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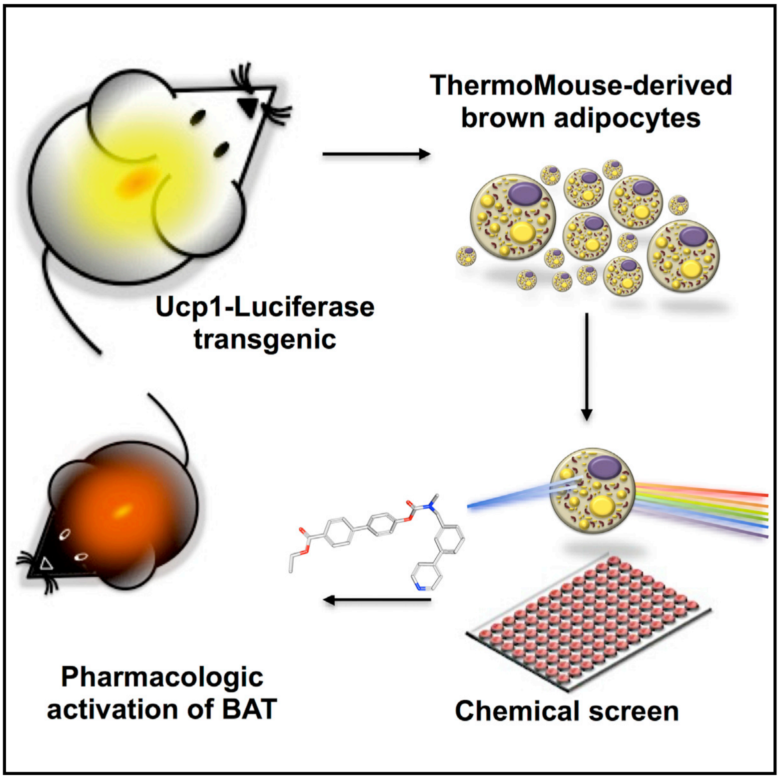
ThermoMouse: An In Vivo Model to Identify Modulators of UCP1 Expression in Brown Adipose Tissue

Graphical Abstract



Highlights

ThermoMouse is a transgenic model that faithfully reports endogenous UCP1 expression

A derived *Ucp1* luciferase brown preadipocyte line retains BAT capacity in vivo

A screen using this line yielded a small molecule that increases UCP1 expression

Compound-treated mice show increased energy expenditure upon adrenergic stimulation

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In Brief

Pharmacological activation of brown adipose tissue (BAT) thermogenesis and energy dissipation, a process mediated by UCP1, may be useful to counter the energy imbalance that engenders obesity. Galmozzi et al. have developed an in vivo model to monitor UCP1 expression in real time and identified a small molecule that increases UCP1 levels. Mice treated with this molecule show greater energy expenditure upon adrenergic stimulation. Discovery of compounds with this ability is an important stride toward enhancing BAT function in obese individuals.

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ThermoMouse: An In Vivo Model to Identify Modulators of UCP1 Expression in Brown Adipose Tissue

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SUMMARY

Obesity develops when energy intake chronically exceeds energy expenditure. Because brown adipose tissue (BAT) dissipates energy in the form of heat, increasing energy expenditure by augmenting BAT-mediated thermogenesis may represent an approach to counter obesity and its complications. The ability of BAT to dissipate energy is dependent on expression of mitochondrial uncoupling protein 1 (UCP1). To facilitate the identification of pharmacological modulators of BAT UCP1 levels, which may have potential as antiobesity medications, we developed a transgenic model in which luciferase activity faithfully mimics endogenous UCP1 expression and its response to physiologic stimuli. Phenotypic screening of a library using cells derived from this model yielded a small molecule that increases UCP1 expression in brown fat cells and mice. Upon adrenergic stimulation, compound-treated mice showed increased energy expenditure. These tools offer an opportunity to identify pharmacologic modulators of UCP1 expression and uncover regulatory pathways that impact BAT-mediated thermogenesis.

INTRODUCTION

The epidemic of obesity poses a dire public health problem, for obesity is a major risk factor for development of insulin resistance, type 2 diabetes, cardiovascular disease, and cancer. Obesity is the result of a sustained energy imbalance in which intake exceeds expenditure. Current antiobesity drugs work by limiting energy intake, either through suppression of appetite or inhibition of intestinal lipid absorption (Kim et al., 2014). These medications are effective, but side effects often associated with long-term use, such as depression or steatorrhea, limit patient compliance. The discovery of brown adipose tissue (BAT) in adult humans and its correlation with body mass index (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) suggests that active BAT may protect from obesity. BAT dissipates energy in the form of heat, thus increasing energy expenditure. It is thought that pharmacological activation of BAT thermogenesis may be an alternative approach to alter energy balance, one complementary to existing obesity medications (Nedergaard and Cannon, 2010; Kajimura and Saito, 2014).

The ability of BAT to produce heat is dependent on expression of the BAT-specific uncoupling protein 1 (UCP1). In response to exposure to cold or a high-fat diet, UCP1 reduces the mitochondrial membrane potential and uncouples cellular respiration from ATP synthesis, thereby generating heat. Because other UCP proteins (e.g., UCP2 and UCP3) do not contribute to adaptive thermogenesis (Golozoubova et al., 2001), UCP1 is thought to be solely responsible for adaptive nonshivering thermogenesis. UCP1-null mice are intolerant to cold (Enerbäck et al., 1997) and develop obesity at thermoneutral conditions (Feldmann et al., 2009). In contrast, transgenic expression of UCP1 in fat increases oxygen consumption in BAT and epididymal white adipose tissue (WAT) and reduces body weight gain (Kopecky et al., 1995). Contrary to the mechanism of action of small-molecule mitochondrial uncouplers such as 2,4-dinitrophenol that proved too toxic as weight loss agents (Grundlingh et al., 2011), UCP1-mediated uncoupling is a highly regulated process that requires direct binding of long-chain free fatty acids to UCP1 in response to physiologic cyclic AMP (cAMP) signaling (Fedorenko et al., 2012). A pharmacological approach to increase UCP1 expression and activity in adipose tissue is thus likely to constitute a safer avenue to enhance whole-body thermogenic capacity and energy expenditure. To test this concept, it is of great interest to identify small molecules that can stimulate UCP1 expression in fat tissue.

Phenotypic screens with adipocytes have proven to be a powerful method to isolate small molecules that ameliorate the symptoms of metabolic syndrome through novel mechanisms of action (Waki et al., 2007; Dominguez et al., 2014). To facilitate the identification of pharmacologic agents to modulate UCP1 expression in adipocytes, we have generated a transgenic reporter mouse, ThermoMouse, in which luciferase activity recapitulates the pattern of expression of UCP1 in vivo and allows real-time visualization and quantification of UCP1 expression in live animals. A chemical screen using brown adipocytes derived from this model yielded a compound that can induce UCP1 expression in cells and enhance UCP1 expression in vivo. In response to adrenergic stimulation, mice treated with this compound show increased energy expenditure. These results demonstrate the utility of these models to identify pharmacological modulators of UCP1 levels. Discovery of compounds with this ability is an important stride toward the goal of enhancing BAT function in obese individuals with drug-like molecules.

RESULTS

Development of a Transgenic Model to Image UCP1 Expression In Vivo

To develop an in vivo reporter system to monitor endogenous UCP1 expression in a noninvasive manner, we generated transgenic mice that express luciferase2 under the control of the *Ucp1* genetic locus. A luciferase2-T2A-tdTomato cassette was inserted at the initiation codon of the *Ucp1* gene in a 98.6 kb bacterial artificial chromosome (BAC) containing the entire *Ucp1* gene locus (Figure S1A) and proper targeting confirmed (Figure S1B). Next, we used the IVIS Spectrum Imaging System to monitor luciferase activity in transgenic mice generated using this BAC construct. 3D imaging detected robust luciferase signals in interscapular BAT, perirenal BAT, and inguinal WAT (Figure 1A). No signals were detected in adipose depots of wild-type mice (Figure S1C). To assess if luciferase activity recapitulated endogenous UCP1 protein levels, we measured luciferase activity and UCP1 protein in BAT, WAT, liver, and muscle. A tight correlation was found between luciferase activity and endogenous UCP1 protein expression (Figures 1B and 1C), indicating that the reporter model mirrors the tissue distribution of UCP1.

To examine whether reporter mice responded to physiologic stimuli known to induce UCP1 expression and BAT activity, we monitored changes in luciferase activity during cold adaptation (Figure 1D). Luciferase signal in interscapular BAT was low in mice maintained at 28°C (Figure S1D) but increased robustly in mice kept at 9°C for 24 hr (Figures 1D and 1E). Luciferase induction correlated with increases in UCP1 mRNA (Figure 1F) and protein levels (Figure 1G). Similarly, subchronic (4 days) or acute (1 day) treatment of reporter mice kept at 28°C with a specific β_3 adrenergic receptor agonist (CL-316,243; 1 mg/kg) dramatically enhanced the luciferase signal and endogenous UCP1 protein levels in BAT (Figures 1H and 1I). To evaluate the response of beige cells in inguinal WAT, imaging was performed from a side angle. Strong luciferase activity was detected in inguinal WAT in response to chronic CL-316,243 treatment (Figure 1J). The increase in signal was paralleled by robust induction of UCP1 protein in this depot (Figure 1K). These results show that our *Ucp1* luciferase reporter accurately indicates changes in UCP1 elicited by physiological responses and is a useful tool to quantify changes in UCP1 expression in vivo.

A Cell-Based Screening Platform to Monitor UCP1 Protein Expression

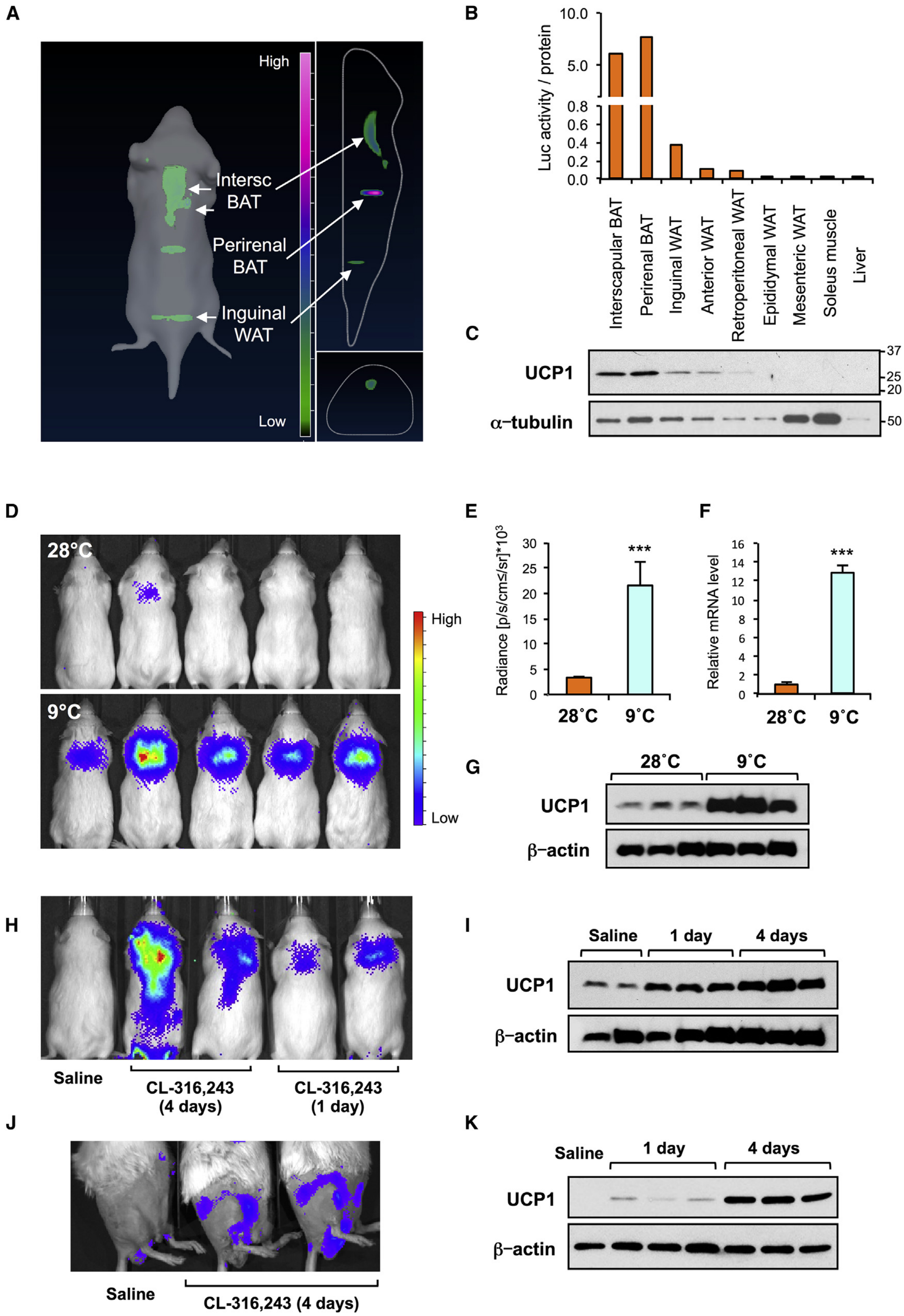
Next, to establish a cell-based system for quantifying UCP1 expression in fat cells suited for screening of small-molecule or genomic libraries, we generated immortalized preadipocyte lines from *Ucp1* luciferase transgenics. From six cell lines derived from interscapular BAT, we selected one that showed high adipogenic capacity and significant levels of UCP1 and luciferase expression. Because PPARγ ligands can stimulate UCP1 expression (Sears et al., 1996), we tested the response of this line to rosiglitazone. Luciferase activity was induced in a dose-dependent manner and correlated tightly with endogenous UCP1 protein levels (Figures 2A and 2B). Treatment with the cAMP-signaling activator forskolin also increased luciferase activity and UCP1 protein expression (Figures 2C and 2D).

To confirm that the *Ucp1* luciferase reporter cell line preserved the response to BAT activators, and that it could report sequential changes in UCP1 expression, nude mice were implanted with *Ucp1* luciferase preadipocytes subcutaneously and treated 6 days posttransplantation with saline or rosiglitazone (10 mg/kg per day) for 7 days. Luciferase activity in transplants of rosiglitazone-treated mice was increased significantly at 4 days of treatment and thereafter (Figures 2E and 2F). Transplanted preadipocytes in mice treated with rosiglitazone formed discrete adipose tissue containing multilocular adipocytes (Figure 2G, upper left). These adipocytes were positive for UCP1 protein (Figure 2G, upper right) and GFP (i.e., tdTomato; Figure 2G, lower left), indicating that transplanted cells retained brown adipogenic capacity in vivo. These observations indicate that this cell-based system is a robust surrogate to assess endogenous UCP1 expression.

A Small-Molecule Screen Identifies a Regulator of UCP1 Expression

To test the ability of our monitoring systems to identify regulators of UCP1 expression, we performed a phenotypic screen using a modestly sized library of small molecules, primarily carbamates and triazole ureas (Adibekian et al., 2011; Bachovchin et al., 2010). *Ucp1* luciferase brown preadipocytes were seeded and differentiated in 96-well plates. Mature adipocytes (day 8) were incubated with compounds and luciferase activity quantified 16 hr later (Figure 3A). To evaluate the power of the assay to identify compounds that modulate UCP1 levels in either direction, four hits with differing properties (activators and inhibitors) were selected for study (Figure 3B). As it is often the case with reporter-based screens, two hits could not be validated and may represent, for example, nonspecific stabilizers of luciferase signal. Of those that confirmed, compound 4 robustly increased luciferase activity (Figure 3C). In contrast, compound 3 significantly reduced luciferase signal. Importantly, compound 4 increased endogenous UCP1 protein expression to a similar level to that induced by rosiglitazone (Figure 3D). None of the compounds had any effect on brown preadipocytes (Figure S2).

Compound 4 (WWL113 in original nomenclature) was also a hit in a different, image-based screen we recently described, in which white preadipocytes were treated chronically (8 days) during differentiation (Dominguez et al., 2014). WWL113 inhibits



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two serine hydrolases expressed in adipocytes, carboxylesterase 3 (Ces3 or Ces1d) and Ces1f (CesML1). However, the ability of WWL113 to induce UCP1 expression in brown adipocytes does not appear to be mediated by Ces3/1f inhibition, for a structurally distinct Ces3 inhibitor (WWL229) failed to have the same effect on UCP1 expression, whereas the urea version of WWL113 (WWL113U), which does not inhibit Ces3, retained the ability to induce UCP1 (Figure S3A). Nonetheless, we concluded that WWL113 could be a valuable chemical probe to further validate our reporter systems.

Induction of UCP1 Expression by WWL113 Relies on PPARα Signaling

To characterize the effect of WWL113 on endogenous UCP1 expression, we treated cultured brown adipocytes with several doses of WWL113. At a dose of 1 μM and higher, WWL113 significantly increased UCP1 protein expression (Figure 4A). The effect of WWL113 on UCP1 protein levels was largely due to activation of *Ucp1* transcription, because WWL113 powerfully increased expression of *Ucp1* mRNA (Figure 4B). WWL113 treatment also stimulated expression of mRNAs for other thermogenic genes, such as *Cidea*, *Pgc1a*, and *Cox7a1* (Figure 4B). WWL113 treatment had no effect on expression of the adipogenic marker *Adiponectin*. WWL113 treatment for 24 hr was sufficient to activate endogenous *Ucp1* mRNA expression without affecting expression of multiple adipogenic markers (Figures 4C and S3B), indicating that WWL113 enhanced the BAT-selective thermogenic gene program in a cell-autonomous manner without affecting adipogenesis per se. To test the functional consequences of WWL113 treatment, we examined the extent to which WWL113 could sensitize brown adipocytes to physiologic activators of BAT such as norepinephrine. WWL113 pretreatment enhanced the increase in *Ucp1* mRNA expression normally induced by norepinephrine (Figure 4D). More importantly, WWL113-treated cells showed greater total and uncoupled respiration in response to norepinephrine and greater uncoupled basal respiration (Figures 4E and S3C).

Next, we explored the mechanism by which WWL113 increased *Ucp1* transcription. Because PPARα is a critical regulator of thermogenic gene expression in BAT (Barbera et al., 2001), we hypothesized that the action of WWL113 in BAT could be mediated via PPARα. To test this notion, primary brown adipocytes were treated with a selective PPARα antagonist (GW6471) and/or a PPARγ antagonist (GW9662) in the presence or absence of WWL113. The capacity of WWL113 to increase *Ucp1* mRNA expression was largely blunted by treatment with GW6471, but not with GW9662 (Figure 4F). The inhibitory effect of GW6471 on WWL113-induced *Ucp1* mRNA expression was not altered when cells were cotreated with GW9662. These results indicate that the ability of WWL113 to enhance *Ucp1* expression is principally dependent on PPARα, but not PPARγ, activity. Because WWL113 is not a direct PPARα activator (Dominguez et al., 2014), we tested the extent to which cotreatment of differentiated brown adipocytes with WWL113 and a PPARα agonist (GW9578) would further boost *Ucp1* mRNA levels. Cotreatment with WWL113 and GW9578 modestly but significantly increased expression of *Ucp1* relative to treatment with either compound alone (Figure 4G), suggesting that WWL113 cooperates with the PPARα pathway to enhance UCP1 expression.

WWL113 Increases UCP1 Expression and the Thermogenic Response In Vivo

We next tested if WWL113 could enhance UCP1 expression in vivo. *Ucp1* luciferase mice were treated with vehicle or WWL113 (50 mg/kg once daily) for 5 days. This dose has been shown to be effective in mice (Dominguez et al., 2014). A robust and significant increase (5-fold) in *Ucp1*-driven luciferase expression was detected in the interscapular BAT depots of transgenic mice treated with WWL113 (Figures 5A and 5B). To confirm these findings, we examined the ability of WWL113 to enhance thermogenic gene expression in C57BL/6J mice treated with the compound for 5 days. WWL113 treatment induced significant increases in mRNA expression of *Ucp1* and thermogenic genes, such as *Pgc1a* and *Dio2*, in the BAT of wild-type mice (Figure 5C). No change in the general marker *Pparγ* was seen. Importantly, UCP1 protein expression was highly induced in vivo by WWL113 (Figure 5D). In contrast, no difference in mRNA expression of *Ucp1*, *Pgc1a*, *Dio2*, and *Pparγ* was observed in inguinal WAT of WWL113-treated mice (Figure S5A), indicating that the effects of WWL113 on the thermogenic gene program may be specific to brown fat.

Finally, we examined the effects of WWL113 on whole-body energy expenditure. Mice treated with WWL113 for 7 days showed no differences in basal energy expenditure, but upon adrenergic stimulation (CL-316,243 injection), they responded with a more robust increase in energy expenditure than controls (Figure 5E). WWL113 did not affect locomotor activity (Figure 5F), food intake (Figure 5G), or heart rate (Figure 5H). These data indicate that WWL113 increased UCP1 levels in vivo to a functionally meaningful degree, increasing the adaptive thermogenic capacity of treated mice.

Figure 1. Luciferase Imaging of UCP1 Expression In Vivo

(A) 3D reconstruction of luciferase signals in *Ucp1* luciferase reporter mice. Adipose depots with specific luciferase signal are indicated.

(B) Quantification of luciferase in adipose depots, skeletal muscle, and liver of mice kept at room temperature. Values normalized to protein content.

(C) UCP1 protein expression in tissue lysates from the mouse in (B).

(D) Luciferase activity in *Ucp1* luciferase reporter mice kept at 28°C and subsequently kept at 9°C for 24 hr. Representative mice are shown.

(E) Quantification of luciferase signal in interscapular BAT of *Ucp1* luciferase reporter mice shown in (D) (n = 9). ***$p < 0.001$. Data are expressed as mean ± SEM.

(F) *Ucp1* mRNA expression in interscapular BAT of *Ucp1* luciferase reporter mice kept at 28°C and 9°C for 24 hr. ***$p < 0.001$. Data are expressed as mean ± SEM.

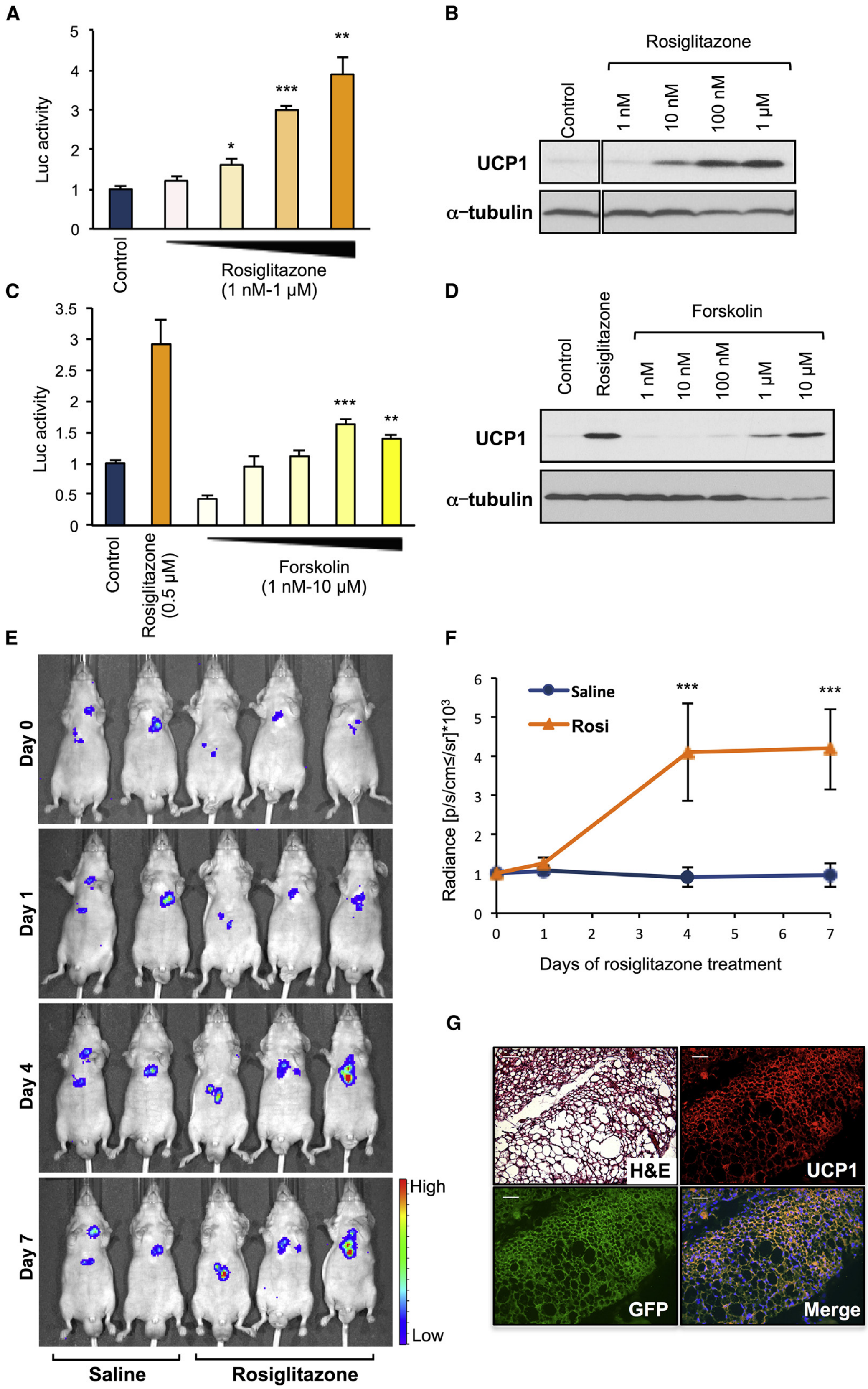
(G) UCP1 protein expression in interscapular BAT of transgenic mice analyzed in (F).

(H) Representative image of luciferase signal in *Ucp1* luciferase reporter mice treated with saline or CL-316,243 for 1 day (acute) or 4 days (subchronic; n = 3).

(I) UCP1 protein expression in BAT of transgenic mice analyzed in (H).

(J) Representative images of luciferase signal in inguinal WAT depots of *Ucp1* luciferase reporter mice treated with saline or CL-316,243 for 4 days.

(K) UCP1 protein expression in inguinal WAT depots of *Ucp1* luciferase mice treated with saline or CL-316,243 for 1 day or 4 days.



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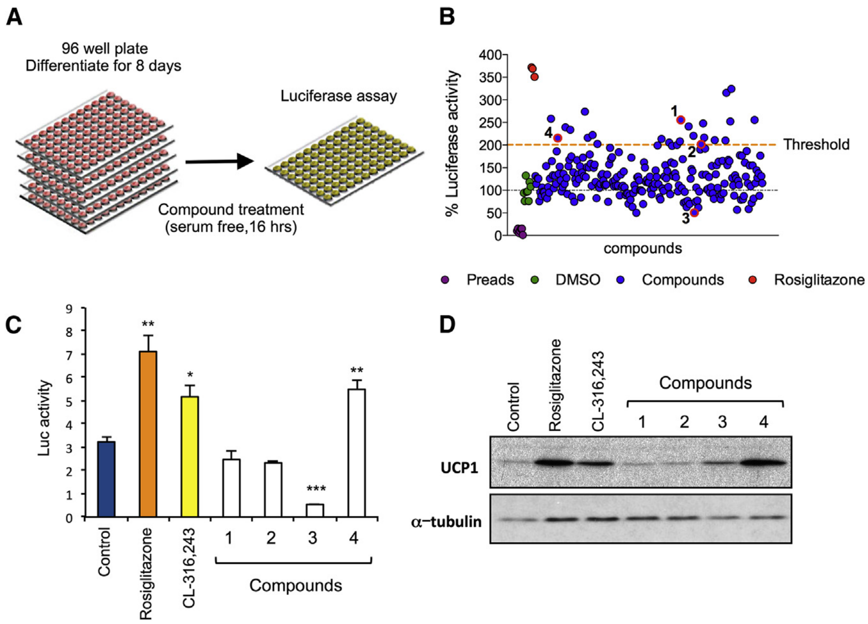

Figure 3. Small-Molecule Screen to Identify Regulators of UCP1 Expression

(A) Screen scheme. *Ucp1* luciferase brown preadipocytes were differentiated in 96-well plates and treated at day 8 with compounds for 16 hr (n = 3).

(B) Screen performance. Mean luciferase activity of compounds (blue circles) plotted relative to the value of DMSO-treated *Ucp1* luciferase adipocytes (100%; green circles). Rosiglitazone served as positive control (red circles). *Ucp1* luciferase preadipocytes (purple circles) were used to determine background and induction of signal upon differentiation. Hits selected for evaluation (blue circles, red outline) are numbered according to the scheme used in validation experiments.

(C) Luciferase activity in differentiated *Ucp1* luciferase brown adipocytes treated with compounds for 5 days. Rosiglitazone (0.5 μM) and CL-316,243 (10 nM) served as positive controls (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. Data are expressed as mean + SD.

(D) UCP1 protein expression in cells from (C).

DISCUSSION

We have developed an in vivo monitoring system that allows to quantitatively track sequential changes in UCP1 expression within the same individual. Because UCP1 expression shows a high level of interindividual variation (Boeuf et al., 2002) and changes dynamically during circadian oscillation (Gerhart-Hines et al., 2013), seasonal changes (Au-Yong et al., 2009), and aging (Rogers et al., 2012), this model may prove of wide utility. ^{18}fluoro-labeled 2-deoxy-glucose positron emission tomography (^{18}FDG-PET) scanning has been applied to assess BAT activity in rodents and humans (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009), but detection of ^{18}FDG-PET signals in BAT depends entirely on glucose uptake. In contrast, the level of UCP1 expression in BAT is a more-direct measure of the thermogenic capacity of this tissue (Nedergaard and Cannon, 2010). Hence, the transgenic UCP1 reporter we describe provides an opportunity to identify signaling pathways and transcriptional events that control thermogenic capacity in brown adipocytes in vivo. Unlike prior UCP1 models (Cassard-Doulcier et al., 1993), it also enables characterization of modulators of BAT function in real time in live animals.

*Ucp*1 luciferase brown adipocyte lines derived from this transgenic retain the characteristics of bona fide brown fat cells. A phenotypic screen using these cells identified a compound, WWL113, that can increase UCP1 expression in vitro and in vivo. It is important to note that UCP1's uncoupling activity is dependent on sympathetic nerve activation and increased intracellular cAMP levels. As BAT activity is stimulated by cold exposure, long-chain free fatty acids supplied from cAMP-induced lipolysis and from the circulation directly bind UCP1 and serve as a substrate to transport protons into the mitochondrial matrix (Fedorenko et al., 2012). Thus, small molecules that solely stimulate UCP1 expression are unlikely to induce substantial thermogenesis. In agreement with this notion, we found that the effect of WWL113 treatment on cellular respiration was greater when cells were stimulated with norepinephrine. In that setting, this chemical probe significantly increased total and uncoupled cellular respiration. More importantly, mice treated with WWL113 showed considerably enhanced energy expenditure, but this increase required adrenergic stimulation. WWL113-treated mice showed no

Figure 2. Cell-Based System to Monitor UCP1 Expression

(A) Luciferase activity in immortalized *Ucp1* luciferase brown adipocytes. Differentiated adipocytes were treated with DMSO (control) or rosiglitazone for 5 days (n = 3).

(B) UCP1 protein expression in cells analyzed in (A).

(C) Luciferase activity in differentiated *Ucp1* luciferase brown adipocytes treated with DMSO (control), forskolin, or rosiglitazone (0.5 μM) for 5 days (n = 3).

(D) UCP1 protein expression in cells analyzed in (C).

(E) Luciferase activity monitored at days 0, 1, 4, and 7 after the start of rosiglitazone treatment in mice implanted with *Ucp1* luciferase immortalized preadipocytes. Saline (n = 4) or rosiglitazone (n = 6; 10 mg/kg) treatment started 6 days postimplantation. Representative mice are shown.

(F) Sequential changes in luciferase activity measured in fat transplants from mice in (E).

(G) Hematoxylin and eosin and immunofluorescent stains for UCP1 or GFP (i.e., tdTomato) in transplants from mice treated with rosiglitazone. Merged UCP1 and GFP image counterstained with DAPI. The scale bar represents 50 μm. H&E, hematoxylin and eosin staining.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data are expressed as mean ± SEM.

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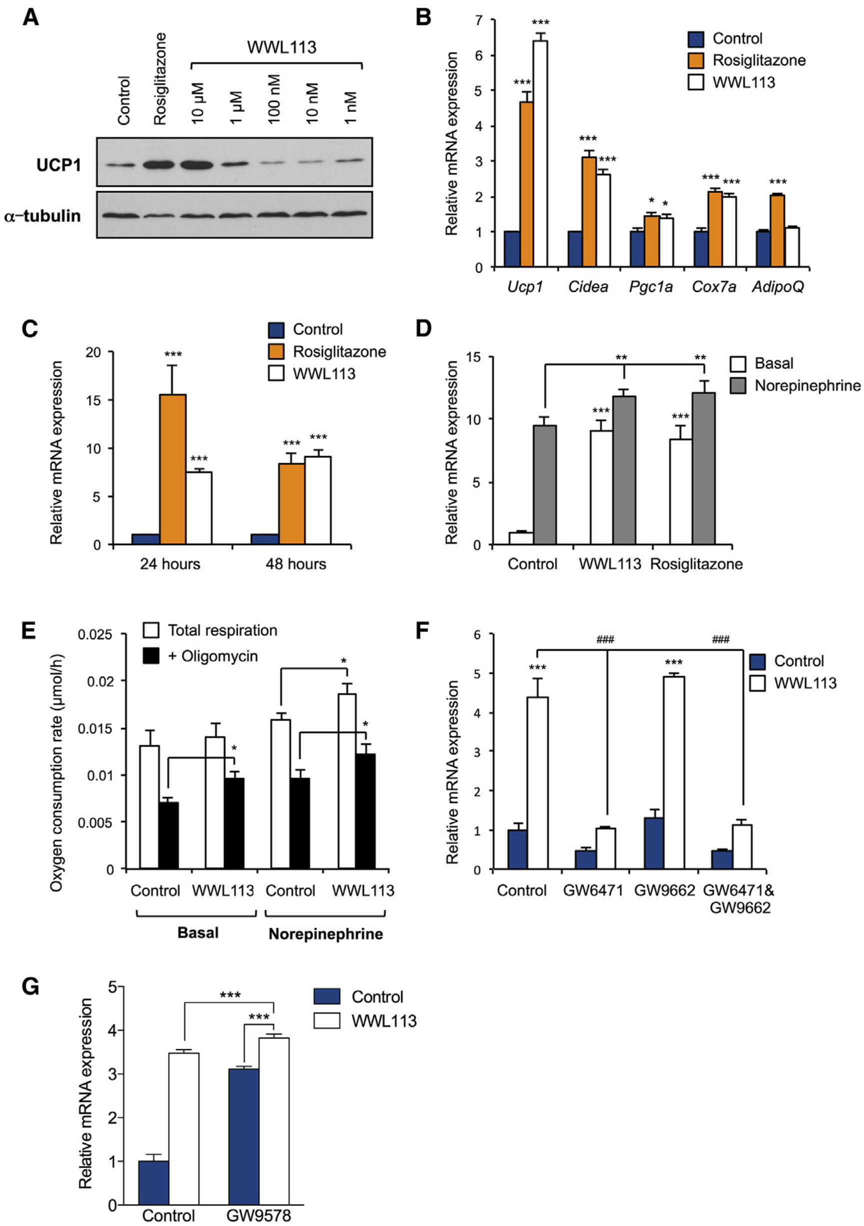

Figure 4. Effect of WWL113 on UCP1 Expression and Cellular Respiration

(A) UCP1 protein expression in differentiated *Ucp1* luciferase brown adipocytes treated with WWL113 for 5 days. Rosiglitazone (0.5 μM) served as positive control.

(B) Expression of thermogenic genes in differentiated *Ucp1* luciferase brown adipocytes treated with WWL113 (10 μM) for 5 days. Rosiglitazone (0.5 μM) served as positive control (n = 3). $^{*}p < 0.05$; $^{***}p < 0.001$ versus control.

(C) *Ucp1* mRNA expression in primary differentiated brown adipocytes treated with WWL113 (10 μM) or rosiglitazone (5 μM) for 24 or 48 hr (n = 3). $^{***}p < 0.001$ versus control.

(D) *Ucp1* mRNA expression in primary differentiated brown adipocytes treated with WWL113 (10 μM) or rosiglitazone (5 μM) for 48 hr. Norepinephrine (0.1 μM) was added 8 hr prior to harvest (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$ versus control.

(E) Total and uncoupled (oligomycin-insensitive) respiration of differentiated brown adipocytes (5×10^5 cells/sample) treated with WWL113 (10 μM) in the presence or absence of norepinephrine (0.1 μM; n = 3 to 4). $^{*}p < 0.05$ versus control.

(F) *Ucp1* mRNA expression in differentiated brown adipocytes treated with vehicle or WWL113 (10 μM) for 24 hr with or without 30 min pretreatment with a PPARα-selective antagonist (GW6471; 3 μM) and/or a PPARγ-selective antagonist (GW9662; 10 μM; n = 3). $^{***}p < 0.001$ versus control. $^{###}p < 0.001$ versus WWL113-treated cells.

(G) *Ucp1* mRNA expression in differentiated brown adipocytes treated with vehicle, WWL113 (10 μM), a PPARα-selective agonist (GW9578), or the combination for 24 hr (n = 4). $^{*}p < 0.05$; $^{***}p < 0.001$.

Data are expressed as mean + SD.

differences in locomotor activity, food intake, or heartbeat, indicating that the compound does not enhance basal sympathetic drive. Together with the finding that WWL113 increases UCP1 expression in brown fat cells in a cell-autonomous manner, these results suggest that WWL113 boosts energy expenditure primarily by increasing the content of UCP1 in BAT that can be activated by physiologic stimuli such as cold.

We previously reported that chronic administration (2 months) of WWL113 to obese-diabetic mice reduced body weight gain, improved systemic glucose and lipid homeostasis, and cleared hepatic steatosis (Dominguez et al., 2014). We ascribed the effects of WWL113 to inhibition of Ces3 in WAT and liver. Using a distinct phenotypic screen as a starting point, in this study, we have found that WWL113 can have Ces3-independent effects in BAT. WWL113 is not a direct activator of PPARα (Dominguez et al., 2014), but its effects on UCP1 expression in brown fat cells depend to a large extent, though not completely, on PPARα signaling. Although the molecular target(s) for WWL113's action in BAT remains to be clarified, this tool compound has nonetheless demonstrated the utility of our UCP1-monitoring systems to identify pharmacologic activators of UCP1 expression.

BAT is the major adipose depot that contains UCP1-positive adipocytes (classical brown adipocytes), but rodents and humans also possess an inducible type of thermogenic fat cells, termed beige or brite adipocytes (Sharp et al., 2012; Wu et al., 2012; Cypess et al., 2013; Lidell et al., 2013). UCP1-positive beige adipocytes emerge within WAT in response to external cues, such as sustained cold exposure or exercise. Beige adipocytes are considered promising reservoirs for enhancing energy expenditure, but current technologies (e.g., ^{18}FDG-PET and MRI scans) do not possess enough resolution to detect beige cells in vivo. Our data indicate that our *Ucp1* luciferase mouse may

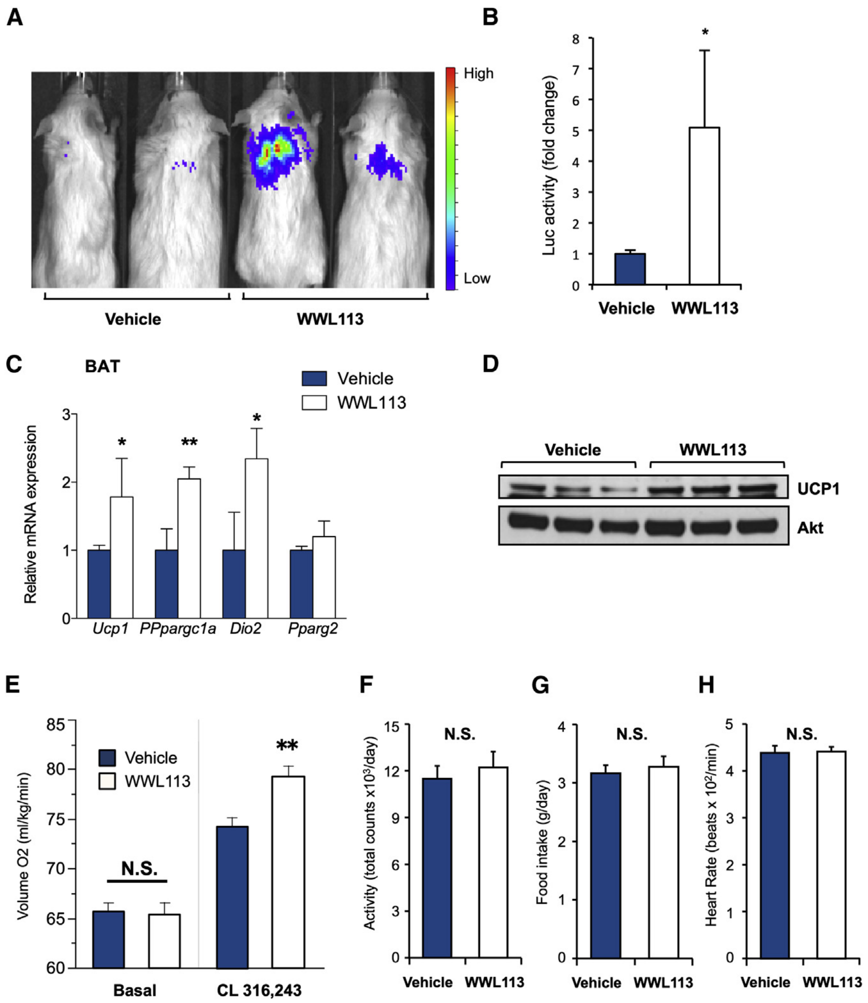

Figure 5. WWL113 Increases UCP1 Expression in Mice

(A) Luciferase activity in *Ucp1* luciferase reporter mice treated daily with WWL113 (50 mg/kg) or vehicle for 5 days (n = 5). Representative mice are shown.

(B) Quantification of luciferase signal in interscapular BAT of mice treated as in (A). Values normalized to protein content and shown as fold change relative to vehicle.

(C) Expression of thermogenic genes in interscapular BAT of C57BL/6 mice treated daily with WWL113 (50 mg/kg) or vehicle for 5 days (n = 5). $^{*}p < 0.05$; $^{**}p < 0.01$

(D) UCP1 protein expression in interscapular BAT of C57BL/6 mice analyzed in (C).

(E) VO_2 of wild-type mice treated daily with WWL113 (50 mg/kg) or vehicle for 7 days (n = 6). CL-316,243 (1 mg/kg) was injected to examine the response to adrenergic stimulation. $^{**}p < 0.01$. N.S., not significant.

(F) Locomotor activity of mice in (E).

(G) Food intake of mice in (E).

(H) Heart rate of mice in (E).

Data are expressed as means ± SEM.

serve as a tool to monitor the effects of compounds and biological factors on beige cells.

Although they share the thermogenic ability of brown adipocytes, beige adipocytes have a distinct, heterogeneous developmental origin that is not fully understood. Beige adipocytes in multiple WAT depots can arise from *Myf5*-positive and negative cells (Sanchez-Gurmaches and Guertin, 2014), and a subset of inguinal beige adipocytes originates from a smooth-muscle lineage (Long et al., 2014). Prior work has shown that regulation of *Ucp1* is distinct in the two types of thermogenic adipocytes (Guerra et al., 1998; Koza et al., 2000; Xue et al., 2007). We found that WWL113 could activate UCP1 expression in BAT, but not in inguinal WAT, implying that WWL113-initiated signaling events that regulate UCP1 expression may be unique to brown adipocytes.

BAT and liver are the major organs responsible for triglyceride uptake (Bartelt et al., 2011), and emerging evidence points to a close connection between BAT activity, liver function, and systemic lipid homeostasis. For example, defects in thermogenesis caused by BAT-specific knockout of the enzyme euchromatic histone-lysine N-methyltransferase 1 resulted in hepatic steatosis and insulin resistance, even when weight-matched mice were compared (Ohno et al., 2013). Conversely, activation of BAT thermogenesis, for example by overexpression of PTEN, reduced hepatic lipid accumulation and enhanced systemic insulin sensitivity (Ortega-Molina et al., 2012). Thus, the therapeutic benefits of increased BAT thermogenesis may not be limited to effects on weight but could include improvements in lipid homeostasis and whole-body insulin sensitivity. The set of tools we have developed should aid the development of pharmacologically tractable approaches to activate BAT function as a therapy against obesity and its complications.

EXPERIMENTAL PROCEDURES

Animals

Experiments were approved by the Institutional Animal Care and Use Committees of the University of California, San Francisco, and The Scripps Research Institute. Unless stated, mice were kept at room temperature. WWL113 was administered either intraperitoneally once daily (50 mg/kg in a 4:1 PEG300:Tween 80 vehicle solution) or orally (50 mg/kg in 0.5% hydroxypropylmethylcellulose). At the conclusion of treatment, BAT, WAT, skeletal muscle, and liver were snap frozen for luciferase assays and RNA and protein analysis. Energy balance studies were performed over the course of 7 days using a Comprehensive Lab Animal Monitoring System (Columbus Instruments) as described (Ohno et al., 2013). Data were normalized to body weight, as there were no differences between groups. Heart rate in the conscious state was measured by the indirect tail cuff method with a SC1000 MSP (Hatteras Instruments).

Generation of Ucp1 Luciferase Reporter Mice

A 98.6 kb BAC (bMQ353d13; Source BioScience) containing the entire *Ucp1* gene locus was obtained and a luciferase2-T2A-tdTomato reporter cassette (Addgene 32904) inserted at the initiation codon of the *Ucp1*-coding sequence located in exon 1 using BAC recombineering techniques (Warming et al., 2005). *Ucp1 luciferase* BAC DNA was microinjected into single-cell FVB embryos and transgenic founders and their offspring identified by PCR (primers provided in Table S1). Segregation patterns indicate that the transgene inserted into the Y chromosome. Transgenics display no decrease in fertility or any other abnormalities.

In Vivo Luciferase Imaging

Luciferase activity was monitored using an IVIS Spectrum Instrument (Caliper Life Sciences). For 3D reconstruction, six images (exposure time: 180 s; binning: L; F/Stop:1; emission filters: 560–660 nm; field of view: C) were collected starting 8 min after injection of 150 mg/kg luciferin (Goldbio). In other experiments, one image per scan (exposure time: 300 s; binning: M; F/Stop:1; emission filters: open; field of view: D) was acquired 15 min after luciferin injection. 3D reconstructions and luciferase activity were calculated using Living Image Software (Caliper Life Sciences).

Immortalized Ucp1 Luciferase Adipocyte Lines

The stromal vascular fraction from interscapular BAT of 3-week-old male *UCP1 luciferase* transgenic mice was isolated (Ohno et al., 2012) and cells infected with a retrovirus expressing large T antigen (pBabe SV40 Large T antigen; Addgene) and selected in puromycin (2 μg/ml). Immortalized preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), penicillin, and streptomycin. Upon 100% confluence (day 0), differentiation was induced with medium containing 10% FBS, 5 μg/ml insulin, 1 nM T3, 0.125 mM indomethacin, 2 μg/ml dexamethasone, and 0.125 mM 3-isobutyl-1-methylxanthine for 2 days. From day 2 on, cells were cultured only in the presence of insulin and T3.

Phenotypic Screen

A library of approximately 300 compounds (Adibekian et al., 2011; Bachovchin et al., 2010) was screened. Immortalized *Ucp1* luciferase brown preadipocytes were seeded in 96-well plates and differentiated as described above. At day 8, cells were exposed to compounds (5 μM for carbamates, 2.5 μM for triazole ureas) in serum-free DMEM. Rosiglitazone (0.5 μM) served as positive control. After 16 hr, media was replaced with Glo Lysis Buffer (Promega) and luciferase activity quantified in a PHERAstar reader (BMG Labtech). Activity was normalized relative to the signal in DMSO-treated cells in each plate. Assays were performed in triplicate. Compounds inducing >50% increase or decrease in luciferase activity were selected for follow-up.

Luciferase Assays

Cells or tissues were lysed in Cell Culture Lysis Reagent (Promega) and luciferase activity quantified using the Luciferase Assay System (Promega). Activity was measured in an Optocomp I reader (MGM Instruments) and normalized to total protein content.

Gene Expression

Total RNA was isolated using RiboZol reagents (AMRESCO) and reversed transcribed using an iScript cDNA synthesis kit (Bio-Rad) and quantitative real-time PCR performed using SYBR green and an ABI ViiA7 machine. Relative mRNA expression was determined by the ΔΔ-Ct method with TATA-binding protein as a normalization control. Primer sequences are provided in Table S1.

Metabolic Studies

Whole-body energy expenditure was measured at ambient temperature using a Comprehensive Lab Animal Monitoring System (Columbus Instruments) at the University of California, San Francisco (UCSF) animal metabolic core facility. Wild-type mice were treated daily with WWL113 (50 mg/kg) or vehicle for 7 days (n = 6). The mice were injected intraperitoneally with a β3-adrenergic receptor-specific agonist CL-316,243 at a dose of 1 mg/kg to examine the response to adrenergic stimulation. VO_2 was normalized by body weight.

Western Analysis

Proteomes were separated by SDS-PAGE. Antibodies used: UCP1 (Abcam; ab10983); Akt (Cell Signaling Technology; 9272); α-tubulin (Sigma; T8203); and β-actin (Sigma-Aldrich; AC-15).

Cellular Respiration

Oxygen consumption rate was measured in a MT200A Cell Respirometer (Strathkelvin), as previously described (Kajimura et al., 2009). Briefly, differentiated brown adipocytes treated with DMSO or WWL113 (10 μM) for 48 hr were trypsinized and incubated in serum-free medium in the presence or absence of norepinephrine. Uncoupled and nonmitochondrial cellular respiration were measured using oligomycin (1 μM) and antimycin A (1 μM).

Fat Transplantation

Preadipocytes were implanted as described (Kajimura et al., 2009). Briefly, cultured immortalized *Ucp1* luciferase preadipocytes were trypsinized, washed, and resuspended in PBS. Preadipocytes in a volume of 300 μl (~4 × 10^7 cells) were injected subcutaneously into NCr nude mice (Taconic). Six days after transplantation, mice were injected with either saline (n = 4) or rosiglitazone (n = 6; 10 mg/kg) twice daily for 7 days. Luciferase activity was monitored on days 0, 1, 4, and 7.

Histology

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (7 μm) were analyzed with hematoxylin and eosin staining and immunofluorescence to detect UCP1 (ab10983 1:1,000 in 5% goat serum) or GFP (GFP-1020 1:500 in 5% goat serum) as described (Sharp et al., 2012).

Statistics

Statistical significance was defined as $p < 0.05$ and determined by two-tailed Student t tests, Wilcoxon, or ANOVA, with Dunne's multiple comparison post hoc analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.066.

AUTHOR CONTRIBUTIONS

A.G., S.B.S., E.S., and S.K. designed experiments. J.W.C. and B.F.C. provided libraries and synthesized compounds. S.K. and S.B.S. generated transgenic model and cell lines. A.G. performed screen and follow-up. A.G., S.B.S., S.A.-K., Y.H., K.S., and S.K. performed in vivo and vitro experiments. L.Z.S. and I.H.N.L. provided technical help. A.G., S.B.S., E.S., and S.K. wrote the manuscript.

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