The Gq signalling pathway inhibits brown and beige adipose tissue

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Brown adipose tissue (BAT) dissipates nutritional energy as heat via the uncoupling protein-1 (UCP1) and BAT activity correlates with leanness in human adults. Here we profile G protein-coupled receptors (GPCRs) in brown adipocytes to identify druggable regulators of BAT. Twenty-one per cent of the GPCRs link to the Gq family, and inhibition of Gq signalling enhances differentiation of human and murine brown adipocytes. In contrast, activation of Gq signalling abrogates brown adipogenesis. We further identify the endothelin/Ednra pathway as an autocrine activator of Gq signalling in brown adipocytes. Expression of a constitutively active Gq protein in mice reduces UCP1 expression in BAT, whole-body energy expenditure and the number of brown-like/beige cells in white adipose tissue (WAT). Furthermore, expression of Gq in human WAT inversely correlates with UCP1 expression. Thus, our data indicate that Gq signalling regulates brown/beige adipocytes and inhibition of Gq signalling may be a novel therapeutic approach to combat obesity.
Brown adipose tissue (BAT) is important for basal and inducible energy expenditure in mammals. Importantly, recent findings indicate that adult humans possess metabolically active BAT and that obese subjects have reduced activity of BAT. BAT uniquely expresses the uncoupling protein-1 (UCP1), a mitochondrial protein which uncouples ATP production from oxidative phosphorylation leading to energy expenditure in a thermogenic manner. In contrast, white adipose tissue (WAT) is mainly responsible for energy storage and is an important endocrine tissue that releases adipokines, which in turn regulate energy intake and expenditure. Brown-fat like cells have recently been described in WAT depots and their number and activity can be strongly induced by cold exposure, a process described as ‘browning’.

G protein-coupled receptors (GPCRs) are a large family of seven transmembrane proteins that regulate important biological processes in diverse tissues including adipose tissue. Approximately 25% of currently marketed drugs target GPCRs, illustrating their importance in disease and therapeutic development. GPCRs are coupled to heterotrimeric G proteins, which are composed of Gα, β and γ subunits. Activation of GPCRs leads to the dissociation of Gα from the Gβγ dimer, allowing the binding and regulation of signalling effectors. The downstream signalling of GPCRs is in part determined by their G protein coupling. There are four main classes of Gα proteins: Gαs, Gαi, Gαq and Gα12/13. Activation of Gαs and Gαi leads to the stimulation or inhibition of cAMP signalling, respectively, while Gαq activates phospholipase C (PLC). Gα12/13 activates the small GTPase Rho, a pathway also known to be modulated by the Gq pathway.

Until now, the analysis of BAT GPCRs has focused mainly on a few Gαq-coupled receptors (for example, β-adrenergic and adenosine receptors) that activate cAMP signalling and UCP1-dependent thermogenesis. The function of other Gαq GPCR families in adipocytes is not as clear. Therefore, in the present study, we investigate the expression pattern of non-odorant GPCRs in brown adipocytes (BAs). Our analysis reveals that Gαq-coupled receptors are highly expressed in brown adipocytes. Moreover, using pharmacological and genetic approaches, we found that Gαq via modulation of RhoA signalling, regulates adipogenesis of BAs in vitro and in vivo.

Results

GPCR expression of brown adipocytes. To identify the expression pattern of non-odorant/tastant GPCRs in BAs, we profiled the transcript levels of 347 GPCRs. We detected 182 GPCRs in preadipocytes and 230 in mature BAs (Supplementary Tables 1,2). 18 GPCRs and 66 GPCRs were uniquely expressed in preadipocytes and mature BAs, respectively (Fig. 1a). We used the IUPHAR database to define GPCRs. Class A and adhesion GPCRs represented the two largest classes in both pre- and mature BAs (Table 1). During differentiation, class A receptors increased from 139 to 188 receptors, whereas 13 out of the 20 adhesion GPCRs expressed in preadipocytes decreased upon differentiation with five of them decreasing below the detection limit (Table 1). With respect to coupling to heterotrimeric G proteins, we found that 14.0 and 31.5% of the GPCRs detected in BAs interact with Gαs and Gαq, respectively (Fig. 1b). Unexpectedly, 21.5% of the GPCRs expressed in BAs are predicted to link with Gαq/11 (Fig. 1b). Preadipocytes exhibited a similar pattern of GPCR linkage (Fig. 1c).

During differentiation, the expression levels of 91 GPCRs changed more than twofold, with 26 and 65 showing a decrease or increase in expression, respectively (Supplementary Fig. 1a,b). During brown adipogenesis, Gαq- and Gαi-coupled GPCRs were the largest group of receptors with known G protein linkages to be down- and upregulated, respectively (Supplementary Fig. 1a,b).

To validate the expression pattern of GPCRs, we focused on six Gαq-coupled GPCRs that are highly expressed in pre- and/or mature BAs as identified by the GPCR array (Supplementary Tables 1,2): Endothelin (ET) receptors type A and B (Ednra and Ednrb), Adrenergic Alpha 1A (Adra1a), Angiotensin II receptor, type 1a and 1b (Agtr1a and Agtr1b) and Cholinergic Receptor, Muscarinic 3 (Chrm3). Both quantitative PCR (qPCR) and TaqMan GPCR array data demonstrated high expression of Ednra mRNA in preadipocytes and an increase of Ednrb during differentiation (Supplementary Fig. 1c). Ednrb increased 332-fold (qPCR) and 322-fold (GPCR array) during differentiation; Adra1a increased 83-fold (qPCR) and 322-fold (GPCR array); Agtr1a increased 3.9-fold (qPCR) and 2.9-fold (GPCR array) (Supplementary Fig. 1c). Gαq and Gα11 are both expressed in preadipocytes, BAs and BAT and their levels of expression did not change significantly during differentiation (Supplementary Fig. 1d). Thus, GPCR/Gαq/11 signalling might contribute to the differentiation and function of BAs; however, virtually no information exists regarding this signalling pathway in BAT.

Regulation of adipogenesis by Gαq. We inhibited Gαq11, signal transduction using pharmacological and genetic approaches. Treatment with FR900359 (FR; 1 μM), a natural inhibitor of Gαq11 (ref. 20), enhanced differentiation of murine BAs, as demonstrated by increased Oil Red O staining (Fig. 1d) and elevated protein levels of the adipogenic markers aP2 and PPARγ as well as of the thermogenic marker UCP1 (Fig. 1e,f). To complement the pharmacological approach, we used lentiviral small hairpin RNA (shRNA) directed only against Gαq (shGαq). Knockdown of Gαq by ~50% (Supplementary Fig. 1e) significantly increased adipogenesis, with increased lipid staining (Fig. 1d) and expression of adipogenic markers and UCP1 (Fig. 1g,h).

Inactivation of Gαq signalling thus promotes differentiation of murine BAs.

To determine the impact of increased Gαq signalling on brown adipogenesis, we first selectively enhanced Gαq signalling using a Gαq-coupled designer GPCR (DREADD) that represents a modified M3 muscarinic receptor (Dq)22. Dq couples exclusively to Gαq and is activated by otherwise pharmacologically inactive clozapine-N-oxide (CNO)22. Brown preadipocytes were transduced with lentiviral vectors expressing Dq. To analyse Gαq-induced responses in living cells in a label-free manner, we used biosensor technology based on dynamic mass redistribution (DMR). Treatment of Dq-expressing cells with CNO (1 μM) induced a concentration-dependent DMR response with half-maximal activation at 269 nM (Fig. 1i). In contrast, no DMR response was observed in buffer- or CNO-treated wild-type cells (Supplementary Fig. 1f). The Dq-induced DMR response was markedly different from the optical traces obtained by stimulation of Gαq with isoproterenol (Supplementary Fig. 1g,h), implying that DMR can be used to monitor different G protein signalling pathways in BAs. Treatment of Dq cells with FR abolished the CNO-induced DMR response, providing further evidence for FR as a Gαq inhibitor (Fig. 1j and Supplementary Fig. 1j).

Dq stimulation during BA differentiation reduced Oil Red O staining (Supplementary Fig. 1j), suppressed expression of adipogenic markers aP2 and PPARγ and of UCP1 (Fig. 1k,l). Treatment of non-transduced control cells or cells transduced with a control virus (LVctrl) with CNO did not affect differentiation (Fig. 1k,l and Supplementary Fig. 1j). Expression of Dq without CNO stimulation did not alter BA differentiation (Fig. 1k,l and Supplementary Fig. 1j).
Secondly, we used lentiviral vectors to express a constitutively active mutant of Gq (LVGqQL) as a means to enhance Gq signalling. We validated the activity of GqQL in brown preadipocytes by measuring IP1 in non-transduced cells and cells that express either GFP or GqQL. IP1 is a downstream metabolite of PLC and IP3, a pathway known to be activated by Gq GPCRs. We found that IP1 levels were highly increased in preadipocytes that express GqQL compared with GFP-expressing or control cells (Supplementary Fig. 1k). GqQL overexpression during adipocyte differentiation reduced Oil Red O staining and inhibited the expression of adipogenic and thermogenic markers (Fig. 1m,n and Supplementary Fig. 1l) in a similar manner as did the DREADD approach.

**Endothelin/Ednra regulation of brown adipocytes.** To identify an endogenous receptor that activates Gq signalling in BAs, we focused on Ednra, one of the most highly expressed Gq-coupled GPCRs in preadipocytes and BAs (Supplementary Tables 1,2). Both preadipocytes and BAs express constituents of the ET pathway, including ET and Ednra. In preadipocytes, we observed that Ednra levels were increased during adipocyte differentiation (Supplementary Fig. 1i). To study the role of Ednra in brown adipocyte function, we used lentiviral vectors to express Ednra in murine brown adipocytes. We found that Ednra expression reduced Oil Red O staining and inhibited the expression of adipogenic and thermogenic markers (Fig. 1m,n and Supplementary Fig. 1l) in a similar manner as did the DREADD approach.
system: Endothelin-1 (ET-1), ET receptors type A and B (Ednra and Ednrb) and ET converting enzyme-1 (ECE-1) (Supplementary Fig. 2a). Although ECE-1 was expressed at low levels in BA, it is not the only enzyme that can catalyse the production of ET-1 (ref. 23). Importantly, ET-1 was released by both cell types (Fig. 2a) and its secretion was inhibited by noradrenaline (NE) (Fig. 2a).

Differentiation of BAs was inhibited by chronic treatment with ET-1 (0.3 nM) but was restored by the selective Ednra antagonist BQ-123 (100 nM) (Fig. 2b–d) and by treatment with FR (1 μM) (Fig. 2e–g). Notably, BQ-123 increased the differentiation of the untreated cells. In contrast, ET-1 effects on differentiation were not restored with the Ednrb antagonist BQ-788 (100 nM) (Supplementary Fig. 2b–d). Moreover, acute treatment of mature BAs with ET-1 reduces lipoprotein lipase gene expression 24.

Gq regulation of BAs via Rho/Rho-kinase. Gq can activate multiple intracellular signalling pathways including IP3/DAG, Ca2+, ERK and also by GTP-binding proteins of the Ras and Rho families1,16,17. Since Rho/Rho-kinase (ROCK) controls brown adipogenesis25,26, we evaluated this pathway. Cells expressing GqQL had a marked increase in phallloidin staining of actin filaments (Fig. 2h) and Rho activity (Rho-GTP pull-down assay) (Fig. 2i). In addition, pharmacological inhibition of ROCK with Y-27632 (10 μM) enhanced differentiation and increased expression of adipogenic and thermogenic markers in GqQL-expressing BAs (Fig. 2j–l). ROCK inhibition also restored adipogenesis of ET-1 treated cells (Supplementary Fig. 2e–g).

In Vivo regulation of BAT by Gq signalling. To study the effects of elevated Gq signalling in vivo, we expressed constitutively active Gq in BAT (Fig. 3a). Lentiviral particles encoding either GqQL (LVGqQL) or GFP (LVGFP) under the control of the CMV promoter were directly injected into BAT of mice that were subsequently cold-exposed for 2 weeks (Fig. 3b). Histological analysis revealed increased size of the lipid droplets with more unilocular, white-like adipocytes in LVGqQL-injected BAT and subsequently cold-exposed for 2 weeks (Fig. 3c). expression (Fig. 3c). In contrast, iWAT and gWAT of GqQL mice exhibited larger lipid droplets with decreased expression of BA markers (UCP1, PGC1α and DIO2) (Fig. 3a,b and Supplementary Fig. 3c,d).

Further evidence for a role of Gq in inducible brown/beige cells was obtained by induction of browning in isolated primary murine adipocytes (Fig. 4c). Using the DREADD system (Fig. 1i–l), we activated Gq signalling with CNO (10 μM), which resulted in a significant reduction in UCP1 expression (Fig. 4c).

To explore the effect of enhanced Gq signalling on white adipogenesis, we used murine white adipocytes isolated from iWAT (WAT). We overexpressed Dq and activated it with CNO throughout differentiation. Gq activation suppressed WAi differentiation (Supplementary Fig. 3e–g).

Role of Gq signalling in human adipocytes. Human BAT also highly expresses Gq protein. We found Gq-coupled receptors that were highly expressed in murine BAs are also highly expressed in the human brown/beige adipocyte cell line (hMADS) (Ednra, Ednrb, Adra1a, Agtr1 and Chrm3) (Supplementary Fig. 3h,i).

In the present study, we screened for non-chemosensory GPCRs in murine brown preadipocytes and mature adipocytes. GPCR profiling revealed that BAs express more than 180 GPCRs and that the expression pattern changes greatly upon differentiation. The number of GPCRs detected is in agreement with previous analysis performed on human subcutaneous adipose tissue and murine metabolic tissues that detected 163 and 198 GPCRs, respectively29,30. In BAs, we found that Gq-coupled receptors were one of the largest groups and that their expression was highly altered during differentiation, indicating a potential role in adipogenesis. Importantly, we show that Gq signalling is a crucial inhibitor of adipocyte differentiation, through activation of the Rho/ROCK signalling cascade, a major regulator of BA differentiation20 (Fig. 4g).

Discussion

In the present study, we screened for non-chemosensory GPCRs in murine brown preadipocytes and mature adipocytes. GPCR profiling revealed that BAs express more than 180 GPCRs and that the expression pattern changes greatly upon differentiation. The number of GPCRs detected is in agreement with previous analysis performed on human subcutaneous adipose tissue and murine metabolic tissues that detected 163 and 198 GPCRs, respectively29,30. In BAs, we found that Gq-coupled receptors were one of the largest groups and that their expression was highly altered during differentiation, indicating a potential role in adipogenesis. Importantly, we show that Gq signalling is a crucial inhibitor of adipocyte differentiation, through activation of the Rho/ROCK signalling cascade, a major regulator of BA differentiation20 (Fig. 4g).

Table 1 | Classification of GPCR families.

| Receptor class | Preadipocytes | Brown adipocytes |
|---------------|---------------|-----------------|
| Class A       | 139 (76%)     | 188 (82%)       |
| Class B       | 5 (3%)        | 7 (3%)          |
| Class C       | 5 (3%)        | 6 (3%)          |
| Adhesion      | 20 (11%)      | 15 (7%)         |
| Frizzled      | 10 (5%)       | 11 (5%)         |
| Other         | 3 (2%)        | 3 (1%)          |

GPCR, G protein-coupled receptor.
Figure 2 | Role of Endothelin-1 in differentiation and downstream signalling of G\textsubscript{q}. (a) Release of ET-1 by preadipocytes, non-stimulated and NE-stimulated BA. ANOVA, *P<0.05. (b) Oil Red O stain of BA chronically treated with ET-1, Ednra inhibitor BQ-123, or both. Scale bar, 1 cm. (c,d) Representative immunoblots (c) and quantification (d) of adipogenic markers and UCP1 after treatment of BA with and without ET-1, ET-1+BQ-123 or both. ANOVA, *P<0.05. (e) Oil Red O stain of BA chronically treated with ET-1, FR, or both. Scale bar, 1 cm. (f,g) Representative immunoblots (f) and quantification (g) of aP2, PPAR\text{\gamma} and UCP1 of BA chronically treated with and without ET-1, FR or both. ANOVA, *P<0.05. (h) Phalloidin staining of F-Actin stress fibres in non-transduced cells and cells transduced with control virus (LVGFP) or virus expressing constitutively active G\textsubscript{q} (LVGqQL) in serum-starved and stimulated state. Scale bar, 50 \mu m. (i) RhoA activation assay in preadipocytes expressing GFP (LVGFP) or GqQL (LVGqQL) ET-1 and/or BQ-123. ANOVA, *P<0.05. (j) Oil Red O stain of non-transduced BA and BA expressing GFP (LVGFP) or GqQL (LVGqQL) differentiated in presence or absence of ROCK inhibitor Y-27632. Scale bar, 1 cm. (k,l) Representative immunoblots (k) and quantification (l) of aP2, PPAR\text{\gamma} and UCP1 in BA non-transduced and transduced with lentivirus expressing GFP (LVGFP) or GqQL (LVGqQL) and differentiated in presence or absence of Y-27632. *P<0.05. All data are shown as mean ± s.e.m.

Treatment with the G\textsubscript{q} inhibitor FR enhanced the adipogenic and thermogenic potential of BAs, indicating either the presence of constitutively active G\textsubscript{q} receptors and/or an autocrine loop of G\textsubscript{q} signalling in BAs. Analysis of highly expressed G\textsubscript{q}-coupled GPCRs indicated that murine BAs and human brown/beige cells express ET receptors. Moreover, murine BAs also express the...
enzymes necessary for ET production and secretion. Stimulation with ET-inhibited adipogenesis, while the inhibition of $G_q$ and Ednra, but not Ednrb, rescued adipogenesis of ET-treated cells. Recently, Xue et al.\textsuperscript{31} identified Endrb as a genetic marker that positively correlates with the thermogenic capacity of cloned human BAs. Inhibition of Ednra alone increased the differentiation of untreated cells, indicating endogenous activity of the ET system in BAs. Thus, our data implicate the ET-1/Ednra signalling axis as an autocrine/paracrine inhibitor of brown/beige adipogenesis via $G_q$ signalling (Fig. 4g). In addition to its function in BAT, ET-1 plays an important role in the vasculature and excessive ET-1 contributes to atherosclerosis\textsuperscript{32} as well as to the disturbed insulin-induced vasorelaxation in obesity\textsuperscript{33}.

Our data also indicate an inhibitory role of $G_q$ in BAT function and browning \textit{in vivo}. Overexpression of GqQL induced a phenotype of BAT reminiscent of ‘whitening’, while preventing the ‘browning’ of WAT in mice exposed to cold. Overall, activation of $G_q$ signalling in brown/beige adipocytes resulted in reduced whole-body energy expenditure of cold-exposed mice. Thus, our data show that the $G_q$ signalling pathway inhibits brown and beige adipose tissue \textit{in vivo} and the studies done using cultured adipocytes show that $G_q$ signalling inhibits adipogenesis in a more general manner. Furthermore, we found a negative correlation between $G_q$ and UCP1 mRNA expression in human abdominal WAT. Although abdominal adipose tissue is predominantly white, it can be induced to brown and inducible brown-like cells have been identified in abdominal fat after $\beta-3$ adrenergic receptor treatment\textsuperscript{34}. Further studies are required to investigate the role of alterations in the expression of BAT marker genes in abdominal fat depots in adult humans. Interestingly, $G_q$ signalling is not only involved in regulation of adipocyte differentiation and non-shivering thermogenesis, but also in the CNS regulation of appetite\textsuperscript{35}.

In conclusion, our results demonstrate a previously unappreciated role in brown/beige adipogenesis for $G_q$, to which $\sim 20\%$ of the GPCRs expressed in these cells are predicted to couple. Our findings suggest that antagonizing $G_q$ signalling by targeting $G_q$-coupled GPCRs and/or by directly inhibiting $G_q$ represent

![Figure 3 | $G_q$ activation negatively affects BAT \textit{in vivo}.](Image)

(a) Relative mRNA expression of $G_q$, Ndufa and Nds5 in BAT of LVGFP and LVGqQL mice. t-test, *$P<0.05$. (b) Representative images of interscapular BAT of mice injected with LVGFP or LVGqQL in bright field (left) and fluorescent microscopy (right). Scale bar, 5 mm. (c) Representative hematoxylin/eosin (HE) and UCP1 staining of BAT in LVGqQL and LVGFP mice after cold stimulation. Scale bar, 100 $\mu$m. (d) Representative immunoblots (top) and quantification (bottom) of UCP1 expression in BAT of LVGFP and LVGqQL mice. t-test, *$P<0.05$. (e) Relative mRNA expression of $G_q$ and UCP1 in BAT of UCP1-GFP and UCP1-GqQL transgenic mice. t-test, *$P<0.05$. (f) Representative HE and UCP1 staining of BAT in UCP1-GFP and UCP1-GqQL mice after cold stimulation. Scale bar, 100 $\mu$m. (g) Representative immunoblots (top) and quantification (bottom) of UCP1 expression in BAT of UCP1-GFP and UCP1-GqQL mice. t-test, *$P<0.05$. (h) Oxygen consumption of UCP1-GFP and UCP1-GqQL mice over 24 h. (i) Area under the curve (AUC) of the oxygen consumption of UCP1-GFP and UCP1-GqQL mice over 24 h t-test, *$P<0.05$. (a-d) 11 animals per group were analysed. (e-g) 6 animals per group were analysed, (f,h,i) 4 animals per group were analysed. All data are shown as mean $\pm$ s.e.m.
previously unexplored ways to enhance the amount of brown/beige fat and thus increase energy expenditure.

**Methods**

**Isolation and differentiation of BAs.** BAT-derived mesenchymal stem cells were isolated from interscapular BAT of newborn wild-type mice. Excised BAT was incubated for 30 min at 37°C in digestion buffer (Dulbecco’s modified Eagle’s medium (DMEM), Invitrogen) containing 123 mM Na⁺, 5 mM K⁺, 1.3 mM Ca²⁺, 131 mM Cl⁻, 5 mM glucose, 1.5% (w/v) bovine serum albumin (BSA), 100 mM Hepes and 0.2% (w/v) collagenase type II (pH 7.4). After digestion, tissue remnants were removed by filtration through a 100 μm nylon mesh and placed on ice for the next 30 min. The infranatant was filtered through a 30 μm nylon mesh and placed on ice for the next 30 min. The pellet containing mesenchymal stem cells was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin, streptomycin (100 μg ml⁻¹), 100 mM Hepes and 0.2% (w/v) collagenase type II (pH 7.4). Approximately 60,000 cells per cm² were seeded on a six-well plate and grown at 37°C and 5% CO₂. Preadipocytes were immortalized using the lentivirus containing the SV40 large T antigen and unselected cells were cultured in DMEM supplemented with FBS and P/S (GM). The cells were expanded in GM at 37°C and 5% CO₂ (ref. 37).

For differentiation, immortalized cells were seeded in GM on a six-well plate in a density of ~180,000 cells per well. 48 h after seeding (day 0), GM was replaced by differentiation medium (DM) supplemented with FBS, P/S, 20 nM insulin and 1 nM triiodothyronine. Confluent cells (day 0) were then treated for 48 h with DM supplemented with 0.5 mM isobutylmethylxanthine (IBMX) and 1 μM dexamethasone. After the induction, cells were treated with DM for the next 5 days, which was replenished every second day. Where indicated, cells were treated every second day with the Gαi inhibitor FR (1 μM), ROCK inhibitor Y-27632 (10 μM), Ednra inhibitor BQ-123 or Ednrb inhibitor BQ-788 (both Tocris, 100 nM). In the experiments with ET-1, FR, Y-27632, BQ-783 or BQ-788 were added to the culture medium 30 min before the addition of ET-1.

For all the preadipocyte experiments, the cells were used on day 2. For mature BA experiments, the cells were used on day 7 after induction.

**Isolation and differentiation of white and beige adipocytes.** WAT-derived mesenchymal stem cells were isolated from inguinal white fat pads of 8- to 12-week-old wild-type mice. Excised WAT was incubated for 30 min at 37°C in digestion buffer (DMEM containing 0.5% (w/v) BSA and 0.15% (w/v) collagenase type II). After digestion, cells were allowed to stand for 10 min at room temperature and centrifuged at 700g for 10 min. The pellet was resuspended in GM and filtered through a 100 μm nylon mesh. Approximately 180,000 cells per well were seeded in GM on a six-well plate at 37°C and 5% CO₂.

For differentiation, cells were seeded 2 days after reaching confluence (day 0) for 48 h, using WAT induction medium (DMEM supplemented with 5% FBS, 1% P/S, 1 μM dexamethasone, 0.5 mM IBMX, 1 mM triiodothyronine, 1 mM D-biotin, 17 mM pantothenate, 1-ascorbate (50 mg ml⁻¹), 1 μM rosiglitazone and 0.172 mM insulin). After the induction, cells were treated with maintenance medium (DMEM supplemented with 5% FBS, P/S, 1 nM triiodothyronine, 1 mM D-biotin, 17 mM pantothenate, 1-ascorbate (50 mg ml⁻¹), 1 μM and 0.172 μM insulin) for the next 7 days, which was replenished every second day. For differentiation experiments, cells were treated every second day with CNO (1 μM).

![Figure 4](image-url)
separated using SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membrane was blocked for 1 h in 5% BSA in Tris-Buffered Saline and 0.1% Tween 20 (TBST) and incubated overnight at 4 °C in different primary antibodies (all primary antibodies diluted 1:1,000). Incubation in secondary antibody was performed the next day, for 1 h at room temperature in 5% milk in TBST. Proteins were visualized with an enhanced chemiluminescence (ECL) reagent and quantified by densitometric analysis with Image J software. Antibodies directed at the following were used: α-P2 (Santa Cruz Biotechnology, Cat#: sc-86661), PPARγ (Santa Cruz Biotechnology, Cat#: sc-7273); UCP1 (Sigma Aldrich, Cat#: sc-6529 and Thermo Fisher Scientific, Cat#: PA1-24894); Tubulin (Dianova, Cat#: MS-719-P0) and GAPDH (Cell Signalling, Cat#: 2118). Secondary horseradish peroxidase-linked antibodies were green for UCP1 (1:700) and red for Tubulin (1:35-147, dilution 1:5,000), mouse (Dianova, Cat#: 115-035-146, dilution 1:10,000) and rabbit (Cell Signalling, Cat#: 7074, dilution 1:5,000) were used. Complete immunoblots of western blot sections are shown in Supplementary Fig. 4.

Label-free dynamic mass redistribution assays. The DMR analysis was carried out using the Corning Epic system (Corning, NY, USA) in conjunction with a Cytbi-SELMa semi-automated electronic pipetting system (Analytik Jena AG, Jena, Germany)3. Briefly, brown preadipocytes stably expressing Dq were seeded on fibronectin-coated 384-well biosensor plates at a density of 3,000 cells per well and cultivated for 48 h in growth medium (37 °C, 5% CO2). Before the measurement, cells were washed twice with assay buffer (Hanks balanced salt solution with 20 mM HEPES). Where indicated, cells were pre-incubated with FR (1 μM) for 1 h or 1 μM FR for 18-24 h before the DMR was measured for at least 5,000 s. The optical DMR recordings were buffer-corrected. To quantify the DMR signals for concentration-effect curves, the maximum response within 1,000 s was calculated. pEC50 value determination and data calculation were performed using GraphPad Prism 5.04 (GraphPad Software, La Jolla, USA).

IP1 assay. Intracellular alteration of the second messenger IP1 was quantified with the HTTR-IP1 kit (Casbio Bioassays) following the manufacturer’s instructions. Briefly, brown preadipocytes non-transduced and transduced with lentiviruses carrying QGQL (LVQGQL) or GFP (LVGFP) were cultivated for 48 h in growth medium (37 °C, 5% CO2). For the assay, the cells were resuspended in stimulation buffer containing 30 mM LaCl₃ and transferred to a 384-well microtiter plate at a density of 12,500 cells per well. After 90 min incubation time, 3 μl of IP1 ELISA substrate followed by 3 μl of europium cryptate-labeled anti-IP1 antibody in lysin buffer were added to the cells to quantify intracellular IP levels. After a further incubation of 1 h at room temperature, time-resolved fluorescence was measured at 620 and 665 nm with the Mithras LB 940 multimode reader.

ET-1 ELISA assay. Cell culture medium was collected from the preadipocytes and mature BAs, with or without 8 h NE (1 μM) stimulation. ET-1 ELISA was performed using the Endothelin-1-ELISA kit (Enzo) according to the manufacturer’s instructions.

F-actin staining of adherent cells in culture. Glass coverslips were placed in 24-well plates and coated with fibronectin (3 μg ml⁻¹). Brown preadipocytes were seeded to a density of 20,000 cells per well and serum-starved for 24 h. Cells were incubated for 30 min with 10% FBS to induce the formation of F-actin stress fibres. Cells were then fixed with 4% PFA, permeabilized with 0.1% Triton X-100, blocked with 1% BSA/PBS and stained with phalloidin-Alexa 546. Coverslips were mounted on glass slides using PermaFluor mounting medium and visualized using a confocal microscope.

Rhøa activation assay. Brown preadipocytes were seeded on a 10 cm tissue plate at a density of 180,000 cells per well and infected with lentiviruses carrying QGQL or GFP (control) for 8 h. Cells were cultured until reaching 50–60% confluence, serum-starved for 24 h and collected for Rhøa activity measurement. Rhøa activation was assessed with an ELISA-based Rhøa G-LISA Activation Assay Kit (Cytoskeleton). The level of Rhøa activity was determined by colorimetric measurement at 490 nm. Active Rhøa was normalized to total Rhøa protein.

Lentiviral injections of constitutively active Gq (CMV-QGQL) into BAT. Four-week-old male mice (C57BL/6, Charles River) were anaesthetized using isoflurane. A small incision was made in the neck region and 1 μl of lentiviruses carrying either QGQL (LVQGQL) or GFP (LVGFP) under control of the ubiquitous CMV promoter were injected directly into each fat pad of BAT. After injections, mice were acclimatized to cold for 1 week at 18 °C following 1 week of cold exposure at 4 °C. During the study, the mice were maintained on a daily cycle of 12 h light (0600 to 1800 hours) and 12 h darkness (1800 to 0600 hours), and were allowed free access to standard chow and water. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany.

RNA isolation and real-time RT-PCR (qPCR). RNA was isolated using Trizol method (Analytik Jena AG). The samples from human retroperitoneal BAT have been previously described46. cDNA was synthesized from 0.5 μg RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time RT-PCR (qPCR) was performed with SYBR-Green PCR master mix (Applied Biosystems) or LightCycler 480 SYBR Green I Master (Roche) using a HT7900 instrument (Applied Biosystems). Fold changes were calculated using relative quantification methods with mHPRT (murine hypoxanthine guanine phosphoribosyl) transferase) serving as an internal control unless otherwise stated. Primer sequences are available in Supplementary Table 3.

GPCR expression analysis. GPCR profiling was performed using TaqMan Mouse GPCR Arrays (Applied Biosystems) for quantitative expression analysis of mouse GPCR genes3. These arrays detect 347 non-chemosensory GPCRs. cDNA was synthesized from 1 μg RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Two nanograms of cDNA was used for each gene in the GPCR arrays. Quantification of GPCR cDNA expression was normalized to 185 rRNA. Data shown are an average of three independent arrays. GPCRs were considered unexpressed if at least two of the arrays had a delta Ct above 25. G protein coupling of GPCRs was determined using the IUPHAR database (http://guidetopharmacology.org/).

Lentiviral infection. Constitutively active Gq (GqQL) was provided by Silvio Gutkind, NIH42. Constitutive activity was achieved by mutating Glutamine to Leucine at the amino acid residue 209. Lentiviral vectors were obtained by cloning constitutively active Gq into the Bam HI and Sal I sites of the vector p156rrlpsinPPTCMV, which carries a murine uncoupling protein-1 promoter (UCPI-QGQL) or ubiquitous CMV promoter (LVGqQL). Control vectors (p156srlpsinPT) contain green fluorescent protein (UCPI-GFP and LVGFP). The shRNA against Gq and the control shRNA were purchased from Sigma Aldrich and were expressed under the U6 promoter (pLKO.1-U6-sh-Gq-287918-3′, pLKO.1-U6-sh-Gq-287918-3′). Whole cells were cultured for 18–20 h before the DMR was monitored for at least 5,000 s. The optical DMR recordings were buffer-corrected. To quantify the DMR signals for concentration-effect curves, the maximum response within 1,000 s was calculated. pEC50 value determination and data calculation were performed using GraphPad Prism 5.04 (GraphPad Software, La Jolla, USA).

Oil Red O staining. Mature BAs were fixed in PBS containing 4% paraformaldehyde (PFA). After washing with PBS, cells were incubated with Oil Red O (Sigma) solution (3 mg ml⁻¹ in 60% isopropl alcohol) for 3 h at room temperature, washed with PBS and visualized under a microscope.

Western blot analysis. Protein lyses from cells and tissues were isolated26,36 using lysis buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA and 1 mM EGTA) supplemented with complete protease inhibitor cocktail (Roche), 1 mM Na₂VO₃ and 10 mM NaF. Protein contents were determined by the Bradford method. Proteins were separated using SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membrane was blocked for 1 h in 5% BSA in Tris-Buffered Saline and 0.1% Tween 20 (TBST) and incubated overnight at 4 °C in different primary antibodies (all primary antibodies diluted 1:1,000). Incubation in secondary antibody was performed the next day, for 1 h at room temperature in 5% milk in TBST. Proteins were visualized with an enhanced chemiluminescence (ECL) reagent and quantified by densitometric analysis with Image J software.

For the C57BL/6 mice the adipocytes were used on day 7 after induction.
Generation of transgenic mice and energy expenditure. Transgenic mice were generated by subcutaneous injections 44 of the lentiviruses UCPI-GqQL and UCPI-GFP into otic vesicles of the donor mouse with C57BL/6 background. Ten-week-old female mice were kept for one week at 18 °C followed by one week cold exposure at 4 °C. Oxygen consumption was measured with Phenomex (TSE Systems) for 120 s every 15 min for 24 h. During the study, the mice were maintained on a daily cycle of 12 h light (0600–1800 hours) and 12 h darkness (1800–0600 hours), and were allowed free access to standard chow and water. The study was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany.

Immunohistochemistry. BAT and WAT was fixed in PBS containing 4% PFA for 48 h and dehydrated using ethanol. Tissue was embedded in paraffin and cut into 5 μm sections. Sections were blocked with 2% normal goat serum-TBS (tris-buffered saline) for 30 min RT and immunohistochemical stainings were performed with a primary antibody (UCPI, 1:1000, Sigma Aldrich) overnight. Secondary antibody-conjugated with horseradish peroxidase (Santa Cruz Biotechnology) was applied for 1 h at RT and sections were visualized using DAB substrate (Vector Laboratories). Standard haematoxylin and eosin (HE) staining was performed on 5 μm tissue sections.

Analysis of human adipose tissue. Gq, UCPI and CIDEA mRNA expression were measured in abdomenal omental and adipose tissue (AT) samples obtained in parallel from 266 donors who underwent open abdominal surgery for Roux-en-Y bypass, sleeve gastrectomy, explorative laparotomy or elective cholecystectomy. All participants gave their written informed consent before taking part in the study. All investigations have been approved by the ethics committee of the University of Leipzig (363-10-13122010 and 017-12-230112) and were carried out in accordance with the Declaration of Helsinki. Human Gq, UCPI and CIDEA mRNA expression was measured by qPCR using Assay-on-Demand gene expression kit (Gq: forward: 5′-TGGGAGAAAGGTGTCTGCTTTTGA-3′; reverse: 5′-ATCTTGTGTCGTTAGG CAGCTTAGG-3′; UCPI: Hs00222453_m1; CIDEA: Hs00154455_m1; Applied Biosystems, Darmstadt, Germany), and fluorescence was detected on an ABI PRISM 7900 Sequence Detector (Applied Biosystems). Gq, UCPI and CIDEA mRNA expression was calculated relative to the mRNA expression of HPRT1 mRNA (Hs003267_m1; Applied Biosystems).

Statistics. For cell culture experiments, ‘n’ indicates the number of cultures grown and differentiated independently. For experiments with mice, ‘n’ indicates number of mice per each group. Single comparisons were analysed using two-tailed student’s t-test. For RhoA activity assay, one-tailed student’s t-test was used. Multiple comparisons were analysed using analysis of variance (ANOVA) with Newman-Keuls post-hoc test. Values below 0.05 were considered significant. Analyses were performed using GraphPad Prism 5 software. All data are represented as mean ± s.e.m. The sample size was chosen based on our previous in vitro and in vivo studies 39.

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Author contributions
K.K. designed and performed most experiments, analysed the data and wrote the manuscript. A.K. planned experiments. T.G. analysed the experiments and wrote the manuscript. B.H. performed DMR experiments. A.W. performed GPCR array experiments and analysed the data. A.B. helped with in vivo experiments. A.G. helped with in vivo experiments. L.M.B. analysed GPCR array data and helped write the manuscript. K.S. performed the IP3 experiments and helped with the DMR experiments. M.E.L. performed qPCR and analysed human PCR data. M.J.B. collected human BAT samples. S.E. analysed human PCR data. J.W. planned experiments. M.F. helped generating transgenic mice. M.B. provided human WAT data. G.K. supplied FR. E.K. planned experiments and analysed DMR data. P.I. planned GPCR screen and analysed data and wrote manuscript. A.P. supervised experiments and wrote the manuscript.

Additional information
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