, β 2-AR, and β 3-AR. β 1-AR and β 2-AR are widely distributed throughout the body (Parichatikanond et al., 2024, Salpeter et al., 2004). β 3-AR is

primarily found in adipose tissue, functioning as a key thermogenic receptor (Krief et al., 1993, Velmurugan et al., 2019). Norepinephrine binds to the β 3-AR on adipocytes, activating the cAMP/PKA pathway and promoting the expression of thermogenic genes, including UCP1 and PGC1 α (Liu et al., 2016). C/EBP α transcriptionally regulates β 3-AR expression, and responsiveness to β 3-adrenergic stimulation depends on β 3-AR levels. In obesity, chronic inflammation downregulates β 3-AR expression (Haji et al., 2021, Valentine et al., 2022), thereby attenuating β 3-adrenergic stimulation-induced adipose browning (Reynisdottir et al., 1994).

Recent studies show that extracellular matrix (ECM) components contribute to β 3-AR signaling. Approximately 40% of proteins secreted by brown adipocytes in response to catecholamines are ECM components (Villarroya et al., 2019). The roles of specific ECM components in adipose tissue have been characterized. Integrins and ECM proteins regulate thermogenic capacity, while laminin α 4 deficiency is associated with sustained adipose browning and improved metabolic function (Gonzalez Porras et al., 2021). Additionally, fibronectin and collagen isoforms modulate β -adrenergic signaling by regulating adenylyl cyclase activity (Freyer et al., 2004).

Transforming growth factor- β -induced protein (TGFB1) is an ECM protein whose expression is upregulated by TGF- β signaling (LeBaron et al., 1995). TGFB1 is synthesized intracellularly and secreted into the extracellular milieu, where it may function as an autocrine factor. Structurally, TGFB1 comprises an EMI domain, a FAS1 domain, and an Arg–Gly–Asp (RGD) motif (Runager et al., 2007). The RGD motif is located near the C-terminus. The FAS1 domain constitutes most of the TGFB1 protein and directly interacts with α v β 3 integrin (Son et al., 2013). The EMI domain is a short, cysteine-rich domain containing conserved motifs and hydrophobic clusters (Callebaut et al., 2003). However, while TGFB1 is a representative ECM protein (Costanza et al., 2019), its functional association with β 3-AR-mediated adipose browning remains unreported.

So far, the role of TGFB1 in obesity focuses on the paracrine effects of macrophage-derived TGFB1 on adipose tissue. A study reports that macrophage-derived TGFB1 suppresses adipose browning through adipsin suppression. This study did not examine the role of TGFB1 in adipocytes independent of macrophage-derived signals or its effects under physiological adrenergic stimulation (Lee et al., 2023). Therapeutic application of this mechanism is limited by two major factors. First, the molecular interface mediating TGFB1–Notch1 signaling has not been defined, limiting the development of strategies that directly disrupt this interaction (Suganami et al., 2005). Second, targeting adipsin, a systemic complement factor D, may inadvertently disrupt host immune homeostasis (Rosen et al., 1989). Therefore, this study investigated the autocrine role of adipocyte-derived TGFB1 in β 3-adrenergic stimulation-induced adipose browning and its underlying molecular and structural mechanisms. TGFB1 constrains adipose browning during physiological and pharmacological β 3-adrenergic signaling activation, such as cold exposure and CL316,243 administration. Collectively, these findings identify adipocyte-derived TGFB1 as an autocrine regulator of adipose browning induced by physiological stimuli.

Methods

Animal study

Male C57BL6/J mice aged 6–8 weeks (Hyochang Science, Daegu, Republic of Korea) were housed under standard conditions ($24 \pm 1^\circ\text{C}$, 10–20% humidity, 12-h light/dark cycle) and acclimated for 1 week. The mice had ad libitum access to chow (Hyochang Science, Daegu, Republic of Korea) and drinking water. Mice were fed a 60% high-fat diet (HFD) (Research Diets, New Brunswick, NJ, USA) for 12 weeks. CL316,243 (CL; 1 mg/kg; MedChemExpress, Monmouth Junction, NJ, USA) or PBS was administered intraperitoneally (*i.p.*) for 7 days. For $\beta 3$ -adrenergic receptor blockade, SR59230A (iADRB3; 5 mg/kg, *i.p.*) (MedChemExpress, Monmouth Junction, NJ, USA) was administered 30 minutes before each CL treatment. Cold exposure was conducted at 4°C for 3 days. During cold exposure, mouse core body temperatures were measured every 6 hours using a K-type thermocouple thermometer (Testo, Baden-Württemberg, Germany) inserted rectally. Mice were subjected to fasting for 12 hours before euthanasia under isoflurane anaesthesia.

Generation of transforming growth factor β -induced protein knockout mice

TGFBI knockout mice (KO; TGFBI^{-/-}) were generated as previously described (Lee et al., 2023). Briefly, a TGFBI-targeting vector was constructed by inserting sequences upstream and downstream of exon 3, and microinjected into C57BL/6J blastocysts. The PGK-neomycin cassette, flanked by FLP recombinase target sites, was subsequently removed by FLP recombinase.

Ethics statement

All animal experiments were conducted following the ethical guidelines of Kyungpook National University (Daegu, Republic of Korea) and approved by its Institutional Animal Care and Use Committee (Approval No. KNU-2023-0592).

Intraperitoneal glucose tolerance test

After fasting, mice received D-glucose (1 g/kg) in PBS, *i.p.* (Sigma, St. Louis, MO, USA). Blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes after administration. Blood samples were collected from the tail vein, and glucose levels were measured using an Accu-Chek Active glucometer (Roche Diagnostics GmbH, Mannheim, Germany).

Mouse bone marrow-derived mesenchymal stem cell isolation and adipogenic differentiation

Bone marrow cells were harvested from the femur and tibia of male mice aged 6–8 weeks. The bones were digested with 1 mg/mL of collagenase II (Worthington Biochemical, Lakewood, NJ, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium-high glucose (DMEM-H) containing 20% FBS, 0.01 M HEPES, and L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Adipogenic differentiation was initiated by differentiation medium I, comprising DMEM-H supplemented with 20% FBS, 172 nM insulin (Sigma, St. Louis, MO, USA), 5 μM dexamethasone (Sigma, St. Louis, MO, USA), 500 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO, USA), 125 μM indomethacin (Sigma, St.

Louis, MO, USA), and 1 μ M rosiglitazone (Sigma, St. Louis, MO, USA). After 2 days, the medium was replaced with differentiation medium II, comprising DMEM-H supplemented with 20% FBS, 172 nM insulin, and 1 μ M rosiglitazone, and differentiation was continued for an additional 12 days.

Cell culture and adipogenic differentiation of 3T3-L1 adipocytes

3T3-L1 mouse preadipocytes (CL-173) were purchased from ATCC (Manassas, VA, USA). Cells were maintained in DMEM-H supplemented with 10% newborn calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Adipogenic differentiation was initiated using medium I (DMEM-H supplemented with 10% FBS, 172 nM insulin, 0.25 μ M dexamethasone, 500 μ M IBMX, and 125 μ M indomethacin). After 2 days, the medium was replaced with differentiation medium II (DMEM-H supplemented with 10% FBS, 172 nM insulin) for 6–8 days.

Gene manipulation

For TGFBI knockdown (KD), Tet-on-*pLKO.1* puro vectors carrying either a control shRNA (5'-ATCTCGCTTGGGCGAGAGTAAG-3') or a TGFBI-targeting shRNA (5'-GCACCAACAAGAAATACTT-3') were obtained from Addgene (Watertown, MA, USA). Lentiviral particles were produced by co-transfecting 293T cells with the shRNA vector, psPAX2, and pMD2.G, followed by infection of 3T3-L1 cells in the presence of polybrene. Puromycin selection was performed 48 hours post-infection.

For TGFBI overexpression (OE), the mouse *Tgfb1* cDNA entry clone (Origene, Rockville, MD, USA) was inserted into pcDNA3.1. Cells were transfected with either empty pcDNA3.1 or pcTGFBI using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting

Protein concentrations were determined, and 30–60 μ g of protein was separated on 10–15% acrylamide gels and transferred to 0.45 μ m nitrocellulose membranes. Membranes were blocked with 5% skimmed milk and incubated with the primary antibody at 4°C, and then with an HRP-conjugated secondary antibody. Table S1 presents the antibodies and their respective dilutions. Protein bands were detected using enhanced chemiluminescence (ECL; Cytiva, Marlborough, MA, USA) and imaged using a Fusion Solo system (Vilber Lourmat, Marne-la-Vallée, France). Western blot band intensities were quantified using ImageJ and normalized to that of β -actin.

Reverse transcription quantitative polymerase chain reaction

Total RNA was extracted using TRIzol® reagent, and cDNA was synthesized using ReverTra Ace™ qPCR RT Master Mix (Toyobo, Osaka, Japan). mRNA expression was quantified using SYBR Green (Toyobo) and gene-specific primers (Table S2) on an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA). Data were visualized and statistically analyzed using Bio-Rad CFX Maestro software (version 4.0). Gene expression ratios were normalized to reference gene levels using the $\Delta\Delta C_q$ method.

Oxygen consumption rate analysis

Oxygen consumption rates were assessed using the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA). The sensor cartridge was loaded with distilled water and incubated overnight at 37°C in a non-CO₂ incubator. The cartridge was then hydrated with XF calibrant. The cell culture medium was replaced with Seahorse XF DMEM assay medium supplemented with 10 mmol/L of glucose, 1 mmol/L of pyruvate, and 2 mmol/L of L-glutamine. The concentrations of oligomycin, FCCP, and rotenone/antimycin A (Rot/AA) loaded into the cartridge were 1, 2, and 1 µmol/L, respectively. The assay comprised equilibration (0–20 minutes), followed by oligomycin (20–40 minutes), FCCP (40–60 minutes), and Rot/AA (60–80 minutes).

Enzyme-linked immunosorbent assay

Serum leptin concentrations were measured by a Mouse Leptin enzyme-linked immunosorbent assay (ELISA) Kit (RayBiotech, GA, USA) following the instructions of the manufacturer. Serum samples were diluted 1:10. Briefly, standards or samples were added and incubated. After washing, 1× biotinylated antibody was added. The wells were washed again and incubated with streptavidin solution. The TMB substrate was added, and the reaction was stopped. Absorbance at 450 nm was measured immediately.

TGFBI concentrations in cell supernatants were determined using the Mouse beta IG H3 ELISA Kit (Abcam, Cambridge, UK) following the instructions of the manufacturer. Briefly, standards and samples were added with the antibody cocktail. After washing, the TMB development solution was added to each well. After adding stop solution, the absorbance was measured at 450 nm. TGFBI levels in the culture medium were normalized to the total cellular protein content of the corresponding samples.

Dual-energy X-ray absorptiometry

Whole-body composition was assessed using an InAlyzer dual-energy X-ray absorptiometry system (Medikors, Gyeonggi-do, Republic of Korea) to determine total, fat, and lean body mass (g). Acquired images were analysed using InAlyzer software.

Haematoxylin and eosin staining

iWAT samples were fixed overnight at 4 °C in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. All sections were imaged using a microscope, and adipocyte areas were quantified with Adiposoft (ImageJ).

Oil Red O staining

Cells were fixed with 4% paraformaldehyde and stained with 40% Oil Red O solution (Sigma, St. Louis, MO, USA). To quantify Oil Red O content, the plate was dried, and 1 mL of isopropanol was added to each well. Absorbance was measured at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland).

Co-immunoprecipitation

Cell lysates were incubated with the primary antibody overnight. Protein G magnetic beads (Roche, Basel, Switzerland) were then added, and the mixture was incubated for 1 h. The protein complexes were eluted and isolated using beads and a magnetic stand (Invitrogen, Carlsbad, CA, USA). Samples were analyzed by western blotting.

Immunofluorescence staining

Cells were cultured on chamber slides, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 1% BSA. They were then incubated overnight at 4°C with 4 µg/mL of HES1 antibody (E-5) conjugated to Alexa Fluor® 594 (1:50; Santa Cruz Biotechnology, Dallas, TX, USA). Nuclei were counterstained with DAPI.

MitoTracker staining

MitoTracker™ Orange dye (Thermo Fisher Scientific, Waltham, MA, USA) was diluted to 25 nmol/L in DMSO. Cells cultured in an 8-well chamber slide were incubated with the diluted dye for 30 minutes. The cells were then fixed with 4% paraformaldehyde (Biosesang, Gyeonggi-do, Republic of Korea), followed by DAPI staining. Fluorescent signals were visualized, and images were acquired under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Flow cytometry

Mitochondrial staining was performed using MitoTracker™ Orange dye (Thermo Fisher Scientific, Waltham, MA, USA). Cells were harvested, washed with PBS, and then incubated in pre-warmed, serum-free minimum essential medium containing 25 nM MitoTracker dye for 45 minutes at 37°C. Excess dye was removed by centrifugation and washing, and the cells were resuspended in DMEM. Fluorescence intensity was measured using an Attune™ NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, 10,000 gated cells were analyzed.

Purification of recombinant transforming growth factor-β-induced protein

Coding sequences for recombinant (rc) full-length (FL) and mutant TGFBI proteins were designed based on sequence information obtained from NCBI and UniProt, and subsequently synthesized and cloned into a pET expression vector containing a 6x His-tag by VectorBuilder (Chicago, IL, USA). Rc proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified using Ni-NTA agarose resin (QIAGEN, Hilden, Germany). The purified proteins were buffer-exchanged into PBS and validated by Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis and Western blotting.

His-tag pull-down assay

Protein G magnetic beads were washed and incubated with anti-His-antibody (sc-8036; Santa Cruz Biotechnology, Dallas, TX, USA). Subsequently, 500 mg of rc proteins (FL, ΔEMI, ΔFAS1, and ΔRGD) were quantified and incubated with the 6x His-tagged rc protein at room temperature for 90 min. The beads–

His-tagged protein complexes were then washed with PBS and incubated with cellular protein. The beads were then collected using a magnetic stand (Invitrogen, Carlsbad, CA, USA), and samples were analyzed by western blotting.

Human patient data analysis

Data on lipid droplet size and TGFB1 expression in human subcutaneous white adipose tissue (scWAT) were obtained from the GTEx Portal (https://storage.googleapis.com/adult-gtex/bulk-gex/v10/rna-seq/counts-by-tissue/gene_reads_v10_adipose_subcutaneous.gct.gz). Datasets containing TGFB1 expression levels and associated clinical parameters in human scWAT were obtained from the Gene Expression Omnibus (GEO). GSE174475 contains RNA-sequencing data from scWAT samples from 46 individuals, along with clinical information, such as BMI, HDL, TG, HOMA-IR, and LDL levels. GSE152991 contains gene expression profiles from 11 normal-weight controls and 34 individuals with obesity.

Bulk RNA sequencing and pathway enrichment analysis

Bulk RNA-sequencing data were obtained from the dataset used in our previous study (Lee et al., 2023). Briefly, bulk RNA from the iWAT (inguinal white adipose tissue) of WT and TGFB1^{-/-} mice was sequenced on an Illumina platform by Macrogen Inc. (Seoul, South Korea). Differentially expressed genes between the two groups were identified using thresholds of $|\log_2FC| > 1$ and adjusted $p < 0.05$ and subjected to pathway enrichment analysis using the g: Profiler platform (<https://biit.cs.ut.ee/gprofiler/>). Detailed quantitative results of the enrichment analysis are presented in Table S3. RNA-seq data for iWAT from CL-treated mice were obtained from GEO (GSE86338).

Statistical analysis

Statistical significance was determined using Student's *t*-tests and analysis of variance, with $p < 0.05$ considered statistically significant. Pearson's correlation analysis was used to assess the relationships between variables. All statistical analyses were performed using GraphPad Prism 9 and IBM SPSS Statistics for Windows, Version 27 (IBM Corp., Armonk, NY, USA). Post hoc tests were applied, where appropriate, to assess differences between specific groups. Experiments were conducted in triplicate unless otherwise indicated.

Results

Adipose TGFB1 is associated with obesity-related metabolic dysfunction and is reduced by β 3-adrenergic stimulation

To examine the relationship between TGFB1 and obesity-associated adipose tissue remodeling, 714 scWAT samples from the GTEx dataset were stratified based on TGFB1 expression, and samples in the top and bottom 10% were selected for analysis. Lipid droplet area was significantly greater in the *TGFB1*-high group than in the *TGFB1*-low group (Fig. 1A, B, S1A). *TGFB1* expression was positively correlated with

BMI and HOMA-IR ($r = 0.523$ and 0.304 , respectively) and negatively correlated with HDL levels ($r = -0.348$), whereas no significant association was observed with LDL levels ($r = 0.032$). Triglyceride levels showed a positive but non-significant trend (Fig. 1C, S1B–G). Bulk RNA-seq analysis of adipose tissue from TGFB1 WT and KO mice revealed significant enrichment of pathways associated with $\beta 3$ -adrenergic stimulation-induced adipose metabolism. Among the KEGG pathways, ‘lipid metabolic process’, including ‘fatty acid metabolic process’ and ‘unsaturated fatty acid metabolic process’, and ‘lipid localization’ were highly enriched, suggesting altered lipid-handling pathways related to lipolysis and adipose browning. Additionally, enrichments of ‘transporter activity’, ‘transmembrane transport’, ‘signaling receptor binding’, and ‘calcium ion binding’ suggest changes in transport- and signaling-related processes potentially influencing $\beta 3$ -adrenergic stimulation-dependent adipocyte responses (Fig. 1D) (Valentine et al., 2022). The selective $\beta 3$ -AR agonist CL is widely used for investigating catecholamine-induced lipid metabolism (Ferrer-Lorente et al., 2005, Himms-Hagen et al., 1994). Analysis of ECM-related genes in iWAT from CL-treated obese mice showed markedly reduced TGFB1 expression (Fig. 1E, F). Adipocytes secreted TGFB1 into the culture medium, and $\beta 3$ -AR stimulation reduced TGFB1 secretion (Fig. 1G).

TGFB1 deficiency enhances $\beta 3$ -adrenergic stimulation-induced adipose browning

Obesity was induced with a 12-week HFD, followed by CL treatment (1 mg/kg/day) for 7 days (Fig. 2A). After CL treatment, KO mice showed greater body weight loss and lower WAT mass than WT mice, while BAT weight remained unchanged (Fig. 2B–D). Lipid accumulation in iWAT was lower (Fig. 2E). Serum triglyceride and free fatty acid levels were also lower in KO mice than in WT mice (Fig. 2F, G), without a reduction in food intake. The post-treatment increase in food intake in KO mice may suggest a compensatory response to increased energy demand (Fig. S3A, B). Analyzing gene and protein expression in iWAT and BM-MSC-derived adipocytes revealed increased expression of the adipose browning markers UCP1, PGC1 α , PRDM16, and PPAR γ in KO mice following $\beta 3$ -AR stimulation. Additionally, elevated GLUT4 expression in KO mice may indicate improved glucose-handling capacity (Emont et al., 2019) (Fig. 2H–M). Consistent with this metabolic adaptation, serum leptin levels—a key regulator of energy balance and thermogenesis (Houseknecht and Portocarrero, 1998, Reidy and Weber, 2000)—were also higher in KO mice following $\beta 3$ -AR stimulation, and *Lep* and *Lepr* expression was elevated in KO mice iWAT (Fig. S3C, D). These findings suggest that TGFB1 deficiency enhances CL-induced adipose browning;

TGFB1 deficiency increases adipose tissue browning during cold exposure

Changes similar to those observed with CL were also evident during physiological cold exposure, inducing norepinephrine release (Collins, 2012, Galitzky et al., 1995, Jimenez et al., 2003) and activating $\beta 3$ -adrenergic stimulation-mediated adipose browning (Li et al., 2021). Obese WT and TGFB1 KO mice were exposed to 4°C for 3 days, with food withdrawn for the final 12 hours (Fig. S2A). KO mice showed greater reductions in iWAT and epididymal white adipose tissue (eWAT) mass than WT mice (Fig. S2B). KO mice showed slightly higher body temperatures during feeding but sharp declines during fasting,

suggesting insufficient energy compensation for the increased thermogenic demand (Fig. S2C). Body weight loss was comparable between WT and KO mice during feeding (0–60 h) but was greater in KO mice during fasting (60–72 h) (Fig. S2D, E). During cold exposure, food intake was greater in KO mice, potentially reflecting a compensatory response to elevated thermogenic demand and masking genotype-dependent differences in body weight (Fig. S3E). Browning-related gene and protein markers were upregulated in KO mice (Fig. S2G). Increased GLUT4 expression and reduced circulating glucose levels may indicate greater glucose utilization during cold exposure (Fig. S2F, G). Additionally, KO mice showed higher circulating leptin levels and elevated *Lepr* expression in iWAT (Fig. S3F, G). These findings suggest that TGFBI deficiency enhances cold-induced adipose browning; however, increased thermogenic activity may impose a higher energetic burden under limited food availability.

Adipocyte-derived TGFBI regulates β 3-adrenergic stimulation-mediated adipose browning in 3T3-L1 adipocytes

Doxycycline (Dox)-inducible TGFBI KD and transient TGFBI OE (pcDNA-TGFBI) models were established in 3T3-L1 adipocytes. CL reduced lipid droplet size, with a greater reduction following TGFBI KD (+Dox) and attenuation following TGFBI OE (Fig. S4A, B). The CL treatment also increased MitoTracker fluorescence and UCP1 expression, with both measures further elevated in KD cells but suppressed in OE cells. PGC1 α expression remained largely unchanged (Fig. 3A, B, E, and F). These findings suggest that TGFBI limits β 3-adrenergic stimulation-mediated mitochondrial activation without markedly affecting mitochondrial biogenesis. At the gene level, TGFBI KD increased CL-induced *Pgc1 α* and *Ucp1* mRNA expression, whereas TGFBI OE reduced their expression (Fig. S4C, D). Oxygen consumption rate analysis showed that TGFBI KD amplified CL-induced increases in basal respiration, ATP production, proton leak, maximal respiration, and spare respiratory capacity (Fig. 3C, D). Given that C/EBP α regulates β 3-AR transcription (Dixon et al., 2001), the effects of adipocyte-derived TGFBI on β 3-AR and C/EBP α expression, and downstream PKA substrate phosphorylation were evaluated. β 3-AR and C/EBP α expression and PKA substrate phosphorylation were higher in TGFBI KD, while TGFBI OE suppressed it (Fig. 3G, H). Similar increases were observed in iWAT and BM-MSC-derived adipocytes from obese TGFBI KO mice. TGFBI deficiency increased β 3-AR and C/EBP α expression, and downstream PKA (Fig. 3I–M). Given that prolonged β 3-AR stimulation may downregulate *Adrb3* transcription through negative feedback (Valentine et al., 2022), the effect of TGFBI on the persistence of *Adrb3* expression was evaluated. *Adrb3* expression was better maintained in TGFBI KD cells but declined rapidly in TGFBI OE cells (Fig. S4E, F).

Notch1 signaling mediates the suppression of β 3-adrenergic stimulation-induced adipose browning by autocrine TGFBI

Autocrine signaling occurs when a secreted ligand acts on receptors expressed by the same cell. Given that TGFBI KD or OE alone cannot distinguish intracellular from extracellular effects of secreted TGFBI, a cell-surface receptor mediating autocrine TGFBI signaling was examined. Macrophage-derived TGFBI binds directly to Notch1 on adipocytes and activates downstream signaling (Lee et al., 2023). Screening

receptor-associated pathways altered by CL treatment identified Notch1 as a candidate mediator based on reduced downstream signaling (Fig. 4A). HES1, a major downstream target of Notch1 signaling, negatively regulates C/EBP α (De Obaldia et al., 2013, Yan et al., 2024). CL treatment significantly downregulated Notch1 downstream signaling and TGF- β signaling components in the iWAT of obese mice and 3T3-L1 adipocytes. TGF- β signaling transcriptionally induced TGFBI (Yokobori and Nishiyama, 2017), and CL treatment reduced its expression (Fig. 4A–C). CL treatment weakened the interaction between adipocyte-derived TGFBI and Notch1 without affecting Notch1 expression (Fig. 4D). CL also reduced nuclear translocation of the Notch1 intracellular domain (NICD), accompanied by decreased expression of Notch1 target genes *Hes1* and *Hey1* (Fig. 4C, E). Overall, these findings suggest that β 3-AR stimulation suppresses Notch1 activation.

The TGFBI dependence of CL-induced Notch1 suppression and the role of Notch1 signaling in TGFBI-mediated adipose browning were examined under β 3-AR stimulation. TGFBI KD enhanced the CL-induced reductions in NICD and HES1 expression, while TGFBI OE attenuated these reductions (Fig. 4F–G). Under β 3-AR stimulation, TGFBI OE increased NICD and HES1 nuclear localization (Fig. 4H). In CL-treated obese mice, NICD and HES1 protein levels were markedly lower in the iWAT of TGFBI KO mice than in that of WT mice. A similar reduction was observed in CL-treated adipocytes derived from BM-MSCs of TGFBI KO mice (Fig. 4J, K). Collectively, these findings indicate that adipocyte-derived TGFBI acts autocrinally to sustain Notch1 signaling and limit β 3-adrenergic stimulation-mediated adipose browning, while CL promotes adipose browning by weakening this inhibitory axis.

Notch1 signaling is required for TGFBI-mediated suppression of β 3-adrenergic stimulation-induced adipose browning

Recombinant Jagged 1 (JAG1), a canonical Notch1 ligand (Hu et al., 2018), was used to activate Notch1 signaling. JAG1 treatment had minimal effects in WT cells, but suppressed TGFBI KD-induced increases in β 3-AR and C/EBP α expression (Fig. 4L). Conversely, pharmacological inhibition of Notch1 signaling with DAPT (iNOTCH1) (Jiang et al., 2011) minimally affected WT cells but restored the TGFBI OE-mediated reduction in β 3-AR and C/EBP α expression (Fig. 4M). These findings suggest that adipocyte-derived TGFBI activates cell-surface Notch1 autocrinally, thereby suppressing C/EBP α and β 3-AR expression.

EMI domain and RGD motif contribute to TGFBI–Notch1 interaction.

To clarify the molecular basis underlying autocrine adipocyte-derived TGFBI regulation of β 3-adrenergic stimulation-induced adipose browning via Notch1 signaling, the TGFBI structural regions responsible for its interaction with Notch1 were examined. Both the EMI domain and RGD motif contributed to the TGFBI–Notch1 interaction (Fig. 7A, B). FL recombinant TGFBI (rcTGFBI) activated downstream Notch1 signaling, reduced C/EBP α and β 3-AR expression, and ultimately suppressed UCP1 expression. However, deletion of the EMI domain or RGD motif abolished these effects (Fig. 5C). Similarly, FL rcTGFBI reduced mitochondrial signals, while EMI or RGD deletion restored these signals. The FAS1 domain did not contribute to the rcTGFBI–Notch1 interaction or β 3-adrenergic responsiveness (Fig. 5D, E, S5A).

Additionally, ASR490-mediated blockade of the Notch1 NRR domain was associated with reduced rcTGFB1–Notch1 binding, suggesting that the NRR region may contribute to this interaction (Fig. S5B, C).

TGFB1 negatively correlates with β 3-AR expression, and adipose browning in TGFB1-deficient mice depends on β 3-AR signaling

In obese scWAT, weak inverse correlations were observed between TGFB1 expression and *ADRB1* ($R = -0.16$) and *ADRB2* ($R = -0.24$). But a substantially stronger inverse correlation was observed with *ADRB3* ($R = -0.53$). These findings indicate a preferential association between higher TGFB1 expression and lower β 3-AR expression (Fig. 6A, B). Obese mice exhibited higher TGFB1 expression and lower β 3-AR expression, with a significant inverse correlation between these genes ($R = -0.4827$) (Fig. 6C, D, S6A).

The β 3-AR dependence of the enhanced browning response in TGFB1-deficient mice was assessed by pretreating WT and TGFB1 KO mice with the β 3-AR inhibitor SR59230A (iADRB3) before CL administration (Ootsuka et al., 2011). CL caused greater reductions in body weight and fat mass in TGFB1 KO mice than in WT mice. β 3-AR inhibition significantly attenuated these reductions without affecting lean mass (Fig. 6E; S6B–E). Similarly, β 3-AR inhibition counteracted the greater reduction in lipid droplet area observed in CL-treated TGFB1 KO mice (Fig. 6F). CL reduced serum TG levels more strongly in TGFB1 KO mice than in WT mice. β 3-AR inhibition abolished this difference, while serum NEFA (Non-esterified fatty acids) levels remained unchanged (Fig. S6F–G). At the molecular level, CL induced UCP1 and PGC1 α expression and PKA phosphorylation in TGFB1 KO mice more than in WT mice. β 3-AR inhibition abolished these differences (Fig. 6G).

Discussion

A major challenge in obesity treatment is restoring energy expenditure while reducing energy intake in impaired adipose tissue. Reduced β 3-adrenergic responsiveness in obesity limits adipose browning; therefore, maintaining β 3-AR expression and signaling may help sustain the adipose browning response to physiological stimuli, such as cold exposure and exercise (Valentine et al., 2022).

This study is the first to show that adipocyte-derived TGFB1 acts autocrinally to activate Notch1 signaling, suppresses C/EBP α and β 3-AR expression, and limits β 3-adrenergic responsiveness in adipose tissue. The EMI domain and RGD motif were also identified as key structural regions mediating this effect. The FAS1 domain, despite constituting the largest portion of TGFB1 (Tumbarello et al., 2012), showed no detectable contribution to Notch1 interaction or adipose browning.

A previous study reports that macrophage-derived TGFB1 activates Notch1 in adipocytes via a paracrine mechanism to regulate adipsin expression and adipose browning. The study reports that altering TGFB1 expression alone in adipocytes under basal conditions does not significantly affect adipose browning (Lee et al., 2023). Therefore, the autocrine function of TGFB1 under physiological stimuli in adipocytes remains unclear. In contrast to the previous study, this study examines the direct effects of adipocyte-derived TGFB1 on β 3-adrenergic responsiveness and adipose browning. Defining this mechanism is

essential for understanding adipocyte regulation of β 3-adrenergic responsiveness and adipose browning. To determine the potential role of secreted TGFBI in autocrine signaling, its cell-surface receptor was evaluated. Notch1 was selected because it binds TGFBI (Lee et al., 2023), while its downstream effector HES1 suppresses C/EBP α and thereby regulates β 3-AR (De Obaldia et al., 2013, Yan et al., 2024). Consistent with these findings, RNA-seq analysis showed that CL treatment reduced Notch1 downstream signaling (Fig. 5). Therefore, Notch1 was examined as a candidate receptor linking adipocyte-derived TGFBI to β 3-adrenergic responsiveness.

In this study, CL exerted a stronger regulatory effect on TGFBI than cold exposure, likely because CL selectively activates β 3-AR, whereas cold exposure induces broader receptor- and tissue-level responses (Byun et al., 2022, Collins and Surwit, 2001, Liu et al., 2019). *TGFBI* was inversely associated with *ADRB3* but showed no strong correlation with *ADRB1* or *ADRB2*, suggesting a preferential association between TGFBI expression and β 3-adrenergic stimulation-mediated adipose metabolism. Pharmacological blockade of β 3-AR abolished the enhanced adipose browning observed in TGFBI KO mice (Fig. 8E–H), indicating that TGFBI does not intrinsically determine basal adipose browning in adipocytes but limits β 3-adrenergic responsiveness by regulating β 3-AR signaling. Furthermore, the loss of TGFBI-dependent effects following Notch1 activation or inhibition supports a Notch1-dependent role for autocrine TGFBI in regulating β 3-AR signaling and adipose browning.

A study reports that SLC35D3 inhibits Notch1 signaling to promote adipose browning and alleviate obesity. SLC35D3 expression was decreased in obesity. Mechanistically, SLC35D3 binds the extracellular domain of Notch1, causing Notch1 to accumulate in the endoplasmic reticulum and preventing its translocation to the plasma membrane, thereby inhibiting Notch1 signaling and promoting adipose browning and obesity alleviation (Wang et al., 2023). Conversely, adipocyte-derived TGFBI activates cell surface Notch1, thereby reducing β 3-AR expression and adipose browning. Thus, reduced SLC35D3 and increased TGFBI expression in obesity may jointly enhance Notch1 signaling and limit adipose browning.

ECM protein–receptor interactions, including TGFBI, often involve multidomain, multivalent surface contacts rather than a single discrete binding site (Hynes, 2009, Son et al., 2013). Consistent with this concept, the EMI domain and RGD motif both contribute to TGFBI–Notch1 binding. FL TGFBI increased Notch1 activation and suppressed β 3-adrenergic stimulation-mediated adipose browning, while deletion of the EMI domain or RGD motif reversed these effects (Fig. 7). ASR490-blocking experiments suggest that the Notch1 NRR region may contribute to the TGFBI–Notch1 interaction (Fig. S5B, C). However, this approach cannot exclude steric or conformational effects; therefore, further domain-specific binding studies are needed to definitively identify the Notch1 interface.

Future research should identify therapeutic candidates that selectively disrupt the interaction between the TGFBI EMI domain and Notch1. Given that Notch1 signaling is also involved in normal cell differentiation and tissue homeostasis across various tissues, selectively inhibiting TGFBI-induced Notch1 activation rather than broadly blocking Notch1 signaling may provide a more precise therapeutic approach (Siebel and Lendahl, 2017). Therefore, future therapeutic development could focus on

competitive peptides or peptidomimetic inhibitors that selectively target the EMI domain-mediated TGFBI–Notch1 interface and block TGFBI-dependent Notch1 activation. These candidates could be optimized for adipose tissue delivery, for example, via conjugation with adipose-homing peptides. Small-molecule inhibitors disrupting the TGFBI–Notch1 interaction could provide an alternative strategy. For example, ASR490 (Tyagi et al., 2020), binding the Notch1 NRR region and blocking the TGFBI–Notch1 interaction, demonstrates the potential of this therapeutic strategy (Fig. S6B, S6C). These approaches may restore β 3-AR responsiveness and adipose browning in obesity while offering broader therapeutic potential for diseases associated with excessive Notch1 signaling.

However, this study has some limitations for direct translation to drug development. Confirming direct binding to the Notch1 NRR requires additional experiments using NRR mutants. Additionally, the detailed transcriptional regulatory mechanism underlying HES1-mediated suppression of C/EBP α remains unclear. Further validation is needed to establish direct HES1 binding to the *Cebpa* promoter and assess indirect suppression mediated by other transcriptional cofactors. Although increased TGFBI expression was inversely correlated with ADRB3 expression in human adipose tissue in obesity, further functional validation is required to determine whether TGFBI blockade restores β 3-AR responsiveness in human primary adipocytes or patient-derived adipose tissue. In summary, these findings suggest that the EMI domain and RGD motif of adipocyte-derived TGFBI could represent a potential obesity therapeutic target for restoring impaired β 3-AR responsiveness and adipose browning in obesity.

Conclusion

This study highlights adipocyte-derived TGFBI as an autocrine regulator that limits adipose browning in response to β 3-adrenergic stimulation in obesity. Adipocyte-derived TGFBI activates Notch1 signaling, suppresses C/EBP α and β 3-AR expression, and thereby limits β 3-adrenergic responsiveness. Accordingly, TGFBI deficiency enhanced CL316,243- and cold exposure-induced adipose browning, whereas β 3-AR inhibition abolished this phenotype. Notch1 signaling activation or inhibition also abolished the effects of TGFBI regulation, supporting a Notch1-dependent mechanism. Domain-mapping analyses further showed that the EMI domain and RGD motif contribute to the TGFBI–Notch1 interaction and suppression of adipose browning. Further studies should identify the specific Notch1 region binding the EMI domain, clarify the mechanism underlying HES1-mediated suppression of C/EBP α , and experimentally validate this regulatory pathway in human adipocyte and adipose tissue models. Collectively, this study identifies a previously unrecognized autocrine TGFBI–Notch1–HES1–C/EBP α – β 3-AR signaling axis restraining β 3-adrenergic responsiveness and adipose browning, highlighting adipocyte-derived TGFBI as a potential therapeutic target for obesity.

Abbreviations

HFD – High-fat diet

CL – CL316,243

iWAT – Inguinal white adipose tissue

β 3-AR (receptor protein) – β 3-adrenergic receptor

ADRB3 (gene symbol) – Adrenergic receptor beta 3

WT – Wild type

KO – Knockout

ECM – Extracellular matrix

BM-MSCs – Bone marrow-derived mesenchymal stem cells

UCP1 – Uncoupling protein 1

PGC1 α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPAR γ – Peroxisome proliferator-activated receptor gamma

eWAT – Epididymal white adipose tissue

KD – Knockdown

OE – Overexpression

Dox – Doxycycline

NICD – Notch1 intracellular domain

NRR – Negative regulatory region

TG – Triglyceride

FL – Full-length

TGFBI – Transforming Growth Factor Beta Induced

C/EBP α – CCAAT Enhancer Binding Protein Alpha

PKA – Protein kinase A

Co-IP – Co-immunoprecipitation

rWAT – Retroperitoneal white adipose tissue

BAT – Brown adipose tissue

Declarations

- **Ethics approval**

All animal experiments were conducted following the ethical guidelines of Kyungpook National University (Daegu, Republic of Korea) and approved by its Institutional Animal Care and Use Committee (Approval No. KNU-2023-0592).

- **Consent for publication**

Not applicable

- **Availability of data and materials**

The data supporting the findings of this study are included in the article and its supplementary materials. The data generated during this study are available from the corresponding author upon reasonable request. Publicly available datasets analyzed in this study were obtained from the GTEx portal (<https://gtexportal.org/>) and the NCBI Gene Expression Omnibus (GEO) database, with accession numbers provided in the manuscript.

- **Competing interests**

No potential conflict of interest relevant to this article was reported.

- **Funding**

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- **Authors' contributions**

Conceptualization, E.L., S.G.L., and J.O.N.; Methodology, E.L., S.G.L.; Investigation, E.L., S.G.L.; Validation, E.L., S.G.L., and J.O.N.; Formal analysis, E.L., S.G.L.; Resources, J.O.N.; Funding acquisition, J.O.N.; Supervision, J.O.N., S.G.L.; Writing-original draft, E.L., S.G.L.; Writing-review and editing, E.L., S.G.L., and J.O.N.; All authors read and approved the final manuscript.

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Not applicable

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Figures

Figure 1

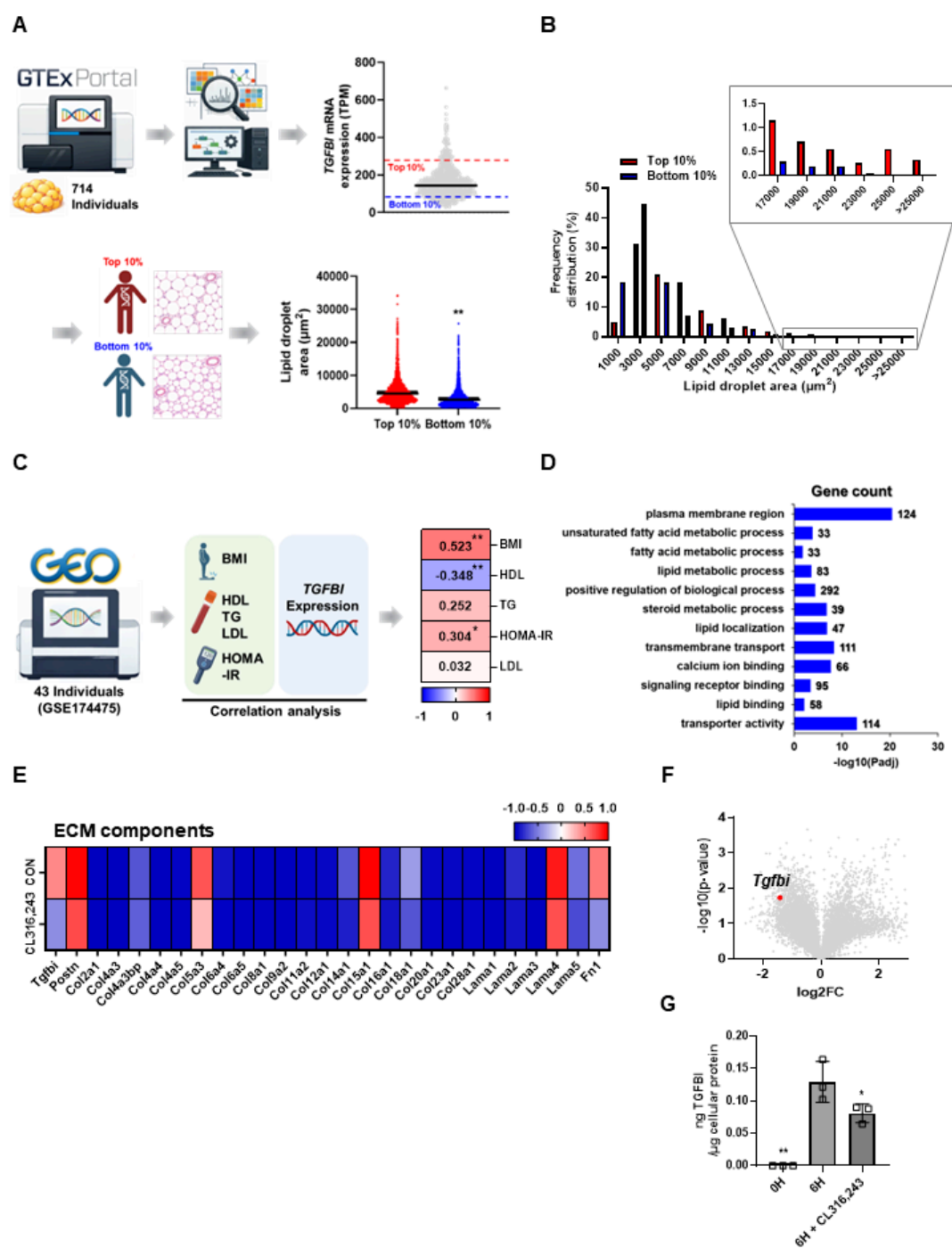


Figure 1

Adipose TGFB1 is associated with obesity-related metabolic dysfunction and is reduced by β 3-adrenergic stimulation.

(A) Schematic of the analysis of human subcutaneous WAT samples from the GTEx dataset ($n = 714$). Samples were ranked based on TGFB1 mRNA expression, and the top and bottom 10% groups were

selected for comparison. Each dot represents an individual lipid droplet. **(B)** Frequency distribution (%) of lipid droplet areas in the top and bottom 10% *TGFBI* mRNA expression groups. **(C)** Correlations between *TGFBI* mRNA expression in scWAT and clinical parameters from the GEO database (accession no. GSE174475, $n = 43$). The heatmap shows Pearson's correlation coefficients for BMI, HDL, TG, HOMA-IR, and LDL. **(D)** GO enrichment analysis of differentially expressed genes in iWAT from obese *Tgfb1* WT and KO (*TGFBI*^{-/-}) mice. **(E)** Heatmap of normalized ECM-related gene expression in adipose tissue from obese mice with or without CL treatment. Data were obtained from the GEO database (accession no. GSE86338). **(F)** Volcano plot of differentially expressed ECM-related genes. **(G)** *TGFBI* levels in the culture medium after 6 h of CL316,243 treatment. Data are presented as mean $\pm$ SEM. Statistical significance is denoted using asterisks: * $p < 0.05$; ** $p < 0.01$.

Figure 2

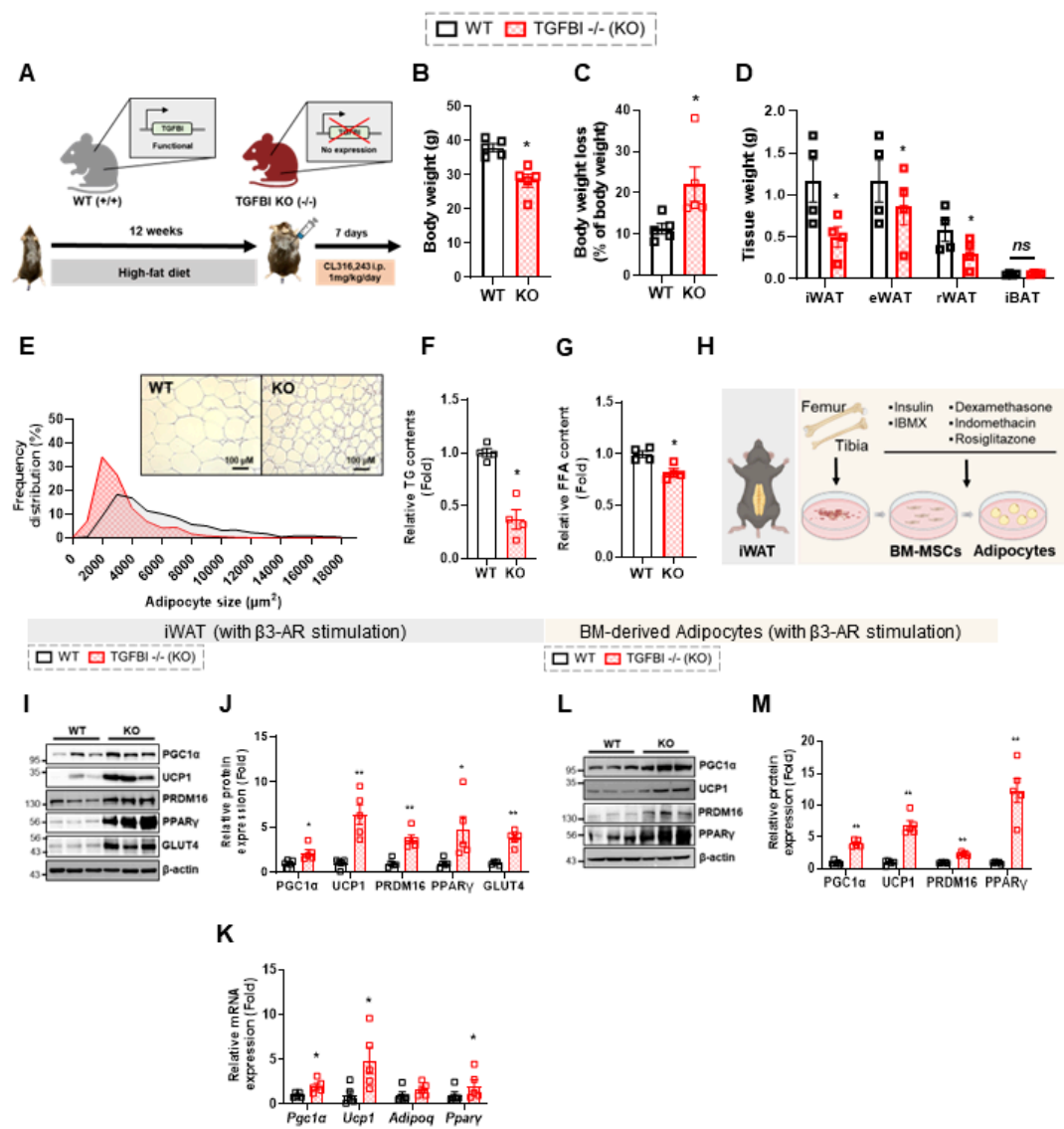


Figure 2

TGFBI deficiency enhances β 3-adrenergic stimulation-induced adipose browning.

(A) Schematic of the *in vivo* experimental design. WT and TGFBI KO mice were fed an HFD for 12 weeks, followed by *i.p.* administration of CL (1 mg/kg/day) to stimulate β 3-AR (n = 5–6). (B) Changes in body weight following CL treatment in WT and TGFBI KO mice. (C) Percentage body weight loss after CL

treatment. **(D)** Weights of four adipose tissue depots: iWAT, eWAT, rWAT, and BAT. **(E)** Representative images of H&E-stained adipose tissues (scale bar = 100 μ m) and frequency distributions of adipocyte sizes. **(F, G)** Serum concentrations of TGs **(F)** and FFAs **(G)** in mouse serum. **(H)** Schematic of the isolation of BM-MSCs from mice and their subsequent adipogenic differentiation into adipocytes. **(I, J)** Western blot bands **(I)** and quantified band intensities **(J)** for browning-related proteins (PGC1 α , UCP1, PRDM16, and PPAR γ) and GLUT4 in iWAT from CL-treated obese WT and KO mice. **(K)** mRNA expression levels of adipose browning-related genes (*Pgc1 α* , *Ucp1*, and *Ppar γ*) and *Adipoq* in iWAT from WT and KO mice. **(L, M)** Western blot bands **(L)** and quantified band intensities **(M)** for browning-related proteins (PGC1 α , UCP1, PRDM16, and PPAR γ) in BM-MSC-derived adipocytes after CL treatment. Data are presented as mean $\pm$ SEM. Statistical significance is denoted using asterisks: * p < 0.05; ** p < 0.01.

Figure 3

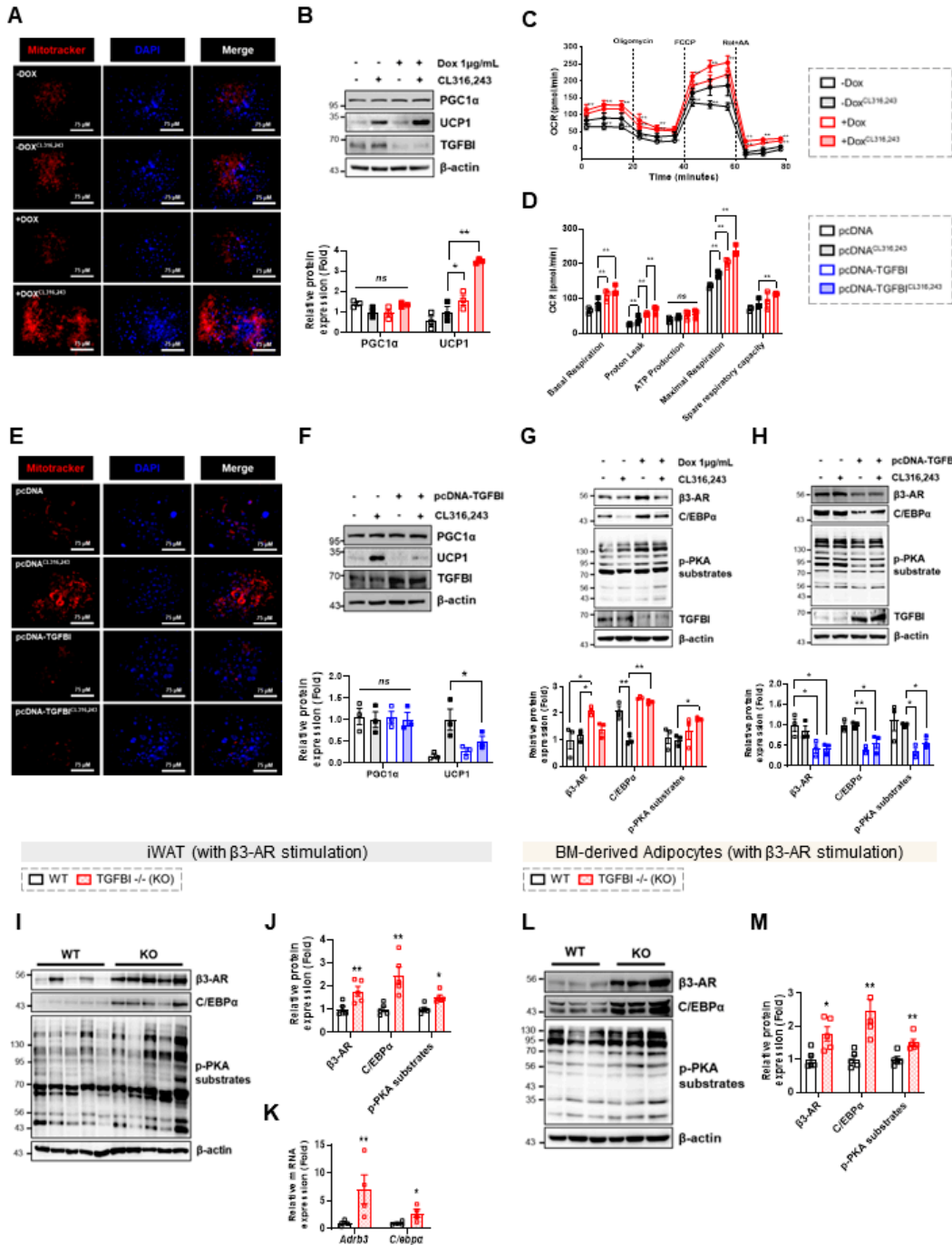


Figure 3

Adipocyte-derived TGFB1 regulates $\beta 3$ -AR expression, PKA phosphorylation, and $\beta 3$ -adrenergic stimulation-induced adipose browning.

(A) Representative images of MitoTracker-stained, Dox-inducible TGFB1 KD 3T3-L1 adipocytes (scale bar = 75 μ m). (B) Western blot bands and quantification of the adipose browning-related proteins PGC1 α and

UCP1 in Dox-inducible TGFBI KD 3T3-L1 adipocytes after treatment. **(C, D)** Seahorse analysis of total cellular oxygen consumption rate **(C)** and basal respiration, proton leak, ATP production, maximal respiration, and spare respiratory capacity **(D)**. **(E)** Representative images of MitoTracker-stained TGFBI OE 3T3-L1 adipocytes (scale bar = 75 μ m). **(F)** Representative Western blot bands and quantification of PGC1 α and UCP1 expression in the TGFBI OE 3T3-L1 adipocytes after CL treatment. **(G)** Representative Western blot bands for β 3-AR, C/EBP α , and p-PKA in TGFBI KD 3T3-L1 adipocytes after CL316,243 treatment, and corresponding quantification. **(H)** Representative Western blot bands for β 3-AR, C/EBP α , and p-PKA in TGFBI OE 3T3-L1 adipocytes after CL316,243 treatment, and corresponding quantification. **(I)** Representative Western blot bands for β 3-AR, C/EBP α , and p-PKA in iWAT from WT and TGFBI KO mice treated with CL316,243 (n = 5), and **(J)** corresponding quantification. **(K)** *Adrb3* and *C/ebp α* mRNA expression in iWAT from WT and TGFBI KO mice (n = 4). **(L)** Representative Western blot bands for C/EBP α , β 3-AR, and p-PKA in BM-MSC-derived adipocytes from WT and TGFBI KO mice after CL treatment (n = 5), and **(M)** corresponding quantification. Data are presented as mean $\pm$ SD for B–D, F–H and mean $\pm$ SEM for I–M. Statistical significance is denoted using asterisks: **p* < 0.05; ***p* < 0.01.

Figure 4

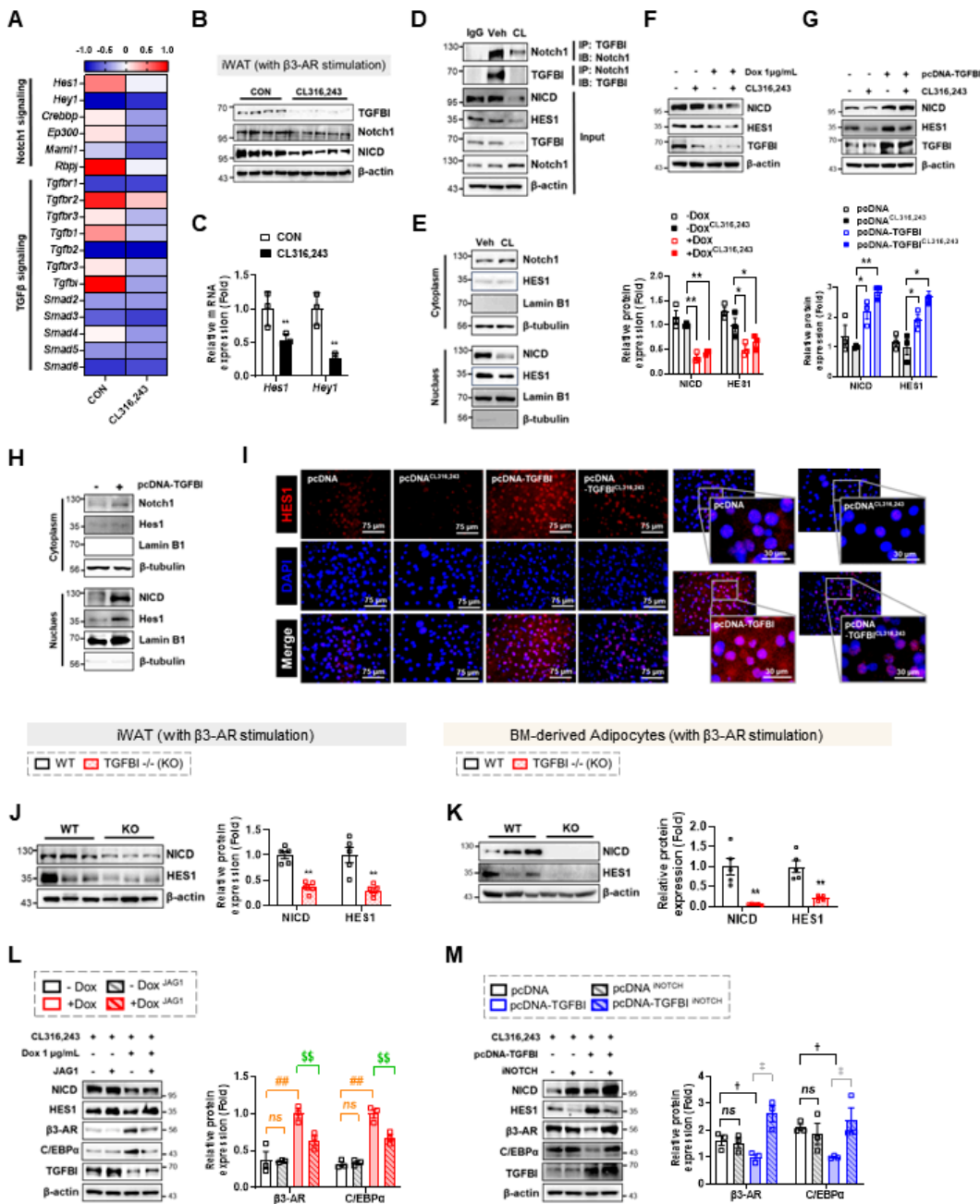


Figure 4

Autocrine TGFBI suppresses β 3-adrenergic stimulation-induced adipose browning via Notch1 signaling.

(A) Heatmap of differentially expressed genes associated with Notch1 and TGF- β signaling in iWAT from obese mice (accession no. GSE86338). (B) Western blots for TGFBI, Notch1, and NICD in iWAT from obese mice (n = 4–5). (C) *Hes1* and *Hey1* mRNA expression in 3T3-L1 adipocytes. (D) Interaction

between TGFBI and Notch1. **(E)** Cytoplasmic and nuclear NICD and HES1 levels after CL316,243 treatment. **(F)** Western blots and quantification of NICD and HES1 in TGFBI KD 3T3-L1 adipocytes. **(G)** Western blots and quantification of NICD and HES1 in TGFBI OE 3T3-L1 adipocytes. **(H)** Western blot analysis of cytoplasmic and nuclear NICD and HES1 levels in TGFBI OE 3T3-L1 adipocytes. **(I)** Representative immunofluorescence images of HES1 in TGFBI OE 3T3-L1 adipocytes (scale bar = 75 μ m). **(J)** Western blots and quantification of NICD and HES1 in iWAT (n = 5). **(K)** Western blots and quantification of NICD and HES1 in BM-MSCs (n = 5). **(L)** Western blots for NICD, HES1, β 3-AR, and C/EBP α in TGFBI KD 3T3-L1 adipocytes treated with and without CL316,243 and JAG1, and corresponding quantification. **(M)** Western blots for NICD, HES1, β 3-AR, and C/EBP α in TGFBI OE 3T3-L1 adipocytes treated with CL316,243, iNOTCH1 (DAPT), or both, and corresponding quantification. Data are presented as mean $\pm$ SD for C, F–G, L– M and mean $\pm$ SEM for J–K. **(C, F, G, J and K)** Statistical significance is denoted using asterisks: * p < 0.05; ** p < 0.01. **(L, M)** Orange # symbols and lines denote comparisons with –Dox as the control group: ## p < 0.01. Green \$ symbols and lines denote comparisons between +Dox and +Dox^{JAG1}: \$\$ p < 0.01. Black † symbols and lines denote comparisons with pcDNA as the control group: † p < 0.05. Grey ‡ symbols and lines denote comparisons between pcDNA-TGFBI and pcDNA-TGFBI^{iNOTCH1}: ‡ p < 0.05.

Figure 5

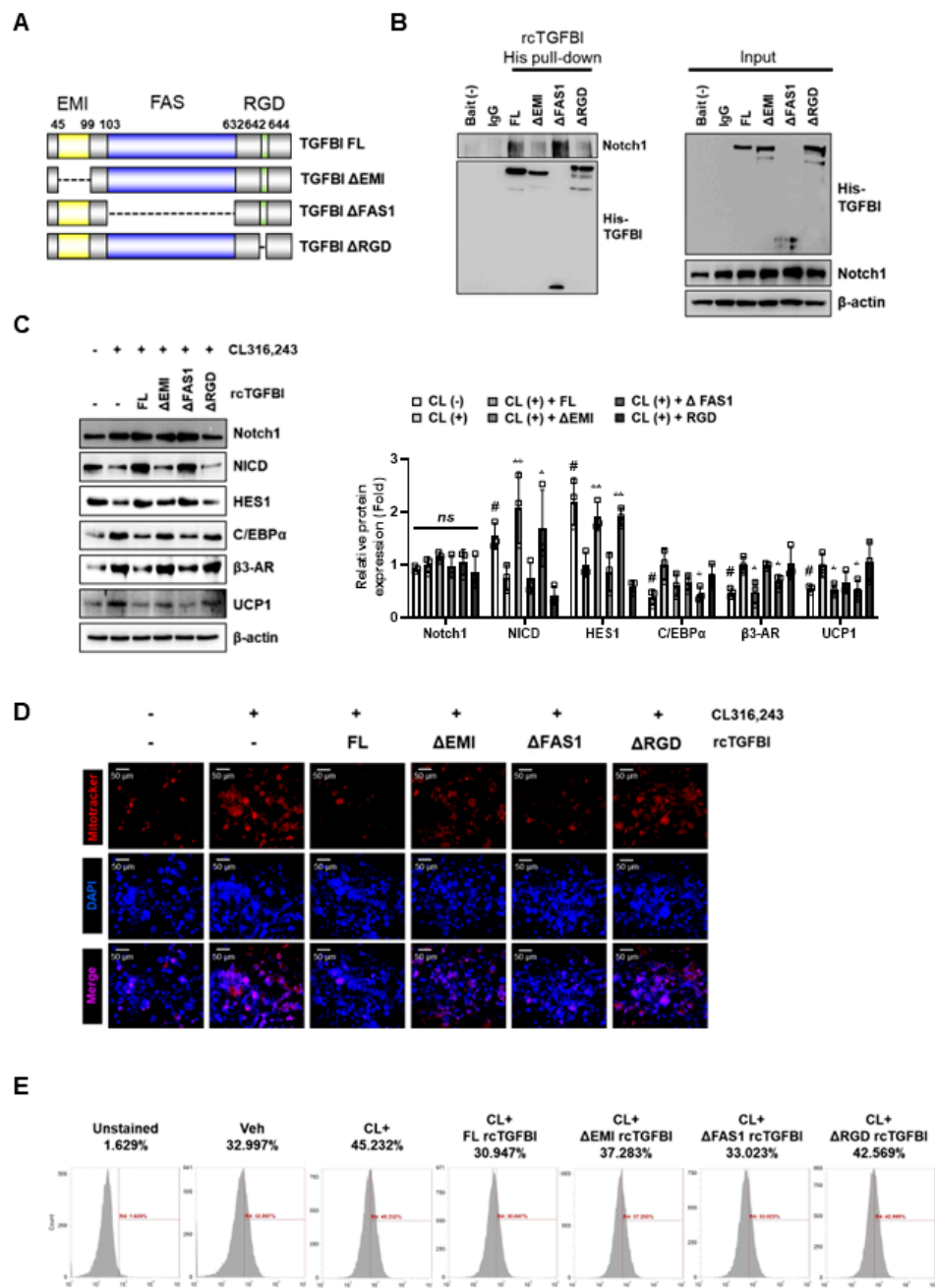


Figure 5

EMI domain and RGD motif provide the principal interface for TGFBI–Notch1 binding.

(A) Schematic of the domain organization of the FL TGFBI and the EMI-, FAS1-, and RGD-deletion mutants. (B) His pull-down assay showing the binding of His-tagged TGFBI to Notch1 for FL and mutant TGFBI proteins. IgG was used as a negative control to assess binding specificity, and input lysates were

included to confirm the presence of total TGFBI and Notch1 proteins. **(C)** Western blot bands and quantification of Notch1, NICD, HES1, C/EBP α , β 3-AR, and UCP1 in control cells, CL-treated cells, and CL-treated cells supplemented with FL or domain-deleted recombinant TGFBI proteins. **(D)** Representative images of MitoTracker-stained control cells, CL-treated cells, and CL-treated cells supplemented with FL or domain-deleted recombinant TGFBI proteins (scale bar = 75 μ m). **(E)** Flow cytometry histograms showing fluorescence intensity in MitoTracker-stained control cells, CL-treated cells, and CL-treated cells supplemented with FL or domain-deleted recombinant TGFBI proteins. Data are presented as mean $\pm$ SD. # p < 0.05 CL- (Veh) versus CL+; * p < 0.05 and ** p < 0.01, versus the CL-treated group (CL+).

Figure 6

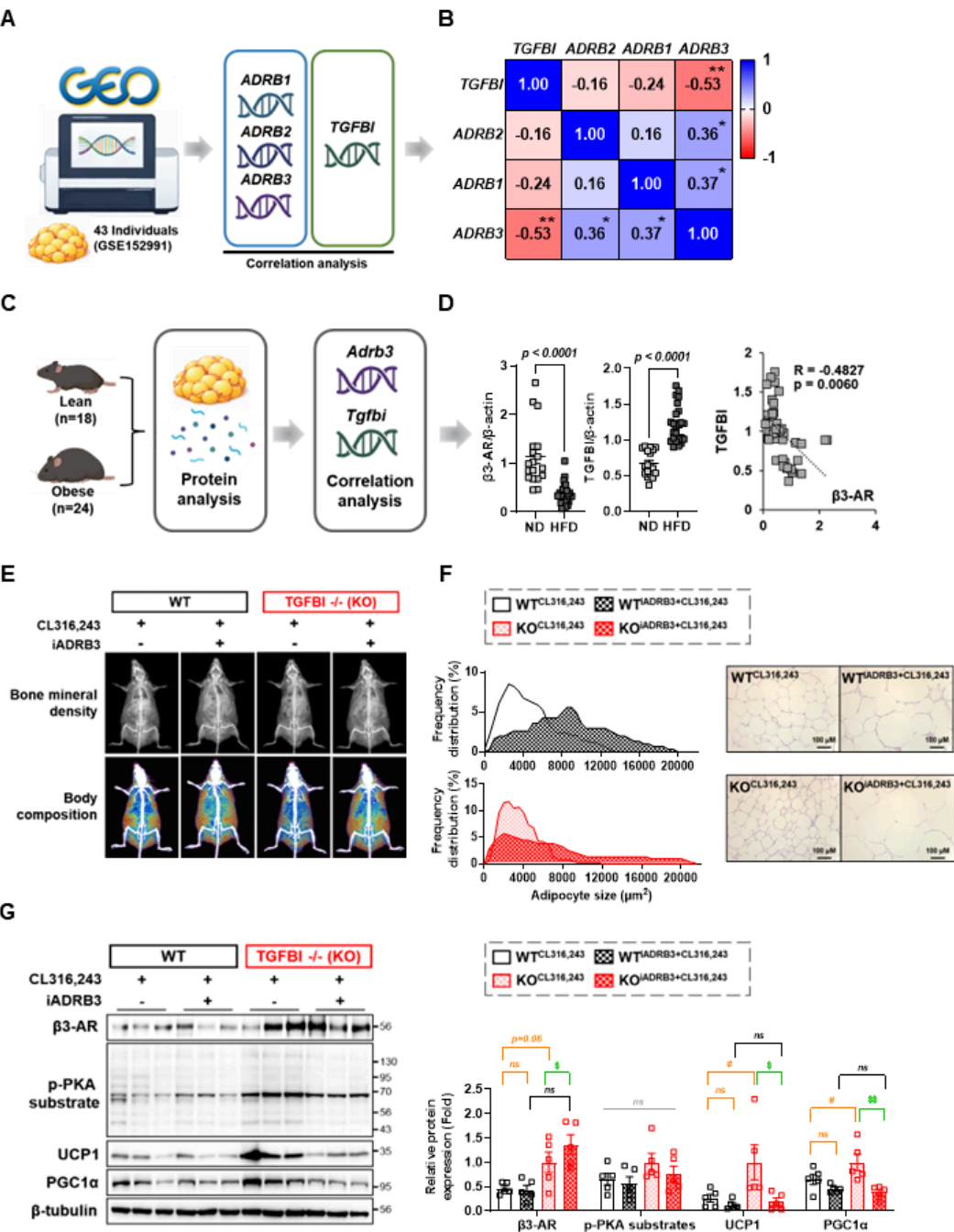


Figure 6

TGFBI negatively correlates with β 3-AR expression, and enhanced adipose browning in *TGFBI*-deficient mice is β 3-AR-dependent.

(A) Workflow for Pearson's correlation analysis of *TGFBI* and adrenergic receptor gene expression in scWAT from patients with obesity (accession no. GSE152991). (B) Heatmap of Pearson's correlation

coefficients among *TGFBI*, *ADRB1*, *ADRB2*, and *ADRB3* expression levels. **(C)** Workflow for analyzing *TGFBI* and β 3-AR expression in iWAT from lean and obese mice. **(D)** *TGFBI* and β 3-AR protein levels based on Western blot band intensities and the Pearson's coefficient for their association. **(E)** Representative dual-energy X-ray absorptiometry images of mice acquired in two modes for each group: bone mineral density (top) and body composition analysis (bottom). In body composition mode, bone structures are displayed in white, intermediate density regions containing mixed fat and lean tissues in green, lean mass in blue, and adipose tissue in red. **(F)** Representative H&E-stained adipose tissue images (scale bar = 100 μ m) and frequency distributions of adipocyte size. **(G)** Western blot images showing β 3-AR, p-PKA, UCP1, and PGC1 α protein expression in iWAT from WT and *TGFBI* KO mice treated with CL and iADRB3, with corresponding quantification. Data are presented as mean $\pm$ SEM. Orange # symbols and lines denote comparisons with WT^{CL316,243} as the control group: #*p* < 0.05. Green \$ symbols and lines denote comparisons between KO^{CL316,243} and KO^{iADRB3+CL316,243}: \$*p* < 0.05; \$\$*p* < 0.01.

Supplementary Files

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