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Visualization and Quantification of Browning Using a Ucp1-2A-Luciferase Knock-in Mouse Model (Postprint)

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Abstract

Both mammals and adult humans possess classic brown adipocytes and beige adipocytes, and the amount and activity of these adipocytes are considered key factors in combating obesity and its associated metabolic diseases. Uncoupling protein 1 (Ucp1) is the functional marker of both brown and beige adipocytes. To facilitate a reliable, easy, and sensitive measurement of Ucp1 expression both in vivo and in vitro, we generated a Ucp1-2A-luciferase knock-in mouse by deleting the stop codon for the mouse Ucp1 gene and replacing it with a 2A peptide. This peptide was followed by the luciferase coding sequence to recapitulate the expression of the Ucp1 gene at the transcriptional and translational levels. With this mouse, we discovered a cold-sensitive brown/beige adipose depot underneath the skin of the ears, which we named uBAT. Because of the sensitivity and high dynamic range of luciferase activity, the Ucp1-2A-luciferase mouse is useful for both in vitro quantitative determination and in vivo visualization of nonshivering thermogenesis. With the use of this model, we identified and characterized axitinib, an oral small-molecule tyrosine kinase inhibitor, as an effective browning agent.

Full Text

Preamble

Visualization and Quantification of "Browning" Using a Ucp1-2A-Luciferase Knockin Mouse Model

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Abstract

Both mammals and adult humans possess classical brown adipocytes and beige adipocytes, and the amount and/or activity of these thermogenic cells are considered key factors in combating obesity and its associated metabolic diseases. Uncoupling protein 1 (UCP1) is the functional marker of both brown and beige adipocytes. To facilitate reliable, easy, and sensitive measurement of Ucp1 expression both in vivo and in vitro, we generated a Ucp1-2A-luciferase knockin mouse by deleting the stop codon of the mouse Ucp1 gene and replacing it

with a 2A peptide sequence followed by the luciferase coding sequence. This design ensures that luciferase expression recapitulates Ucp1 expression at both transcriptional and translational levels.

Using this mouse model, we discovered a cold-sensitive brown/beige adipose depot underneath the skin of the ears (uBAT). Due to the sensitivity and high dynamic range of luciferase activity, the Ucp1-2A-luciferase mouse enables quantitative determination in vitro and visualization in vivo of non-shivering thermogenesis. Using this model, we identified and characterized axitinib, an oral small-molecule tyrosine kinase inhibitor, as an effective "browning" agent.

Key words: uncoupling protein 1; knockin; browning; thermogenesis; luciferase; in vivo imaging
Running title: Knockin mouse model to monitor adaptive thermogenesis

Introduction

The obesity epidemic has intensified research efforts to understand adipose tissue development and function. Currently, three distinct types of adipocytes are recognized: classical multilocular brown adipocytes in brown adipose tissue (BAT), classical unilocular white adipocytes (the main cell type in white adipose tissue, WAT), and beige/brite adipocytes found within WAT. While WAT stores excess energy as fat, brown and brite/beige adipocytes can dissipate energy as heat.

Recent studies have demonstrated that adult humans possess functional BAT that can be activated by mild cold exposure (1-3) and treatment with 3-agonists (4). In humans, BAT activation reduces elevated blood triglycerides and alleviates obesity (5). In mice, BAT transplantation decreases body weight and improves glucose homeostasis and insulin sensitivity in both chow-fed and high-fat-fed animals (6, 7). Therefore, enhancing thermogenic function in BAT and/or recruiting BAT-like brite/beige adipocytes in WAT may represent an effective therapeutic strategy to combat obesity and its associated disorders.

The energy-dissipating, thermogenic function of BAT and beige adipocytes is primarily mediated by uncoupling protein 1 (UCP1). UCP1, located in the inner mitochondrial membrane, allows protons to flow back into the mitochondrial matrix, thereby uncoupling electron transport from ATP production. By enhancing this futile cycle of proton and electron transport, UCP1 increases energy expenditure in the form of heat. Consequently, increasing UCP1 expression and activity in adipose tissues is regarded as a safe and promising approach to enhance whole-body energy expenditure and combat obesity. Under physiological conditions, brown and beige adipocytes readily respond to stimuli such as chronic cold exposure and 3-adrenergic agonists by increasing UCP1 expression and activity. Pharmacological administration of cytokines or compounds such as FGF21, adenosine A2A receptor agonists, and berberine has also been shown to enhance energy expenditure by inducing UCP1 expression in BAT and/or WAT (8-10).

Despite these advances, more efficacious reagents and detailed understanding of the underlying mechanisms regarding recruitment and activation of Ucp1-positive adipocytes are still needed. To gain deeper insight into this area, several transgenic mouse models have been established. UCP1-GFP and UCP1-CreER; ROSA-tdRFP mice have been used to transiently or permanently label UCP1-expressing cells in vivo, respectively. Combined use of these two transgenic models allows tracing of current and past UCP1-positive cells to investigate the interconversion of brite/beige adipocytes (11). Galmozzi et al. generated the "thermo mouse,"where a Ucp1 promoter-driven luciferase reporter was integrated into the Y chromosome (12), restricting this model' s utility to male mice. To obtain a more comparative and comprehensive understanding of "browning" in both male and female mice during development and/or disease, a more reliable functional readout is needed to monitor metabolic changes in fat pads induced by cold exposure or drugs (11). Here, we report the generation of Ucp1-luciferase reporter mice, our discovery of an unappreciated brite/beige fat pad underneath the ears, and our identification and characterization of an effective agent for "browning."

Research Design and Methods

Generation of the Ucp1-2A-Luciferase Construct

Standard homologous recombination procedures were used to target firefly luciferase following exon 6 of the endogenous Ucp1 gene. A left arm of 4.3 kb before the Ucp1 stop codon and a right arm of 2.5 kb immediately after the stop codon, flanking a 2A peptide (GSGATNFSLLKQAGDVEENPGP) and firefly luciferase coding sequence followed by a floxed neomycin selection cassette, were used to generate targeted insertion at the Ucp1 locus. Correctly targeted ES clones were identified, and the floxed neomycin cassette was removed before use in chimera production. The 129 mouse strain was used for construction of the knockin mice, which were backcrossed with C57BL/6J for 10 generations before use in this study. Primers and conditions for Ucp1-Luc genotyping were as follows: F1: CAACAGCGGGCTCTGCAC; R1: AACCGTAGGTTGCGCACTC; R2: CACGGTAGGCTGCGAAATG; 95°C for 15 minutes, 36 cycles of [94°C for 30 s, 57°C for 30 s, and 72°C for 60 s], followed by 72°C for 5 minutes. The resulting PCR products are 500 bp (wild-type) and 1000 bp (UCP1) in length.

Animal Studies

Male Ucp1+/LUC mice (7 weeks old) on a C57BL/6 background were housed in specific-pathogen-free facilities, maintained on a 12 h light-dark cycle at 22°C, and fed standard chow (15.9 kJ/g, 10% energy as fat, 20% energy as protein, 70% energy as carbohydrate). For cold challenge experiments, mice were housed at 4°C for 3, 6, 9, or 12 h. For thermoneutrality experiments, mice were housed at 4°C or 22°C for 12 h, then at 30°C for 48 h. For drug challenge experiments, mice received CL316243 (1 mg/kg) for 2 days before in vivo luciferase imaging. Alternatively, mice were fed a high-fat diet (21.9 kJ/g, 60% energy as fat, 20%

energy as protein, 20% energy as carbohydrate; D12492; Research Diet, New Brunswick, NJ, USA) for 8 weeks. Concurrently, mice received axitinib (Pfizer Inc, manufactured in Freiburg, Germany, 10 mg/kg body weight) or phosphate-buffered saline (PBS) daily by oral gavage for 8 weeks. Energy expenditure was measured by indirect calorimetry, with oxygen consumption (VO2) measured using the Oxymax system (Columbus Instruments).

In Vivo, Ex Vivo, and In Vitro Luciferase Imaging

Bioluminescence imaging was performed using the In Vivo Imaging System 50 (Xenogen Corp.). D-luciferin (Promega) was resuspended at 15 mg/ml in D-PBS. For in vivo imaging, mice were injected intraperitoneally (ip) with 150 mg/kg body weight D-luciferin solution. Ten minutes later, mice were placed in a clear Plexiglas anesthesia box (2.5–3.5% isoflurane) allowing unimpeded visual monitoring. The anesthesia supply tube was split to deliver the same concentration to the manifold inside the imaging chamber. After full anesthesia (approximately 5 minutes), mice were transferred to nose cones attached to the manifold in the imaging chamber and luciferase signals were acquired. Imaging time was set between one and five minutes per side (dorsal/ventral) depending on the experiment. Luminescence was quantified using Living Image software.

For ex vivo imaging of various fat depots, mice were ip injected with 150 mg/kg D-luciferin solution for 20 minutes before fat depots were harvested and imaged. For in vitro imaging of cultured cells, D-luciferin was resuspended at 30 mg/ml in sterile water as stock solution. At day 10 of adipogenic differentiation, D-luciferin was added to cell medium at 150 g/ml and images were captured 10 minutes later.

In Vitro Analysis of Luciferase Activity

In vitro luciferase activity was measured using the Steady-Glo® Luciferase Assay System E2510 (Promega). Cells and mouse tissues were washed twice with ice-cold PBS, homogenized, and lysed with Lysis buffer (Promega) for 30 minutes on ice. Cell lysates were centrifuged at 12,000g for 15 minutes at 4°C, and supernatants were collected. Twenty l of supernatant and 20 l of Dulbecco' s Modified Eagle Medium (DMEM) were mixed with 40 l of Steady-Glo® reagent in 96-well solid-bottom white plates (CulturPlateTM-96, PerkinElmer, MA, USA), and signals were measured in a Veritas TM microplate luminometer (Turner Biosystems, CA, USA). Protein concentration was quantified by BCA method, and relative luminescence activity was normalized accordingly.

Isolation of Preadipocytes from Adipose Tissues

Isolation of stromal cells from fat tissue was performed as previously described (13-15). Subcutaneous, epididymal, and interscapular brown adipose tissue were dissected, rinsed in PBS, minced, and digested for 40 minutes at 37°C in 0.1%

(w/v) collagenase solution (Collagenase type I, dissolved in D-Hanks buffer). Digested tissue was filtered through a 100- m nylon mesh to remove undigested material before centrifugation at 1000g for 5 minutes. The pellets (preadipocytes) were collected, washed in PBS, and resuspended in 5 mL DMEM supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) by pipetting to obtain single-cell suspensions. Cells were cultured in 4-cm dishes with medium changed daily.

For white adipocyte differentiation assays, preadipocytes from subcutaneous adipose tissue were plated in 6-well plates and cultured in DMEM with 10% FBS. Two days after reaching confluence (day 0), differentiation was induced by incubation in differentiation medium containing 5 g/ml insulin (Sigma), 1 M dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine (Sigma). After 2 days, media were replaced with DMEM supplemented with 10% FBS, 5 g/ml insulin, and 1 M rosiglitazone, with re-feeding every 2 days until day 8. AM580, A-769662, CL316243, fmoc-leu-OH, retinoic acid, -aminoisobutyric acid, SD19, axitinib, masitinib, tanduinib, cediranib, and BIBF1120 were dissolved in dimethylsulfoxide (DMSO), and FGF21 was dissolved in PBS for cell culture studies. At day 8, cells were exposed to compounds at indicated concentrations in DMEM supplemented with 10% FBS for an additional two days.

Half-lives of UCP1 and Luciferase in Brown Adipocytes

Brown adipocytes isolated from iBAT were cultured and differentiated as described above. Mature brown adipocytes were treated with CL316,243 for 2 days, then with 10 M cycloheximide at time zero to arrest protein translation. At various time points thereafter, cells were pelleted by centrifugation, washed twice in ice-cold PBS, and lysed on ice for 30 minutes with lysis buffer. Insoluble material was removed by centrifugation.

Seahorse Analysis

The oxygen consumption rate (OCR) of ingWAT cells was analyzed using the XF24 Seahorse bioanalyzer as previously described with minor modifications (16). Briefly, one day prior to analysis, cells were treated with axitinib (1 M) or DMSO. Twenty-four hours later, cells were equilibrated in sodium bicarbonate-free DMEM for 1 hour in a CO2-free incubator. The following drugs were sequentially loaded to each well to measure basal, norepinephrine (NE)-stimulated (10 M NE), ATP production (2 M Oligomycin), maximal (2 M FCCP), and non-mitochondrial (1 M Antimycin A + 3 M Rotenone) respiration. Uncoupled OCR was calculated as the difference between stimulated and non-mitochondrial OCRs.

Glucose and Insulin Tolerance Tests

For glucose tolerance tests (GTT), nine pairs of mice were fasted overnight and injected intraperitoneally (i.p.) with 10% glucose at 1 g/kg body weight. For

insulin tolerance tests (ITT), mice were starved for 6 h and i.p. injected with 0.5 U/kg body weight recombinant human insulin (Sigma). Blood glucose was monitored from tail vein blood using a glucometer (ACCU-CHEK Advantage; Roche Diagnostics China, Shanghai, China) at various time points.

Histochemistry and Immunohistochemistry

Mice were euthanized by cervical dislocation under sodium pentobarbital anesthesia (50 mg/kg, i.p.), and adipose tissues were isolated and fixed in 4% formaldehyde overnight at room temperature immediately after sacrifice. Tissues were paraffinized, sectioned by microtome, and slides were stained with hematoxylin and eosin (HE) (Sigma) following standard protocols. For UCP1 immunohistochemistry, paraffin-embedded sections (6 m) were incubated with anti-UCP1 antibody (1:500; Abcam; ab10983) and examined by light microscopy.

Immunofluorescence Staining Assay

For immunofluorescence staining, coverslips plated with white adipocytes derived from MEF cells were fixed in 4% formaldehyde, blocked with 5% bovine serum albumin (BSA) in PBS at room temperature for 1 h, then incubated with rabbit anti-UCP1 antibody (1:500; Abcam; ab10983) and anti-luciferase (1:200; Abcam; 181640) at 4°C overnight. Sections were subsequently incubated with Alexa Fluor 647-conjugated anti-rabbit secondary antibody (1:200; Abcam; ab150079) and Alexa Fluor 488-conjugated anti-goat secondary antibody (1:200; Abcam; ab150129) at room temperature for 1 h, and nuclei were stained with DAPI. Coverslips were mounted onto glass slides and imaged using a Leica microscope.

Western Blot

Cells were washed twice with ice-cold PBS and lysed with RIPA buffer (Beyotime) for 30 minutes on ice. Cell lysates were centrifuged at 12,000g for 15 minutes at 4°C, and supernatants were collected. Forty g of cellular proteins were resolved by 12% SDS-PAGE and transferred to PVDF membrane (Millipore). Membranes were probed overnight with specific antibodies at 4°C, washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBST), then incubated with horseradish-peroxidase-conjugated secondary antibody for 4 hours at 4°C. Membranes were developed using ECL Plus developing agent (GE Healthcare), stripped with stripping buffer (Comwin biotech), and reprobed with other antibodies when necessary. Primary antibodies used were: UCP1 (1:1000; Abcam; ab10983); luciferase (1:1000; Abcam; 181640); -actin (1:2000; Abcam; ab8227); P-STAT3 (1:1000; 9145; CST); and STAT3 (1:1000; 9132, CST).

RNA Extraction, Reverse Transcription, and Quantitative PCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen). First-strand cDNA synthesis was performed with Superscript™ III Reverse Transcriptase (Invitrogen). mRNA levels were quantified using SYBR® Premix Ex Taq™ (TaKaRa) under optimized conditions following the manufacturer' s protocol. 18S ribosomal RNA was used as the reference gene. All primers are listed in Supplemental Table S1.

Statistical Analysis

Data are expressed as means ± SEM. All comparisons were analyzed by unpaired, two-tailed Student' s t-tests. A p value of less than 0.05 was considered significant.

Results

Generation of Ucp1-2A-Luciferase Knockin Mice

To generate the Ucp1-luciferase reporter knockin mouse, we created a construct containing the firefly luciferase coding sequence preceded by a 2A peptide encoding sequence from Hand, Foot, and Mouth disease virus and followed by a floxed neomycin cassette. The construct was targeted into the last coding exon of the Ucp1 gene in mouse embryonic stem (ES) cells, replacing the endogenous Ucp1 stop codon with the 2A peptide plus luciferase encoding sequence (Fig. 1A [Figure 1: see original paper]). The 18-amino-acid 2A peptide undergoes cleavage between its C-terminal glycine and N-terminal proline through a ribosomal-skipping mechanism (17). Linking proteins with 2A peptide sequences thus enables cellular expression of multiple discrete proteins in essentially equimolar quantities (18). Consequently, the resulting knockin luciferase was brought under control of the endogenous Ucp1 transcriptional unit following Cre-mediated excision of the floxed neomycin gene. Correctly targeted ES clones were injected into blastocysts of 129 strain mice, and germline-transmitted mice were identified by PCR analysis (Fig. 1B). Expression of luciferase and cleavage of the 2A peptide were validated by Western blotting (Fig. 1C), luciferase activity assay (Fig. 1D), whole-body imaging (Fig. 1E), and immunohistochemical analysis (Fig. 1F). Figure 1C showed that UCP1 expression was not compromised by insertion of the 2A-luciferase cassette. Moreover, wild-type (WT) mice did not express luciferase in their BAT, while Ucp1-2A-luciferase knockin mice showed robust luciferase protein expression with the predicted molecular mass, indicating that the 2A peptide was recognized and fully functional (Fig. 1C). Additionally, the luciferase staining pattern overlapped that of UCP1 protein, suggesting co-localization of luciferase and UCP1 (Fig. 1F). Luciferase was functionally active, as its substrate luciferin was converted to the appropriate product with emission of luminescence (Fig. 1D and 1E).

Ucp1-2A-Luciferase Recapitulates the Expression Pattern of UCP1

We examined how Ucp1-reporter mice responded to treatment with a 3-adrenergic agonist and cold exposure. Luciferase imaging demonstrated robust signal increases in areas corresponding to interscapular BAT (iBAT) and inguinal WAT (ingWAT) (Fig. 2A [Figure 2: see original paper]). Similarly, luciferase enzymatic activity in mice housed at 4°C for 6 hours increased significantly compared to mice kept at room temperature (Fig. 2B). Furthermore, we dissected fat pads from mice kept at room temperature (22°C) or exposed to 4°C for 12 hours. Upon cold exposure, luciferase activity was greatly enhanced in dissected fat pads, including iBAT, cervical BAT (cBAT), axillary BAT (aBAT), and ingWAT, but not epididymal WAT (eWAT) (Fig. 2C). To confirm that luciferase activity reflected UCP1 expression patterns, we compared Ucp1 mRNA levels with luminescence measurements. qPCR analyses demonstrated that Ucp1 mRNA levels mirrored luciferase activity in all fat pads (Fig. 2D and 2E). Western blot analyses revealed UCP1-positive bands only in fat depots containing brown or brite/beige adipocytes, including BATs and ingWAT, confirming that luciferase activity serves as a sensitive surrogate marker for UCP1 (Fig. 2F-G).

We performed experiments to determine luciferase and UCP1 expression in vivo at various time points. Relative luciferase activity, Ucp1 RNA, and protein expression were measured in iBAT and ingWAT after mice were kept at 4°C for 0, 3, 6, 9, and 12 hours. As shown in Fig. S1, Ucp1 RNA and protein expression were concordant with luciferase activity. Fluorescence intensity remained stable and continuous within 2 hours by in vivo imaging. The half-lives of UCP1 and luciferase were measured and found to be 10 hours and 5 hours, respectively (Fig. S2). Expression of UCP1 and luciferase was stable and sustained in normal cells, allowing luciferase activity to mirror UCP1 expression in vivo and in vitro.

To verify the reliability of this mouse model, we studied Ucp1 expression during interconversion from white adipocytes toward the brite phenotype under thermoneutral conditions (30°C). As shown in Fig. S3, UCP1 expression in iBAT, cBAT, ingWAT, and eWAT increased at 4°C and decreased at 30°C. These observations support that our Ucp1-luciferase knockin mouse accurately reflects UCP1 expression and serves as a useful tool to quantify changes in UCP1 expression in vivo.

Ucp1-2A-Luciferase Mice Show an Age-Dependent Decline in Luminescence

BAT helps maintain body temperature in neonatal rodents, and its function decreases with age (19-21). To monitor UCP1 activity with age and determine an optimal age for imaging "browning" in Ucp1-2A-luciferase knockin mice, we compared mice at 3, 6, 10, and 18 weeks of age. Live imaging intensity decreased with age, with 3-week-old mice showing the highest UCP1 expression and luciferase activity (Fig. 3A [Figure 3: see original paper]-3E).

Identification of a Novel Beige/BAT Fat Depot Underneath the Ears

By detecting Ucp1-driven luciferase activity, we identified a novel fat depot underneath the ears that exhibited strong luciferase activity, which we named uBAT (underneath ear brown adipose tissue). As illustrated in Figures 1E, 2A, and 3A, luciferase imaging intensity was highly concentrated around the ears and further enhanced upon cold exposure or treatment with a 3-adrenergic agonist. To characterize the precise anatomical location of uBAT, we dissected tissues from this area and performed immunohistochemical and molecular analyses. As shown in Figure 4A [Figure 4: see original paper]-C, the positive tissue was not the ear itself but a diffuse fat pad underneath the ears. H&E histological staining revealed that the fat pad contained both unilocular and multilocular adipocytes at room temperature (Fig. 4D). Moreover, Ucp1 expression was readily detected in multilocular adipocytes even at room temperature, and the percentage of these UCP1-positive multilocular adipocytes further increased upon cold exposure (Fig. 4E). This observation was consistent with luciferase assay results showing that uBAT luciferase activity was relatively high—approximately 25% of that in iBAT and 10-fold higher than in ingWAT (Fig. 4E). After cold exposure, luciferase activity increased further (by 3.5-fold) to levels comparable to iBAT at room temperature (Fig. 4E). Brown and beige-related gene expression levels in uBAT were examined by qPCR. Similar to ingWAT and iBAT, brown-related genes including Ucp1, Prdm16, and Cidea were significantly induced in uBAT in response to cold exposure (Fig. 4F-H). We examined expression of a series of classical brown or beige adipocyte markers to further identify whether uBAT represents classical brown or beige adipocytes. Beige adipocyte markers including Tbx1, Tmem26, Cd137, and Hoxc9 were expressed at levels comparable to ingWAT (Fig. 4F-H) and were further induced in uBAT. In contrast, Zic1, a marker for classical brown adipocytes, was detected in uBAT and induced approximately 3.5-fold during cold exposure (Fig. 4F-H). Compared with iBAT and ingWAT, uBAT displayed an intermediate expression pattern between iBAT and ingWAT. We also studied UCP1 expression under thermoneutral conditions (30°C). As shown in Fig. S3A and 3E, UCP1 expression in uBAT increased at 4°C and decreased at 30°C. Taken together, these results strongly suggest that UCP1-positive cells in uBAT represent a mixture of beige adipocytes and classical BAT. Thus, our Ucp1-2A-luciferase knockin mice revealed a previously unrecognized adipose tissue underneath the ears that efficiently undergoes browning and/or activation in response to cold exposure or 3-adrenergic activation (Fig. 2A-B).

Ucp1-2A-Luciferase System Serves as a Convenient Reporter Model for Drug Screening

Because we could easily and faithfully detect Ucp1 induction in Ucp1-2A-luciferase knockin mice, we explored the possibility of using primary cells from these mice as a screening platform to identify compounds or peptides with browning capacity. To this end, we isolated the stromal vascular fraction

(SVF) from ingWAT and differentiated precursor cells in vitro. To validate the screening platform, we tested several known browning agents: the 3-adrenergic receptor agonist CL316,243, fibroblast growth factor 21 (FGF21) (8), the 5' -AMP-activated protein kinase (AMPK) agonist A-769662 (22), the peroxisome proliferator-activated receptor (PPAR) activator fmoc-Leu-OH (23), -aminoisobutyric acid (BAIBA) (24), all-trans-retinoic acid (ATRA), and the synthetic retinoic acid analogue AM580. These agents increase energy expenditure and/or alleviate insulin resistance in obese mouse models (25-27). Two screening strategies were adopted: test compounds were present either during the entire differentiation period or added to mature adipocytes during the final two days of differentiation (Fig. 5A [Figure 5: see original paper]). When compounds were included throughout differentiation, all except BAIBA significantly increased luminescence (Fig. 5A), suggesting they may promote differentiation of beige precursor cells and/or possess browning activities. More importantly, adding them to mature adipocytes also significantly increased luminescence (Fig. 5A), indicating that cells in the differentiated adipocyte population could induce UCP1 expression or "browning." ATRA induces Ucp1 expression and increases uncoupled respiration in adipocytes (28-31) and also increases Ucp1 expression in WAT of mice (32). ATRA and AM580 inhibit adipocyte differentiation (33-35), and we observed that both compounds increased luminescence more when added to mature differentiated adipocytes than when present throughout differentiation (Fig. 5A). Both ATRA and AM580 dose-dependently increased luciferase activity (Fig. 5B and 5D), and the browning effect was visible via luminescent imaging in vitro (Fig. 5C and 5E).

Axitinib Increases UCP1 Expression and Thermogenesis in Ucp1-2A-Luciferase Mice

Axitinib, a potent and selective inhibitor of vascular endothelial growth factor (VEGF) receptors, effectively treats patients with metastatic renal cell carcinoma in phase II and III trials (36). However, its effect on Ucp1 expression and energy metabolism was unknown. Using our luciferase reporter system, we found that axitinib possessed browning activity (Fig. 6A [Figure 6: see original paper]). In contrast, other tyrosine kinase inhibitors including cediranib, masitinib, tanduinib, and BIBF1120 showed no browning effect (Fig. 6A). Axitinib' s effects on luciferase and Ucp1 expression were dose-dependent (Fig. 6B and 6C). Interestingly, axitinib-enhanced Ucp1 expression was suppressed by activation of signal transducer and activator of transcription 3 (STAT3) with a STAT3-specific activator SD19 (Fig. 6D), suggesting that the STAT3 pathway inhibits axitinib-induced Ucp1 expression. Consistent with this finding, phosphorylation of STAT3 was suppressed in axitinib-treated cells (Fig. 6E and 6F).

The browning activity of axitinib was further tested in vivo. Ucp1-2A-luciferase mice were treated with vehicle or axitinib for 8 weeks while fed a high-fat diet

(HFD). Body weight was monitored during treatment, and axitinib-treated mice gained less weight than vehicle controls (Fig. 7F [Figure 7: see original paper]). Axitinib-treated mice also exhibited improved glucose disposal as determined by glucose tolerance and insulin tolerance tests (Fig. 7G and 7H). H&E staining showed that axitinib-treated mice had smaller adipocytes than untreated mice (Fig. 7K). Additionally, fatty liver symptoms were alleviated by axitinib treatment, evidenced by reduced size and number of lipid droplets in liver sections (Fig. 7L). Consistent with these observations, weights of ingWAT, eWAT, and liver were lower in axitinib-treated mice (Fig. 7I). In vivo luciferase imaging data showed marked increases in luciferase activities in iBAT, ingWAT, and uBAT of axitinib-administered Ucp1-2A-luciferase reporter mice (Fig. 7A-E). Luciferase activities in iBAT, ingWAT, and eWAT were also elevated (Fig. 7J). To determine whether axitinib affected energy expenditure, indirect calorimetry was performed by measuring oxygen consumption and carbon dioxide production. Axitinib reduced fat mass and significantly increased oxygen consumption and carbon dioxide production compared to control mice (Fig. S4A-4C) without affecting food intake or activity (Fig. S4E and 4G). Neither heart rate nor blood pressure increased (Fig. S4H-4J), suggesting that sympathetic nervous system activation was not the main mechanism for axitinib' s browning effects. Furthermore, rectal temperature was significantly increased by axitinib (Fig. S4D). Axitinib also increased oxygen consumption in cultured adipocytes. Seahorse bioanalyzer showed that basal, stimulated, uncoupled, and maximal respiration oxygen consumption rates were all increased in cultured adipocytes treated with axitinib (Fig. S4K and S4L). Consistent with higher oxygen consumption and rectal temperature, axitinib-fed mice displayed enhanced energy expenditure. These results indicate that axitinib treatment enhanced thermogenesis and strongly suggest that axitinib may represent a novel browning agent both in vitro and in vivo.

Discussion

The 2A peptide sequences from different picornavirus family members share a highly conserved 18-amino-acid motif that mediates cleavage between the C-terminal glycine and N-terminal proline of the 2A sequence (17). In our mouse model, the 2A peptide leaves a Pro on the N-terminus of luciferase, but this does not affect luciferase activity since it is distal to the enzyme' s active site, as demonstrated in our previous study (37). Luciferase functionality is also corroborated by readily detectable luminescence in our mouse model. Recognition and processing of the 2A peptide creates a C-terminal 20-amino-acid tag on UCP1 with sequence GSGATNFSLLKQAGDVEENPG (GSG is a flexible linker between UCP1 and the 2A peptide), but this C-terminus does not appear to abolish UCP1 biological function since homozygous mice behave normally compared to wild-type controls, in contrast to Ucp1 null mutants exposed to cold temperature.

By inserting the luciferase gene into the last coding exon of the mouse Ucp1 gene,

we generated a knockin mouse model that detects luciferase enzymatic activity in situ with high sensitivity, creating an accurate proxy for UCP1 expression. Our imaging data clearly showed that these mice responded robustly to 3-adrenergic agonist and cold exposure. At room temperature (22°C), only iBAT exhibited strong luciferase activity; however, with cold exposure (4°C), iBAT and ingWAT showed significant increases in luciferase activity. Interestingly, rWAT also showed increased luciferase activity, albeit to a lesser extent than iBAT and ingWAT. Additionally, we discovered an area underneath the ears that exhibited strong luciferase activity after cold exposure or 3-adrenergic agonist treatment. We named this depot uBAT. The biological function of this adipose depot is unclear at present. Its small size presumably precludes major metabolic significance; however, its sensitive response to temperature changes and/or adrenergic stimuli may prove important for rapid organismal adaptation to internal and external changes. We further characterized uBAT as a mixture of brite/beige and classical BAT based on expression patterns of signature genes for both adipocyte types.

During the final stages of this study, Galmozzi et al. reported generation of a Ucp1-luciferase reporter mouse line using BAC transgenic techniques, which they termed ThermoMouse (12). The segregation pattern indicated transgene insertion into the Y chromosome, restricting that model to male mice. In contrast, we inserted the luciferase coding sequence into exon 6 of the Ucp1 gene, integrating the luciferase reporter into a genomic location that promotes expression of a UCP1-luciferase fusion protein at the same location as endogenous UCP1. The Ucp1-luciferase reporter knockin mouse is amenable to various studies. Obesity accelerates adipose tissue aging by increasing reactive oxygen species formation, ultimately leading to inflammation and increased insulin resistance (38). Additionally, brown adipocytes are gradually lost in mouse BAT and subcutaneous white adipose tissue during aging (19, 39). Using our reporter mouse, we demonstrated a pronounced decrease in UCP1 activity as mice aged from 3 to 18 weeks (Fig. 3), although the reason for this decline remains unknown.

We plan to use our reporter mice to examine the extent to which anti-aging compounds maintain high metabolic function in fat or whether compounds with browning effects delay aging. Indeed, our Ucp1-2A-luciferase reporter mice provide a highly reproducible platform for screening compounds that modulate adipocyte browning. We identified positive regulators for browning, including known UCP1-activating agents such as the 3-adrenergic agonist CL316,243 and retinoids, and previously unrecognized compounds such as the AMPK agonist A-769662 and the VEGF receptor inhibitor axitinib. Since axitinib has been used in clinical trials and potently inhibits VEGF receptors, we characterized it further and found it has browning effects both in vitro and in vivo, possibly through inhibiting the STAT3 pathway. This is supported by a recent report that the JAK/STAT pathway regulates metabolic conversion of white adipocytes to brown adipocytes in humans (40). Interestingly, other VEGFR-targeting drugs such as masitinib, tanduinib, cediranib, and BIBF120 had no

browning effect (Fig. 6A), suggesting axitinib may have other targets beyond VEGFR that induce browning. Using live imaging, we demonstrated that axitinib significantly increased signals in fat pads, and axitinib-treated mice exhibited reduced weight gain, improved glucose tolerance, and enhanced insulin sensitivity. Furthermore, as shown in this study, axitinib treatment increased oxygen consumption and rectal temperature. Thus, axitinib decreased body weight gain and fat accumulation by increasing energy expenditure and enhancing thermogenesis. Regarding food intake, we observed no significant differences between groups, consistent with previous animal reports (41), although appetite loss has been reported as a possible side effect in human subjects. Axitinib offers several advantages over other drugs, including a favorable toxicity profile and the ability to be administered on a constant, manageable schedule to limit toxicity (42). Thus, axitinib may be an effective drug for treating metabolic disease.

Finally, a recent study demonstrated that cancer-associated cachexia in a mouse model was associated with browning of subcutaneous WAT, leading to increased energy expenditure (43). This highlights another potential application of our luciferase reporter mice: developing "browning" inhibitors as treatments for cachexia.

Our study establishes a sensitive, non-invasive, and convenient system to monitor UCP1 function that can be used to screen drugs and help identify and validate compounds with browning effects in vitro and in vivo.

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Duality of Interest

The authors declare that there is no duality of interest associated with this manuscript.

Author Contributions

L.M., D.W., and B.N. researched data and wrote the manuscript. L.M., T.N., and K.L. participated in generation of the Ucp1-2A-Luciferase mouse model. L.M. performed Western blots, histological experiments, immunostaining, luciferase imaging, and luminescence activity assays. L.M., R.Y., and X.T. performed mRNA expression analyses. L.M., T.N., X.H., X.L., Y.X., and Z.Z. per-

formed energy expenditure experiments, Seahorse analysis, and measurement of blood pressure and heart rate. T.N., X.H., A.X., X.G., X.L., P.L., K.D., Y.W., W.H., P.L., and S.D. reviewed the manuscript and contributed to discussion. D.S., D.W., T.N., and J.F. conceived research ideas and reviewed and edited the manuscript. X.H. and K.K. reviewed and edited the manuscript. L.M., S.D., and D.W. conceived research ideas, supervised the project, and wrote the manuscript. D.W. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for data integrity and analysis accuracy.

Figure Legends

Figure 1. Generation and characterization of Ucp1-luciferase mice.
A: Schematic of the Ucp1-2A-luciferase knockin strategy showing homologous recombination used to generate firefly luciferase linked to a 2A peptide sequence from Hand, Foot, and Mouth virus to replace the stop codon in exon 6 of the Ucp1 gene.
B: Identification of luciferase-knockin mice by genomic PCR. PCR products were 500 bp for wild-type (WT) and 1000 bp for Ucp1-2A-luciferase (U+/LUC).
C: UCP1 and luciferase proteins in interscapular BAT (iBAT) of wild-type and knockin mice analyzed by Western blot.
D: Luciferase enzymatic activity in iBAT of wild-type and luciferase-knockin mice.
E: Whole-animal live imaging showing 30 s luminescence images of Ucp1-2A-luciferase and wild-type mice.
F: Immunohistochemical staining of UCP1 and luciferase in iBAT showing co-localization of UCP1 and luciferase immunoreactivities.

Figure 2. Patterns of luciferase enzymatic activity and endogenous UCP1 levels in adipose tissues of knockin mice.
A: Luminescent images of Ucp1-2A-luciferase mice in response to non-treated control (NC), CL316243 (a 3-adrenergic agonist), or B: cold exposure.
C: Luminescence images of adipose tissues dissected from Ucp1-2A-luciferase mice in B. iBAT, interscapular BAT; aBAT, axillary BAT; cBAT, cervical BAT; ingWAT, inguinal WAT; eWAT, epididymal WAT.
D: Relative Ucp1 RNA levels in different adipose tissues measured by qPCR at 22°C and 4°C for 12 hours. tWAT, thoracic WAT; rWAT, retroperitoneal WAT; mWAT, mesenteric WAT.
E: Relative specific luciferase activity from adipose tissues described in D.
F and G: UCP1 protein levels from adipose tissues described in D. *$p < 0.05$, **$p < 0.01$ compared with control mice, n=5.

Figure 3. Declined luciferase activity supports decreased "browning" ability with age.
A: Luminescence images of Ucp1-2A-luciferase mice at ages 3, 6, 10, and 18 weeks at 22°C.
B-E: Quantification of luminescence in A for dorsal, ventral upper body, ventral

lower body, and side view images at ages 3, 6, 10, and 18 weeks at 22°C. *p < 0.05, **p < 0.01 compared with control group mice, n=5.

Figure 4. uBAT revealed underneath the ears by live imaging of Ucp1-2A-luciferase knockin mice.
A: Luminescence images of a Ucp1-2A-luciferase mouse at 22°C and at 4°C for 12 hours.
B: Luminescence images of the region containing uBAT in a Ucp1-2A-luciferase mouse at 22°C and at 4°C for 12 hours.
C: Ears and attached uBAT.
D: Hematoxylin and eosin (HE) and immunohistochemical staining of UCP1 in uBAT, showing both classical BAT and beige adipose tissue.
E: Relative specific luciferase activity in uBAT, ingWAT, and iBAT after Ucp1-2A-luciferase mice were kept at 22°C and at 4°C for 12 hours.
F-H: Relative expression of Ucp1, Prdm16, Cidea, Zic1, Tbx1, Cd137, Tmem26, and Hoxc9 mRNAs in uBAT, ingWAT, and iBAT after Ucp1-2A-luciferase mice were kept at 22°C and at 4°C for 12 hours. *p < 0.05, **p < 0.01 compared with controls, n=5.

Figure 5. Drug screening in white adipocytes from Ucp1-2A-luciferase knockin mice.
A: Relative "browning" induced by selected molecules (1 M) during the entire period (days 0-10) and during the final two days (days 8-10) of in vitro adipocyte differentiation of primary inguinal preadipocytes.
B: Relative luciferase activities in response to different concentrations of all-trans-retinoic acid (ATRA).
C: Luminescence images of luciferase activities in response to different concentrations of ATRA.
D: Relative luciferase activities in response to different concentrations of AM580.
E: Luminescence images of luciferase activities in response to different concentrations of AM580. #p < 0.05, *p < 0.05, **p < 0.01 compared with control group, n=5.

Figure 6. Axitinib increases "browning" in ingWAT.
A: Relative "browning" induced by selected tyrosine kinase inhibitors (1 M) in primary ingWAT.
B: Relative luciferase activities in response to different concentrations of axitinib.
C: Relative mRNA expression in response to different concentrations of axitinib.
D: STAT3 suppresses UCP1 mRNA expression induced by axitinib.
E-F: Phosphorylation of STAT3 was reduced in cells treated with axitinib. STAT3 was activated by the specific activator SD19. *p < 0.05, **p < 0.01 compared with DMSO, n=5.

Figure 7. Axitinib increases thermogenesis in vivo.
A: Live visualization and quantification of luciferase activity in Ucp1-2A-luciferase mice treated with vehicle or axitinib for 8 weeks. Representative

mice are shown.
B-E: Quantification of luminescence in A for dorsal, ventral upper body, ventral lower body, and side view images.
F: Axitinib limited body-weight gain in Ucp1-2A-luciferase mice fed HFD.
G-H: Axitinib improved glucose clearance in Ucp1-2A-luciferase mice (n=9).
I: Axitinib reduced weights of ingWAT, eWAT, and liver in Ucp1-2A-luciferase mice.
J: Relative luciferase activities in response to axitinib showing enhanced Ucp1 expression.
K: UCP1 immunostaining in iBAT and ingWAT from Ucp1-2A-luciferase mice fed HFD and treated with vehicle or axitinib.
L: HE staining of fatty liver from mice treated with vehicle or axitinib. *$p < 0.05$, **$p < 0.01$ compared with control group, n=9.

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Online Only Materials

Figure S1. UCP1 expression level is in concordance with luciferase activity.
(A-D) Ucp1-2A-luciferase mice were housed at 4°C for various time periods as indicated. (A) and (C) Relative luciferase activity (RLU) and mRNA level in (A) iBAT and (C) ingWAT; (B) and (D) Western blot analysis of UCP1 and luciferase in (B) iBAT and (D) ingWAT; (E) Representative luminescence images of UCP1+/LUC mice after substrate injection for 20 min, 40 min, and 120 min. n=8, #p<0.05, *p<0.05, **p<0.01.

Figure S2. Half-lives of UCP1 and luciferase protein.
Brown adipocytes were stimulated with CL316,243 (1 M) for 2 days, followed by incubation with cycloheximide (10 M at time zero). Relative protein levels of UCP1 and luciferase were examined. (A) Western blot and (B) densitometry analysis of UCP1 and (C) luciferase activity in treated cells at various time points after cycloheximide treatment. n=6.

Figure S3. UCP1 expression was reduced upon thermoneutrality.
Ucp1-2A-luciferase mice were housed at 4°C or 22°C for 12 h, then at 30°C for 48 h. (A) Luminescence images of mice; (B-E) Quantification of luminescence in (A) at various body regions; (F) Relative Ucp1 mRNA levels and (G) relative luciferase activity in different adipose tissues; (H) Immunostaining of UCP1 in iBAT and iWAT. n=8. #p<0.05, *p<0.05, **p<0.01, ND, not detected.

Figure S4. Axitinib promotes energy expenditure in vivo and in vitro.
(A-I) Ucp1-2A-luciferase mice were orally gavaged with axitinib (10 mg/kg body weight) or PBS daily for 8 weeks. (A) Body composition, (B) O2 consumption, (C) CO2 production, (D) Rectal temperature, (E) Food intake, (F) Serum insulin, (G) RER, (H) Activity, (I) Blood pressure, and (J) Heart rate in normal control (NC) and axitinib-treated mice. (K-L) SVF-derived mature adipocytes were treated with DMSO or axitinib (1 M) for 24 h. Oxygen consumption rate (OCR) was measured by Seahorse bioanalyzer. (K) OCRs upon sequential compound injections measuring basal, stimulated (10 M norepinephrine, NE), ATP production (2 M Oligomycin), maximal (2 M FCCP), and non-mitochondrial (1 M Antimycin A and 3 M Rotenone) respiration. (L) Basal, stimulated, maximal, and uncoupled OCRs. Uncoupled OCR was calculated as the difference between stimulated and non-mitochondrial OCRs. *p<0.05, ND, not detected, n=4.

Figure S5. Browning is reduced in female mice after giving birth.
(A-B) (A) Luminescence images of virgin mice and after-birth (AB) mice at the same age; (B-D) Quantification of luminescence in (A) at various body regions; (E) Body weight, (F) Relative Ucp1 mRNA levels, (G) Luciferase activity in various adipose tissues; (H) UCP1 immunostaining in iBAT and ingWAT. *p<0.05, **p<0.01, ND, not detected, n=6.

Table 1 . Primers used for gene expression

Accession number	Gene	Primers
AF349658.1	PRDM16	GGCAGGCAGACGAATGTTC / TTGTCATCTACGGGCACAAAG
NM_027504	Tmem26	CAGCACGGTGAAGCCATTC / GCGTGCATCCGCTTGTG
BC096649.1	Cidea	ACCCTGTCATCCCACAGAG / TGTTTGGTGGAGTCCTAAGGTC
DQ832278.1	Cd137	TGCTCTTCTGTATCGCCCAGT / GCCGTGTTAAGGAATCTGCTG
NM_009463	Hoxc8	CGTGCAGAACTCCTGTGATAAC / GTCCACCTATGCTGGAGAAGG
BC060247.1	Hoxc9	GGCATTCAGAGGCAAATCAGCT / CAATGAACACTGCCACACCTC
NM_010466.2	18S	AACCTCAAGATCCACAAAAGGA / CCTCGAACTCGCACTTGAA
NM_013536.2	Ucp1	GTCTCCCAGCCTCATGTTTC / TCTGATACCGGCTGTAAGTTTGT
		GTAACCCGTTGAACCCCATT / CCATCCAATCGGTAGTAGCG

Note: Figure translations are in progress. See original paper for figures.

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