GPR180 is a component of TGFβ signalling that promotes thermogenic adipocyte function and mediates the metabolic effects of the adipocyte-secreted factor CTHRC1

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Activation of thermogenic brown and beige adipocytes is considered as a strategy to improve metabolic control. Here, we identify GPR180 as a receptor regulating brown and beige adipocyte function and whole-body glucose homeostasis, whose expression in humans is associated with improved metabolic control. We demonstrate that GPR180 is not a GPCR but a component of the TGFβ signalling pathway and regulates the activity of the TGFβ receptor complex through SMAD3 phosphorylation. In addition, using genetic and pharmacological tools, we provide evidence that GPR180 is required to manifest Collagen triple helix repeat containing 1 (CTHRC1) action to regulate brown and beige adipocyte activity and glucose homeostasis. In this work, we show that CTHRC1/GPR180 signalling integrates into the TGFβ signalling as an alternative axis to fine-tune and achieve low-grade activation of the pathway to prevent pathophysiological response while contributing to control of glucose and energy metabolism.
Obesity and the associated diseases such as type 2 diabetes, dyslipidaemia or cardiovascular complications represent a major health burden. Since obesity is a consequence of chronic positive energy balance, effective strategies to combat the disease must target food intake and/or energy expenditure. In contrast to white adipose tissue (WAT), which stores excessive energy in the form of lipids, brown adipose tissue (BAT) is a highly metabolically active tissue capable of dissipating chemical energy in the form of heat\(^1\). In response to certain environmental, hormonal and pharmacological stimuli, beige/brite adipocytes that morphologically and functionally resemble brown adipocytes arise in WAT deposits\(^2\)-\(^4\). The unique capacity of these adipocytes to burn energy is enabled by uncoupling protein 1 (UCP1) present in the inner mitochondrial membrane, which uncouples the proton gradient generated by the respiratory chain from ATP synthesis\(^5\). Brown and beige adipocytes are dependent on glucose and free fatty acids to cover the high-energy demands of the rich mitochondrial network. Thus, active brown/beige adipocytes serve as a metabolic sink for these substrates and therefore are suggested as a target tissue which can be activated to ameliorate metabolic disease\(^6\). Importantly, physiologically relevant amount of brown/beige adipocytes exist in adult humans\(^7\)-\(^9\) and their appearance is associated with increased energy expenditure, lower adiposity and reduced risk of insulin resistance\(^10\),\(^11\), further supporting its role in the control of metabolic homeostasis. So far, the most potent stimulus to activate thermogenesis is noradrenaline released by sympathetic nerve endings upon cold exposure\(^1\). Pharmacological activation of brown/beige adipocytes by the use of a selective \(\beta\)-adrenergic receptor agonist mirabegron requires high doses, which is accompanied by adverse side effects such as an increase in blood pressure and heart rate\(^12\),\(^13\). Therefore, identification of alternative pathways to increase thermogenic activity of brown/beige adipocytes is of great interest.

Transforming growth factor \(\beta\) (TGF\(\beta\)) signalling pathway controls cellular homeostasis in multiple tissues and its aberrant responsiveness is associated with wide range of human pathologies including autoimmune, gastrointestinal and fibrotic diseases, as well as cancer\(^14\),\(^15\). Recently, several studies demonstrated that TGF\(\beta\) pathway inhibits adipogenesis and formation of brown adipocytes\(^16\)-\(^18\). Nevertheless, its function in mature adipocytes is unexplored. Pharmacological targeting of the canonical TGF\(\beta\) cascade is controversial due to the pleiotropy of TGF\(\beta\) action. However, it is well accepted that the TGF\(\beta\) signalling machinery is complex and involves SMAD-independent signalling and many co-receptors including G protein-coupled receptors (GPCRs), which further fine-tune the activity of the pathway\(^19\).

Here, we show that the orphan GPR180 receptor mediates the thermogenic action of Collagen triple helix repeat containing 1 (CTHRC1) protein to control metabolic homeostasis. We propose that CTHRC1/GPR180 signalling integrates with the TGF\(\beta\) signalling machinery to induce low-grade activation of the pathway and to fine-tune the TGF\(\beta\)1 response, to maintain the activity of SMAD3 within the hormetic range and preserve the physiological function of the tissue.

**Results**

**GPR180 is required for proper beige adipocyte function.** To identify novel membrane receptors with the potential to promote thermogenic adipocyte activity, we compared the transcriptome of human supraclavicular BAT (scBAT) and adjacent subcutaneous WAT of six healthy young volunteers, and cross-analysed it with the transcriptome of human multipotent adipose-derived stem (hMADS) cells differentiated into beige and white adipocytes. The overlap of these two datasets revealed 1012 differentially expressed genes (DEGs) specific for mature adipocytes, which were further filtered for surface receptors (Fig. 1a; Supplementary Data 1). Using this approach, we identified 29 receptors differentially expressed between human BAT and WAT. One of the top hits was GPR180, which is upregulated in brown fat on both cellular and tissue level (Supplementary Fig. 1a and 1b). As GPCRs represent one of the most important integral membrane protein families and serve as attractive drug targets, due to their relevance in the treatment of various diseases, we decided to focus on GPR180 to investigate the role of this orphan receptor in the regulation of thermogenic adipocyte function.

Silencing of the gene encoding GPR180 (Supplementary Fig. 1c) in mature beige hMADS cells led to a reduction of UCP1 expression on both mRNA and protein level (Fig. 1b and Supplementary Fig. 1d), which was associated with attenuated cAMP-stimulated uncoupled respiration to the extent that cells with ablated GPR180 resembled white adipocytes (Fig. 1c). Consistently, several brown and beige adipocyte markers were decreased following GPR180 knockdown, while the expression of general adipocyte markers was not affected (Supplementary Fig. 1d). We found that lack of GPR180 signalling affected specifically UCP1 content, independent of any changes in the percentage of mature adipocytes (Supplementary Fig. 1e and f) or expression of mitochondrial protein complexes mediating oxidative phosphorylation (Supplementary Fig. 1g). Suppression of the brown adipocyte phenotype following Gpr180 knockdown was observed also in immortalized murine brown adipocytes (Supplementary Fig. 1h, i and j). Contrarily, overexpression of GPR180 in mature human white adipocytes (Fig. 1d) resulted in an increase in UCP1 protein level (Fig. 1e), as well as in higher cAMP-stimulated uncoupled and maximal mitochondrial respiration (Fig. 1f). A detailed quantification demonstrated that knockdown of GPR180 in preadipocytes did not affect the process of adipogenesis (Supplementary Fig. 1k and l) in line with its low expression in undifferentiated preadipocytes (Supplementary Fig. 1m). These data suggest that GPR180 is essential for thermogenic function of mature brown and beige adipocytes and its ablation shifts brown and beige adipocytes towards a white-like phenotype.

**Metabolic derangements in Gpr180 knockout mice.** To study the physiological relevance of GPR180 signalling, we generated a Gpr180 global knockout mouse by the CRISPR/Cas9 technology (Supplementary Fig. 2a). In accordance with our previous observations, UCP1 expression was downregulated in both interscapular BAT (iBAT) (Fig. 2a and Supplementary Fig. 2a) and inguinal WAT (iWAT) (Fig. 2b) of Gpr180 ablated mice, although we did not observe any difference in body weight (Supplementary Fig. 2b) or body composition (Supplementary Fig. 2c and d) when animals were fed with a chow diet. Concomitant with a decrease in UCP1 protein in both iBAT and iWAT, a significant reduction in energy expenditure for several timepoints with an overall strong trend related to genotype \((p = 0.08)\) was observed in Gpr180 knockout mice (Fig. 2c), while the locomotor activity (Supplementary Fig. 2e), food intake (Supplementary Fig. 2f), as well as substrate utilization (Supplementary Fig. 2g) remained unchanged. In line with these results, we observed a significantly reduced surface temperature in Gpr180 knockout mice in response to administration of a selective \(\beta\)-adrenoreceptor agonist, suggesting impaired brown adipocyte thermogenic activity (Fig. 2d and e). Importantly, mice lacking Gpr180 showed elevated fasting blood glucose levels (Supplementary Fig. 2h) and displayed impaired glucose tolerance (Fig. 2f), although fasting insulin levels were not altered (Supplementary Fig. 2i). When challenged with high-fat diet...
Statistical analysis was performed by two-sided Student’s t-test (Fig. 2i) or two-way ANOVA with Dunnett’s post-hoc test (Fig. 2c, f). Significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001. cAMP cyclic adenosine monophosphate, GPR180 G protein-coupled receptor 180, hMADS human multipotent adipose-derived stem cells, OCR oxygen consumption rate, RFP red fluorescent protein, scBAT supraclavicular brown adipose tissue, UCP1 Uncoupling protein 1.

(HFD), Gpr180 knockout mice gained more weight (Fig. 2g) and this was accompanied by higher fat mass accumulation (Supplementary Fig. 2j and k), pronounced liver steatosis and increased plasma ALT activity indicating an early stage of liver disease (Fig. 2h, Supplementary Fig. 2l and m). Based on the results of the molecular analyses of adipose tissues and the metabolic phenotyping of the global knockout mice we assumed that dysfunctional iBAT and/or impaired adipose tissue browning might be responsible for the accelerated development of metabolic disturbances in Gpr180 knockout mice. To test this hypothesis, we housed animals at thermoneutrality (30 °C, chow diet) for 8 weeks, followed by a glucose tolerance test and a 12-week HFD feeding regime. In this experimental setting, Gpr180 knockout mice did not exhibit any difference in glucose tolerance (Fig. 2i), body weight gain (Fig. 2j) or hepatic lipid accumulation (Fig. 2k) indicating that the adverse metabolic phenotype in this mouse model is most likely mediated by decreased thermogenic activity of brown and beige fat. To confirm our findings, we generated an inducible adipocyte-specific Gpr180 (Gpr180fl/fl × Adip-CreERT2) knockout mouse (Supplementary Fig. 2n). In agreement with our previous observations, ablation of Gpr180 specifically in mature adipocytes resulted in reduced UCP1 protein levels in iWAT (Fig. 2l and m), reduced energy expenditure (Fig. 2n) and surface temperature (Fig. 2o and p). This effect was not due to changes in locomotor activity or food intake (Supplementary Fig. 2o and p). Interestingly, mice with adipocyte-specific ablation of Gpr180 utilized less lipids as indicated by a shift in the respiratory quotient (Supplementary Fig. 2q), which was supported by significantly reduced levels of random fed blood glucose and increased free fatty acid levels (Supplementary Fig. 2r and s). Furthermore, adipocyte-specific Gpr180 knockout mice developed impaired glucose tolerance (Fig. 2q) and showed significantly higher body weight gain when challenged with HFD (Fig. 2r). Altogether, we show here that loss of GPR180 receptor in adipose tissue diminishes both brown and beige adipocyte function, which leads to impaired metabolic control.

GPR180 is not a GPCR but a component of the TGFβ signalling pathway. To study the mechanism of GPR180 action and the downstream signalling cascade, we performed a pathway analysis of DEGs obtained by RNA-seq of beige hMADS cells following GPR180 silencing. Surprisingly, there was no evidence for GPCR mediated signalling (Supplementary Fig. 3a and Supplementary Data 2). In addition, knockdown of GPR180 had no effect on cAMP levels, phosphorylation of PKA substrates, IP1 and calcium levels in mature beige adipocytes (Supplementary Fig. 3b–f). However, results of the above-mentioned analysis suggest an involvement of GPR180 in TGFβ signalling (Fig. 3a). As TGFβ signalling is mediated by SMAD3, we analysed SMAD3 phosphorylation in response to GPR180 knockdown. In line with
the RNA-seq data, we observed a significant reduction in phosphorylation of SMAD3 protein at serine 423 upon GPR180 ablation in beige adipocytes (Fig. 3b). We could not confirm the regulation of other pathways predicted by the DEG analysis, such as MAPK signalling, nor did we observe any difference in ERK or P38MAPK phosphorylation (Supplementary Fig. 3g and h). To validate the role of GPR180 in TGFβ signalling, we tested whether the activity of TGFβ1, the key regulator of SMAD3 phosphorylation, is dependent on presence of GPR180. Interestingly, TGFβ1-induced phosphorylation of SMAD3 was attenuated in beige adipocytes in absence of GPR180 (Fig. 3c). Similar results were obtained also in HEK-293T cells (Supplementary Fig. 3i). On the other hand, overexpression of GPR180 in white adipocytes increased basal SMAD3 phosphorylation (Fig. 3d), which indicates that GPR180 is required to achieve full TGFβ signalling. Based on this data and the fact that we could not find any changes in G protein-coupled signalling (Supplementary Fig. 3b–f), we hypothesized that GPR180 is not a GPCR and therefore its topology might diverge from the one characteristic for canonical GPCRs. To investigate the GPR180 orientation in plasma membrane, we overexpressed GPR180 fused with either a V5 tag at carboxy terminus or a HA tag at amino terminus in hMADS cells. We detected C-terminal V5 tag in both permeabilized and non-permeabilized conditions suggesting its extracellular localization (Fig. 3e and f). In contrast, HA tag could be detected only if the adipocytes were permeabilized indicating intracellular localization of the N-terminus. Taken together, our data demonstrate that GPR180 is not a GPCR but rather a component of the TGFβ signalling pathway.

**TGFβ enhances mature beige adipocyte function.** Since we could show that GPR180 is a component of the TGFβ signalling pathway, we next investigated the expression profile of other receptors of this pathway in mature adipocytes. We found that both white and beige human adipocytes express all three TGFβ receptors and while TGFβR1 and TGFβR2 are abundantly expressed during the whole differentiation, mRNA levels of TGFβR3 are transiently induced after induction of adipogenic differentiation (Supplementary Fig. 3j). Although recent studies...
extensively explored the effect of TGFβ/SMAD signalling cascade on adipocyte formation16-18, experimental evidence on its action in mature adipocytes is missing. Interestingly, treatment of hMADS cells-derived mature beige adipocytes with TGFβ1 dose-dependently increased SMAD3 phosphorylation (Supplementary Fig. 3k), which resulted in UCP1 upregulation (Fig. 3g) along with a marked increase in cAMP-stimulated uncoupled and maximal mitochondrial respiration (Fig. 3h), suggesting that the function of TGFβ signalling in regulation of mature beige adipocyte activity and beige adipogenesis might be different. We therefore investigated how manipulation of the individual components of TGFβ pathway affects beige adipocyte function. Pharmacological blockade of TGFβ pathway with SB-431542, a selective TGFβRI kinase inhibitor, led to reduced SMAD3 phosphorylation (Supplementary Fig. 3i) and UCPI protein expression (Fig. 3i), while mitochondrial respiration was not altered (Supplementary Fig. 3m). In addition, genetic inhibition of the TGFβ pathway with the aid of siRNAs targeting either TGFβRI, a receptor kinase responsible for SMAD3 phosphorylation, or the downstream signal transducers SMAD3 and SMAD2, downregulated UCPI protein level and mitochondrial respiration in human hMADS cells (Fig. 3j–o and Supplementary Fig. 3n–p). Silencing of TGFβR2, the ligand-binding receptor, decreased UCPI expression (Fig. 3k), but did not affect the mitochondrial oxygen consumption rate in human hMADS cells (Fig. 3m), which might be due to a compensation by increased lipolysis (Fig. 3i), as high free fatty acid levels can directly uncouple the inner mitochondrial membrane29,30. Based on these data, we conclude that inhibition of the TGFβ signalling pathway at the level of receptors, or the post-receptor signalling cascade using pharmacological and genetic means suppresses adipocyte thermogenic function, thus emphasizing necessity of TGFβ signalling activation to promote the brown/beige adipocyte phenotype.

Since we could show that GPR180 might be another component of TGFβ signalling pathway, we studied the role of GPR180 in chronic TGFβ signalling. Unlike the effect of GPR180 loss on acute TGFβ signalling which was reduced by approximately 25% (Fig. 3c), long-term TGFβ1 treatment (72 h) increased SMAD3 phosphorylation and UCPI protein levels in beige adipocytes even in absence of GPR180 (Supplementary Fig. 3q and r), suggesting that partial activation of the TGFβ machinery in the absence of GPR180 is sufficient to induce browning. Taken together, we conclude that GPR180 is required for full activation of the TGFβ signalling machinery, however even submaximal activation is sufficient to enhance adipocyte browning.

GPR180 transduces signal of adipokine CTHRC1 to induce SMAD3. Several arguments speak for the existence of a novel endogenously produced ligand activating GPR180 in human adipocytes in auto/paracrine manner. First, we observed basal SMAD3 phosphorylation in unstimulated conditions in hMADS cells that are cultured in serum-free medium; next, genetic manipulation of GPR180 levels affected SMAD3 phosphorylation directly. To investigate contribution of endogenous TGFβ secretion to SMAD3 phosphorylation, we quantified TGFβ levels in hMADS cells-conditioned media using a highly sensitive ELISA kit. We did not observe any secretion of TGFβ isoforms by mature human adipocytes into media (Fig. 4a and b, Supplementary Data 3), pointing to species differences in TGFβ secretion by preadipocytes and adipocytes between human and mouse31. In addition, we observed a persistent increase of SMAD3 phosphorylation in GPR180 overexpressing adipocytes, which were treated with TGFβ neutralizing antibody (Fig. 4c), further supporting this hypothesis. To identify potential ligands of GPR180, we first analysed the transcriptome of hMADS cells differentiated into mature adipocytes. We identified 315 genes that are significantly expressed in human mature adipocytes and encode for a secreted protein, as evidenced by the presence of a signal peptide sequence (Supplementary Data 4). In addition, proteomic analysis of cell-conditioned media using liquid chromatography coupled to tandem mass spectrometry revealed 322 proteins secreted into media in relevant amounts (Supplementary Data 3). By cross-analysis of transcriptomic and proteomic datasets, we could identify 82 secreted molecules. After exclusion of proteins annotated as extracellular matrix components or involved in regulation of immune response, we obtained 36 potential ligands (Fig. 4a). Since we showed that ablation of GPR180 leads to downregulation of UCPI protein levels, we performed a screen aimed at identification of secreted proteins whose knockdown will decrease UCPI levels. Using this
approach, we found that silencing of CTHRC1, FSTL1 and IGFBP7 reduced UCP1 protein content (Supplementary Fig. 4a), however only CTHRC1 and FSTL1 increased phosphorylation of SMAD3 in beige adipocytes, (Supplementary Fig. 4b). While the stimulatory effect of FSTL1 on SMAD3 phosphorylation was independent on GPR180 (Supplementary Fig. 4c), the effect of CTHRC1 could be completely attenuated by knockdown of this receptor (Fig. 4d), suggesting that CTHRC1 is a regulator of GPR180 function. We could confirm that CTHRC1 protein promotes SMAD3 phosphorylation in beige hMADS cells in a time-dependent manner (Fig. 4e) and that this effect was not due to TGFβ secretion or contamination, utilizing a neutralizing TGFβ antibody (Fig. 4f). Furthermore, CTHRC1 increased percentage of phosphorylated SMAD3 positive nuclei (Fig. 4g and h), and at the same time, immunostaining of total SMAD3 was increased in the nucleus following CTHRC1 treatment (Fig. 4g and i) indicating translocation of SMAD3 from the cytosol into the nucleus and thereby functional and transcriptionally active SMAD3 signalling. To demonstrate a direct interaction between cell surface GPR180 and CTHRC1, we generated a stable GPR180 knockout HEK-293T cell line using CRISPR/Cas9 and a construct to overexpress CTHRC1 fused with a HiBiT tag at C terminus, which is secreted into media and thus can be used for binding studies. Using this approach, we could demonstrate that...
and to < 0.0001 for maximal respiration). Effect of SMAD2 (p rate following knockdown of individual TGF

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SMAD3 at serine 423 by CTHRC1. Similarly, silencing of on SMAD3 phosphorylation (Fig. 4m). Since CTHRC1 was shown to stimulate different pathways in various cell lines22

immunostaining (green), nuclei stained by Hoechst (blue), e N-terminal HA tag and f C-terminal V5 tag in hMADS cells overexpressing modified GPR180; scale bar 100 μm. Experiment was performed 3 times with similar results. Long-term TGFβ1 treatment (72 h) dose-dependently promotes uCP1 protein (n = 6; p = 0.0084 for 0.1 ng/ml and p < 0.012 for 1 ng/ml; maximal respiration p = 0.0012 for 1 ng/ml; maximal phosphorylation p = 0.0012 for 1 ng/ml TGFβ1, p = 0.0178 for 10 ng/ml, p < 0.0001 for 100 ng/ml and p = 0.0127 for 1 ng/ml TGFβ1) in mature human beige adipocytes. Effect of i pharmacological (p = 0.0062 for 1 μM and p = 0.0017 for 10 μM) and j genetic (p = 0.0002) inhibition of TGFβ1 on UCP1 protein level in beige hMADS cells (n = 6). Effect of TGFβ2 silencing on k UCP1 expression (n = 6; p < 0.0001) and l HSL phosphorylation at serine 660 (n = 6; p = 0.0126). m Mitochondrial oxygen consumption rate following knockdown of individual TGFβ receptors in mature beige adipocytes (n = 5; p = 0.0082 for cAMP-stimulated uncoupled respiration and p < 0.0001 for maximal respiration). Effect of SMAD2 (p = 0.0001) and SMAD3 (p < 0.0001) knockdown on n UCP1 protein level (n = 6) and o mitochondrial respiration (n = 5; p = 0.0039 for SMAD3 and p = 0.0044 for SMAD2 cAMP-stimulated uncoupled respiration; p = 0.0001 for SMAD3 and p = 0.0011 for SMAD2 maximal respiration) in beige hMADS cells. Data are shown as average ±SEM. Statistical analysis was performed using two-sided Student’s t-test (b, j–l), one-way ANOVA with Dunnett’s post-hoc test (c, d, g, i, n) or two-way ANOVA with Tukey post-hoc test (h, m, o) and significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001. cAMP cyclic adenosine monophosphate, GPR180 G protein-coupled receptor 180, HSL Hormone sensitive lipase, HSPP90 Heat shock protein 90, OCR oxygen consumption rate, RFP red fluorescent protein, SMAD3 Mothers against decapentaplegic homolog 3, TGFβ Transforming growth factor β1, TGFβ1R Transforming growth factor β receptor type 1, TGFβ2R Transforming growth factor β receptor type 2, UCP1 Uncoupling protein 1.

CTHRC1 binding requires the presence of GPR180, as it was strongly reduced in GPR180 knockout HEK-293T cells. These data indicate that CTHRC1 might be a ligand of GPR180, suggesting interaction of these two components of TGFβ signalling pathway (Fig. 4j).

As we had demonstrated that GPR180 is part of the TGFβ signalling machinery, we next examined the dependency of CTHRC1-induced SMAD3 phosphorylation on the presence of TGFβ receptors. Pharmacological (Fig. 4k) or siRNA-mediated (Fig. 4l) inhibition of TGFβ1R fully abolished phosphorylation of SMAD3 at serine 423 by CTHRC1. Similarly, silencing of TGFβ2R could almost completely reverse the effect of CTHRC1 on SMAD3 phosphorylation (Fig. 4m). Since CTHRC1 was shown to stimulate different pathways in various cell lines22–25, we also studied its effect on Gs (Supplementary Fig. 4d–f), Gi (Supplementary Fig. 4f–i) and Gq (Supplementary Fig. 4j and k) signalling as well as other kinases (Supplementary Fig. 4l and m) and SMADs (Supplementary Fig. 4n), however, we did not observe any evidence which would support a role of CTHRC1 in G-protein signalling. These results are in agreement with demonstrated inverted membrane topology of GPR180 receptor. Based on these data, we propose that CTHRC1 activates SMAD3 and together with GPR180 represent components of the TGFβ signalling machinery. This mechanism of SMAD3 regulation is not restricted to adipocytes, as increased phosphorylation of SMAD3 (Supplementary Fig. 4o) in response to CTHRC1 was evident also in HEK-293T cells, and dependent on TGFβ1R kinase (Supplementary Fig. 4p). Similarly, SMAD3 activated by CTHRC1 translocated to the nucleus as shown by increased luciferase activity reflecting higher responsiveness of SMAD binding elements (Fig. 4n) in HEK-293T cell line and, importantly, also this effect could be prevented by genetic deletion of GPR180. Surprisingly, we found that co-treatment of human beige adipocytes by CTHRC1 blunted the TGFβ1-induced phosphorylation of SMAD3 (Fig. 4o and p). When we compared the stimulatory potential of CTHRC1 and TGFβ1, we found that the maximal level of SMAD3 phosphorylation induced by 20 nM CTHRC1 corresponds to a low-level stimulation with 40 fM TGFβ1 (Fig. 4q). Moreover, we observed increased CTHRC1 expression and secretion in response to long-term TGFβ1 treatment in beige hMADS cells (Fig. 4r) suggesting the existence of a feedback loop to modulate the TGFβ1 response. Taken together, we identified CTHRC1 as an adipokine that signals via GPR180 in human mature adipocytes to increase SMAD3 phosphorylation. Importantly, this alternative CTHRC1/ GPR180 axis of TGFβ pathway represents a feedback system to fine-tune TGFβ1 response and mediate low-grade activation of SMAD3 signalling.

CTHRC1 enhances beige adipocyte functionality via GPR180. In accordance with the secretome screening data, we confirmed expression of CTHRC1 on protein level and its secretion into media by both white and brown human adipocytes (Fig. 5a–c). Although CTHRC1 is more abundantly expressed and secreted by white hMADS cells, it is not differentially expressed between supraclavicular BAT and subcutaneous WAT of human volunteers (Fig. 5d). Therefore, we next investigated the effect of CTHRC1 on beige adipocyte metabolism. Long-term CTHRC1 treatment (72 h) increased UCP1 protein levels (Fig. 5e) and cAMP-stimulated uncoupled as well as maximal mitochondrial oxygen consumption (Fig. 5f), and this effect was evident also in presence of a neutralizing TGFβ antibody (Fig. 5g), ruling out the involvement of endogenous TGFβ or recombinant protein contamination. Importantly, the stimulatory action of CTHRC1 on UCP1 levels (Fig. 5h) and mitochondrial respiration (Fig. 5i) in beige hMADS cells required the presence of GPR180. On the other hand, siRNA-mediated CTHRC1 silencing in mature beige adipocytes downregulated UCP1 protein (Fig. 5j) along with a reduction of basal and cAMP-stimulated uncoupled respiration (Fig. 5k). In addition, we could show that unlike TGFβ1R, CTHRC1 does not affect adipogenesis and beige adipocyte formation (Supplementary Fig. 5a–c). Altogether, these data indicate that GPR180 is indispensable for CTHRC1-induced stimulation of beige adipocyte functionality.

GPR180 mediates the beneficial effect of CTHRC1 on energy metabolism. To test the physiological relevance of CTHRC1 in vivo, we generated an AAV8 expressing CTHRC1 under the control of a liver-specific LP-1 promoter to increase circulating levels of this factor. As expected, we found elevated CTHRC1 protein in plasma of mice injected with AAV–Cthrcl1 compared to AAV-stuffer (Fig. 6a). Importantly, CTHRC1 overexpression in male C57BL/6 N mice attenuated the HFD-induced body weight
gain (Fig. 6a and Supplementary Fig. 6a), which was mainly due to reduced accumulation of WAT mass (Supplementary Fig. 6b). CTHRC1 overexpression lowered the fasting glycaemia (Fig. 6b) without altering plasma insulin (Supplementary Fig. 6c) or lipid profile (Supplementary Fig. 6d and e), although non-esterified fatty acids levels tended to be elevated (Fig. 6c, \( p = 0.09 \)). A trend towards an increase in phosphorylation of HSL in both iBAT (Fig. 6d) and iWAT (Fig. 6e) depots suggests that higher fatty acid flux in adipose depots might account for increased tissue activity and contribute to lower body weight gain in CTHRC1 treated mice. This is further supported by upregulated expression of brown adipocyte markers in iWAT (Fig. 6f), while these effects were less prominent in iBAT (Fig. 6g and h). Consistent with these findings, CTHRC1 overexpression in HFD-fed mice led to...
an increase in cumulative energy expenditure and a shift in RER towards fatty acid oxidation (Fig. 6i and j). Although we utilized a liver-specific promoter to overexpress Cthrc1, we did not notice any detrimental effects of the overexpression, since liver mass (Supplementary Fig. 6f), plasma ALT activity (Supplementary Fig. 6g) or expression of inflammatory (Supplementary Fig. 6h) and fibrotic (Supplementary Fig. 6i) markers were either unchanged or decreased. Importantly, we observed an improved glucose tolerance in mice with elevated CTHRC1 (Fig. 6k), which was independent of body weight loss (Supplementary Fig. 6j and k) and was observed only in wild-type but not in global Gpr180 knockout mice, further supporting our in vitro findings that GPR180 mediates the beneficial metabolic effects of CTHRC1.

Human GPR180/CTHRC1 is associated with an improved metabolic profile. To study the relevance of these two components of TGFβ signalling pathway in humans, we analysed the regulation of GPR180 and CTHRC1 in participants with normal weight, with obesity and normal glucose tolerance (NGT), with obesity and prediabetes and in individuals with obesity and newly diagnosed type 2 diabetes. The expression of GPR180 in subcutaneous WAT was significantly lower in participants with obesity, independent of glycaemic control (Fig. 7a). In a subset of study participants, for which we had paired samples of adipose tissue and isolated mature adipocyte fraction, we could show that the expression of GPR180 was 2.5-fold higher in adipocytes than in the whole tissue (Fig. 7b), indicating that adipocytes account to large extent for GPR180 tissue level. Interestingly, we observed a negative correlation of GPR180 expression in WAT with cell size (Fig. 7c) and suppression of fatty acids release during euglycemic hyperinsulimic clamp, reflecting the adipocyte insulin sensitivity (Fig. 7d). Importantly, adipose tissue GPR180 expression positively correlated with basal resting energy expenditure (REE) (Fig. 7e). Next, we measured plasma concentration of CTHRC1 in the same cohort of men. Since the circulating levels of this hormone are low, in several individuals the levels were below the detection limit (sensitivity of the assay 30 pg/ml; lower limit of detection 10 pg/ml). When we stratified the whole measured population into two subgroups based on the CTHRC1 levels, we found that individuals with higher CTHRC1 are younger and more metabolically healthy as evidenced by a lower BMI, smaller adipocyte size, higher insulin sensitivity and better plasma lipid profile as well as lower ectopic lipid deposition (Supplementary Tab. 1). In line with this observation, CTHRC1 prevalence was significantly lower in individuals with diabetes (Fig. 7f). In addition, a positive association of circulating CTHRC1 and energy expenditure was observed (Fig. 7g and h).

In conclusion, our data indicate that GPR180 and CTHRC1 are metabolically relevant also in humans and might play an important role in regulation of adipose tissue and whole-body energy metabolism.

Discussion

BAT is considered an important regulator of systemic glucose and lipid homeostasis, therefore, its activation represents a prospective strategy to increase energy expenditure and ameliorate metabolic diseases. In an unbiased transcriptomic analysis of human brown and white adipose tissue samples, we identified GPR180 as a receptor enriched in human BAT compared to WAT and demonstrated its significance in the regulation of brown and beige adipocyte function and glucose homeostasis by utilizing genetic and pharmacological tools. Mechanistically, we
**Fig. 5** CTHRC1, an adipokine that requires GPR180 to enhance the beige adipocyte phenotype. 

a Representative blots and b quantification of CTHRC1 protein in human adipocytes (n = 4; p = 0.0011) and c cell-conditioned media (n = 7; p = 0.0408). d Expression of CTHRC1 in supraclavicular brown and subcutaneous white adipose tissue of 6 healthy volunteers with detectable BAT (p = 0.3008). Effect of long-term (72 h) CTHRC1 treatment on e UCP1 protein (n = 4; p = 0.0001 for 100 ng/ml and p = 0.0064 for 500 ng/ml) and f mitochondrial respiration (n = 5; basal p = 0.0102, CAMP-stimulated uncoupled p < 0.0001 for 100 ng/ml and p = 0.0021 for 500 ng/ml, maximal p = 0.0207) in mature human beige adipocytes. g Effect of long-term (72 h) CTHRC1 (500 ng/ml) and TGFβ1 (1 ng/ml) on mitochondrial respiration in beige hMADS cells in presence of IgG (control) and neutralizing anti-TGFβ antibody (1 μg/ml) (n = 6; basal CTHRC1 IgG p = 0.0376, CTHRC1 anti-TGFβ p = 0.0489, TGFβ IgG p = 0.0474; uncoupled CTHRC1 IgG p = 0.0372, CTHRC1 anti-TGFβ p = 0.0079; coupled TGFβ IgG p = 0.0449; cAMP-stimulated uncoupled CTHRC1 IgG p = 0.0407, CTHRC1 anti-TGFβ p = 0.0440, TGFβ IgG p = 0.0317; maximal CTHRC1 IgG p = 0.0478, CTHRC1 anti-TGFβ p = 0.0310). h UCP1 levels (n = 6; siGPR180 in PBS p = 0.0016, CTHRC1 in ctrl siRNA p = 0.0001 and siGPR180 in CTHRC1 treatment p < 0.0001) and i mitochondrial respiration (n = 10; p < 0.0001 basal, p < 0.0001 coupled, p = 0.0094 for CTHRC1 treatment in ctrl siRNA and p = 0.0013 for siGPR180 in PBS treated in cAMP-stimulated uncoupled respiration, p < 0.0001 maximal respiration) in adipocytes following long-term (72 h) CTHRC1 treatment (500 ng/ml) in combination with GPR180 knockdown. Effect of CTHRC1 silencing on j UCP1 protein (ctrl siRNA n = 8, siCTHRC1 n = 9; p = 0.0002) and k mitochondrial oxygen consumption (n = 5; p = 0.0311 for uncoupled respiration, p = 0.0326 for cAMP-stimulated uncoupled respiration) in hMADS cells. Data are shown as mean ± SEM. Statistical analysis was performed by unpaired Student’s t-test (b, c, j), paired Student’s t-test (d), one-way ANOVA with Dunnett’s post-hoc-test (e) or two-way ANOVA with Sidak and Tukey post-hoc tests (f-i, k). Significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001. cAMP cyclic adenosine monophosphate, CTHRC1 Collagen triple helix repeat containing 1, GPR180 G protein-coupled receptor 180, hMADS human multipotent adipose-derived stem cells, IgG immunoglobulin G, OCR oxygen consumption rate, PBS phosphate buffered saline, scBAT supraclavicular brown adipose tissue, TGFβ Transforming growth factor β, UCP1 Uncoupling protein 1, WAT white adipose tissue.
show that GPR180 is in fact not a classical GPCR, but a component of TGFβ signalling hub, which binds and mediates the effects of the circulating factor CTHRC1 to regulate thermogenic adipocyte function. Furthermore, we provide evidence that both components are physiologically relevant and participate on the regulation of the metabolic phenotype in mouse and human.

GPCRs are structurally characterized by seven transmembrane domains with extracellular N-terminus and intracellular C-terminus localization. GPR180 was originally described as a Rhodopsin-like orphan GPCR involved in vascular remodelling. Interestingly, several highly conserved motifs characteristic for GPCRs such as tripeptide (D/E)R(Y/W) at the intracellular end of transmembrane domains with extracellular N-terminus and intracellular C-terminus localization. GPR180 was originally described as a Rhodopsin-like orphan GPCR involved in vascular remodelling. Interestingly, several highly conserved motifs characteristic for GPCRs such as tripeptide (D/E)R(Y/W) at the intracellular end of transmembrane
helix III or NPXXY sequence near cytoplasmic end in helix VII are absent in the GPR180 sequence. Besides the fact that we did not observe any changes in G-protein signalling, we provide evidence that GPR180 has a reverse orientation in the plasma membrane which is similar to the adiponectin receptor. This is further supported by the lack of GXXXY motif in the first transmembrane domain of GPR180 that was recently discovered to be present in many GPCRs and being one of the mechanisms responsible for canonical orientation in the plasma membrane.

The TGFβ signalling pathway is involved in regulation of diverse physiological processes such as cellular proliferation, differentiation and growth, immune response, extracellular matrix deposition and tissue repair. TGFβ pleiotropy is mainly based on its immunosuppressive and pro-fibrogenic properties. Deficiency in TGFβR1 and TGFβR2 results into progressive wasting syndrome or embryonal lethality, respectively. Conditional knockout of both receptors in different tissues or global deletion of SMAD3 is associated with increased tumorigenesis demonstrating the importance of this signalling pathway in cellular homeostasis. Defective TGFβ signalling due to increased expression of inhibitory SMAD7 leads to attenuation of SMAD3 phosphorylation, which is characteristic for gut mucosa of patients with Crohn’s disease. Moreover, antisense oligonucleotide based therapy targeting SMAD7 seems to be effective in restoring TGFβ signalling and suppressing mucosal inflammation in these patients. Interestingly, SMAD3 deficient mice are protected from obesity and diabetes, however, these mice display striking differences in body weight and length already at 8 weeks of age indicating developmental defects. On the other hand, it is well established that TGFβ pathway over-activation results in excessive accumulation of extracellular matrix and tissue fibrosis, which is associated with organ dysfunction. Moreover, tumour progression is associated with transformation of cellular response to TGFβ and blockade of the pathway is beneficial. Altogether, these data indicate that intermediate response or fine-tuning of the TGFβ signalling pathway, in agreement with the concept of hormesis, is essential to maintain whole-body homeostasis.

The TGFβ signalling machinery is complex with multiple levels of regulation to achieve signal transduction. The simple model of TGFβR1 and TGFβR2 dimerization in response to ligand is outdated since multiple co-receptors regulating ligand presentation, availability and ligand-independent pathway activation have been identified in the last years. Such an example is GPR50, a GPCR that forms an alternative complex with TGFβR1 in a TGFβR2-independent manner to enhance its basal capacity to induce SMAD3 phosphorylation via interaction with FKBP1241. Identification of GPR180 and CTHRC1 as components of the TGFβ signalling pathway activating SMAD3 further increases the complexity of the TGFβ signalling pathway and provides the first adaptive pathway that is involved in low-grade activation of the signalling pathway and modulation of TGFβ response. In addition, we show here that GPR180 is required for full activation of the TGFβ signalling machinery; however, even submaximal activation is sufficient to drive browning of the cells. Currently, we do not know whether GPR180 is a downstream modulator of TGFβ activity, or whether it directly interacts with either the whole receptor complex or solely with the TGFβR1 kinase. Given that the induction of SMAD3 signalling by TGFβ1 is blunted in presence of CTHRC1, together with our finding that the effect of CTHRC1 is dependent at least partially on both TGFβ receptors, argue in favour of a function as a components of TGFβ receptor machinery.

Several studies have linked CTHRC1 to TGFβ signalling albeit only in an intracellular context. According to these reports, CTHRC1 directly interacts with and accelerates the degradation of phospho-SMAD3 in the cytosol of rat PAC1 smooth muscle cells and human stellate LX-2 cells, indicating a cytosolic action of endogenous CTHRC1. This is in stark contrast to the function of extracellular or circulating CTHRC1, which targets GPR180 and TGFβR1. Here we demonstrate that extracellular recombinant CTHRC1 increases phosphorylation of SMAD3 as well as its shuttling into the nucleus in beige hMADS-derived adipocytes and HEK-293T cells by activating GPR180. Similar observations were made following treatment of hepatic stellate cells (HSCs) with CTHRC1. Interestingly, CTHRC1 expression is regulated by TGFβ in fibroblasts and HSCs since the SMAD binding elements were identified in the promoter of Cthrc1 gene and, and here we confirm the responsiveness of CTHRC1 to TGFβ in mature human adipocytes. These data suggest the existence of a feedback loop, in which CTHRC1 upregulated in response to TGFβ activation tightly controls intracellular stability of SMAD3 as well as fine-tunes high TGFβ stimulatory potential, thereby maintaining only a certain level of SMAD3 phosphorylation. In addition, it should be noted that CTHRC1 induces only a submaximal SMAD3 phosphorylation, suggesting that CTHRC1 and GPR180 represent an alternative axis to achieve a low-grade activation of TGFβ signalling within a hormetic range, which is required to maintain physiological functionality of the tissue and prevent its pathological remodelling.

As mentioned above, the TGFβ pathway is involved in regulation of cellular proliferation and differentiation. Recently,
role of this pathway in adipogenic commitment and thermogenic adipocyte differentiation was extensively investigated and these studies unanimously demonstrated inhibitory effect of activated TGFβ studies. In paired samples of SAT and isolated adipocytes (n = 21; p = 0.0077). Correlation of adipocyte GPR180 level with adipocyte size (n = 54), d suppression of fatty acid release during EHC (n = 55) and e resting energy expenditure (n = 31). f Incidence of circulating CTHRC1 in normal weight individuals, and patients affected by obesity but NGT, patients having obesity and prediabetes and/or type 2 diabetes (n = 85; p = 0.02533) and its association with basal energy expenditure and g energy expenditure measured during the steady state of EHC (n = 20). h Schematic illustration of the signalling mechanism identified in this study. Data are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Dunnett post-hoc test (a), paired Student’s t-test (b) or Fisher’s exact test (f). i For association of GPR180 expression in WAT or circulating CTHRC1 with metabolic parameters Pearson’s correlation coefficient was calculated (c–e, g, h). Significant differences are indicated as *p < 0.05, **p < 0.01 and ***p < 0.001. CTHRC1 Collagen triple helix repeat containing 1, EHC euglycaemic hyperinsulinaemic clamp, FFA free fatty acids, GPR180 G protein-coupled receptor 180, REE resting energy expenditure, SAT subcutaneous adipose tissue, SMAD3 Mothers against decapentaplegic homolog 3, TGFβ Transforming growth factor β1, TGFβR1 Transforming growth factor β receptor type 1, TGFβR2 Transforming growth factor β receptor type 2, UCP1 Uncoupling protein 1.

CTHRC1 acting through GPR180, does not affect formation of human adipocytes, but specifically promotes brown adipocyte activity and adipocyte browning, which could be attributed to low GPR180 expression in progenitor cells and its abundance in mature adipocytes. Activin, another ligand of TGFβ family which can signal via SMAD3, was shown to enhance UCP1 expression in mature murine adipocytes and to increase energy expenditure by stimulation of BAT thermogenesis. Interestingly, TGFβ2 treatment was recently also shown to upregulate UCP1 expression and increase glucose uptake in BAT. These opposing effects of TGFβ signalling on progenitors and mature adipocytes are not unusual. For example Janus kinase or Rho kinase display similar behaviours.

Attenuated CTHRC1 signalling as a consequence of GPR180 ablation was associated with deterioration of the metabolic profile, while CTHRC1 administration exerts protective effects. This is in line with the phenotype of CTHRC1 null mice, which display increased fat mass, liver steatosis and reduced energy expenditure. Although we cannot exclude the contribution of other organs to the observed phenotype due to systemic elevation of CTHRC1 and ubiquitous expression of GPR180, we clearly show that adipose tissue thermogenic activity is the key effector of this phenotype, which is supported by the adipocyte-specific knockout and in vitro functional studies. In addition, our data indicate that the observed changes in adipose tissue thermogenic activity cannot be attributed exclusively to alterations in UCP1 content, but might rather involve changes in alternative thermogenic mechanisms, such as fatty acid flux. Therefore, targeting GPR180 could improve glucose tolerance and modulate body weight by promoting adipose tissue thermogenic activity, although CTHRC1 mediated low-grade SMAD3 activation is clearly not the main driver of this process. Even modest changes in energy expenditure in response to CTHRC1/GPR180 manipulation have a profound effect on whole-body energy metabolism in a long term, similarly to other interventions. However, the potential of CTHRC1/GPR180 axis to affect metabolic health is
further supported by the positive association of both ligand and receptor with energy expenditure in humans. We and others5,5 have shown that circulating levels of CTHRC1 in human population are low, supporting para- or autocrine action of this secreted factor. While the percentage of CTHRC1 detectability in plasma is similar in our and Duarte study, there is a discrepancy in its regulation in type 2 diabetes. This could be due to differences in sample processing time, which was within 30 min in our cohort in contrast to 6–24 h in the other work5,5. Moreover, it is important to note that type 2 diabetic individuals recruited in our study were newly diagnosed and did not receive any medication at the time of examination.

In conclusion, we identify here GPR180 and CTHRC1 as components of TGFβ signalling pathway, which regulate low-grade SMAD3 phosphorylation required for proper thermogenic adipocyte function and control of whole-body energy and glucose homeostasis.

Methods

Contact for reagent and resource sharing. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Christian Wolfrum (christian-wolfrum@ethz.ch).

Clinical transcriptome study. The clinical study for cohort 1 was approved by the Ethics Committee of the Hospital District of Southwest Finland and conducted according to the principles of the Declaration of Helsinki. All study participants provided written consent prior to entering the study. The subjects were screened for medical history and status, and only healthy volunteers were enrolled in the study. PET-CT scan and tissue biopsies were performed as described in the previous publication5,5. Briefly, the subjects underwent a PET-CT examination after an overnight fast. On the cold exposure day, the subjects spent 2 h wearing light clothing in a room with an ambient temperature of 17 ± 1 °C before moving into the PET-CT room, which had an air temperature of 23 °C. During the PET-CT session, one foot of the subject was placed intermittently (5 min in/5 min out) in cold water at a temperature of 8 ± 1 °C. Detailed description of PET-CT examination is available in5,5. The site of the biopsy was selected based on the cold exposure14-FDG-PET-CT image that showed activated BAT. A subcutaneous WAT sample was collected from the same incision. The biopsies were obtained under local lidocaine anaesthesia by a plastic surgeon at normal room temperature (20 °C) one week after the PET-CT examination. Immediately after removal, the tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until further processing. The RNA from adipose tissue was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN), according to the manufacturer’s protocol including the DNase treatment step. Primary outcomes of this study have been previously published.

Study with normal weight participants and participants with obesity and differing metabolic control. The clinical study was approved by the Local Ethics Committee of the University Hospital in Bratislava, Slovakia and it conforms to the ethical guidelines of the 2000 Helsinki declaration. All study participants provided witnessed written informed consent prior entering the study. Eighty-five middle-aged sedentary men were recruited. Screening measurements of BMI, fasting, and/or 2-h glucose (oGTT) were used to stratify them into following groups: (i) metabolically healthy normal weight individuals and patients with similar levels of obesity and (ii) normal glucose tolerance; (iii) prediabetes and (iii) newly diagnosed, yet untreated, type 2 diabetes. Patients with chronic disease or regular use of pharmacotherapy were excluded. Subcutaneous adipose tissue samples were taken by aspiration with needle biopsy from abdominal region in the fasted state under local sedation (1% Meoxicaine, Prague, Czech Republic). The sample was quickly washed in saline to eliminate blood and frozen in liquid nitrogen prior RNA extraction. Patients underwent complex metabolic phenotyping5,5.

Mouse experiments. All the authors of the study who participated in mouse experiments have compiled with ethical regulations for animal testing and research. All animal procedures in this study were approved by the cantonal ethics committee of the veterinary office of the Canton of Zürich. Sample size was determined based on previous experiments in our lab and similar studies reported in the literature. All mice used for the experiments were male, housed 3–4 littermates per cage in individually ventilated cages at standard housing conditions (22 °C, 12 h reversed light/dark cycle, dark phase starting at 7am, 40% humidity), with ad libitum access to chow (18% proteins, 45% fibers, 45% fat, 6.3% ashes, Provimi Klbu SA) and water. Health status of all mouse lines were regularly monitored according to FELASA guidelines.

Generation of the GPR180 global knockout. The GPR180 null allele was obtained by Cas9/CrisPR. The Cas9/CrisPR target sequence tgcagcagagcccccccggg (position of in brackets) is cloned in the plasmid of the GPR180 was modified directly in mouse cell embryos by electroporation. C57BL/6/J female mice underwent ovulation induction by i.p. injection of 5 IU equine chorionic gonadotropin (PMSG; Folligon–Inter Vet), followed by i.p. injection of 5 IU human chorionic gonadotropin (Pregnyl–Essex Chemie) 48 h later. For the recovery of embryos, C57BL/6/J females were mated with males of the same strain immediately after the administration of human chorionic gonadotropin. Embryos were collected from oviducts 24 h after the human chorionic gonadotropin injection and were then freed from any remaining cumulus cells by a 1–2 min treatment of 0.1% hyaluronic acid/L–algin (Aldrich) dissolved in M2 medium (Sigma). Prior to electroporation, the zona pellucida was partially removed by brief treatment with acid Tyrode’s solution and the embryos were washed and briefly cultured in M16 (Sigma) medium at 37 °C and 5% CO2. Electroporation with a mixture of 16µM cr:trcrRNA hybrid targetting GPR180 and 16µM Cas9 protein (all reactants from IDT) was carried out using 1 mm gap electroporation cuvette and the ECM380 electroporator (BTX Harvard) apparatus. Two square 3 ms pulses of 30 V with 100 ms interval were applied as these conditions provide efficient genome editing with embryonic survival5,5. Surviving embryos were washed with M16 medium and transferred immediately into the oviducts of 18–16-wk-old pseudopregnant C57BL1/6J (ICR) females that had been mated with sterile genetically gynandromorph males5,5 the day before embryo transfer (0.5 dpc). Pregnant females were allowed to deliver and raise the pups until weaning age. In total 100 embryos were electro- porated and 97 surviving embryos were transferred into 4 foster mothers. All foster mothers produced live litters with a total of 26 viable F0 pups. Five F0 pups carried in/del modification in the GPR180 gene.

Generation of the adipocyte-specific inducible GPR180 knockout. The Generation of the GPR180 mouse strain used results from a study by Vivien Duarte et al. (ES) cell clone EPD0539_4_D02, obtained from the KOMP Repository (www.komp.org) and generated by the Wellcome Trust Sanger Institute (WTSI). Targeting vectors were used generated by the WTSI and the Children’s Hospital Oakland Research Institute as part of the Knockout Mouse Project (3U01HG009008-09). Chimeric mice were generated by injection of ES into blastocysts (TE) in ETH Phenomics Center Zurich (epic.ethz.ch). Conditional allele was achieved via flipase (Flp) recombination of knockout first allele. F1p deleter mouse was kindly provided by Prof. Markus Stoßfeld (ETH Zurich). Inducible adipocyte-specific ablation of GPR180 was completed after crossing GPR180fl/f mice to Adip-CreERT2 mice. At 12 weeks of age, recombination of the floxed allele was induced by oral tamoxifen gavage (2 mg/mouse in sunflower oil, Sigma–Aldrich).

Adeno-associated virus (AAV) to overexpress CTHRC1. The expression cassette consisting of a Kozak sequence followed by the coding sequence for murine CTHRC1 (UniProt Q9D1D6) was codon-usage optimized for expression in mice and synthesized (Thermo Fisher Scientific/Geneart, Regensburg, Germany) before being cloned into a pFB vector. The vector contains AAV2 ITRs, from which one is lacking the terminal resolution site and a L-P promoter60 to drive liver-specific promoter. As a control, a P-Fb/Stuffer vector45 was used for rAAV production. Recombinant rAAV8 vectors were produced by calcium phosphate transfection of human embryonic kidney HEK-293T cells using the pFB_LPI- mCTHRC1 or the pFB_Stuffer plasmid in combination with the pDP8 (Plasmid Factory, Bielefeld, Germany) and the pHelper plasmid (Thermo Fisher Scientific), both of which were transfection based by purified methylphosphorylated linear DNA (8000 solution), iodixanol gradient and ultrafiltration (Amicon Ultra-15 MWC0 100,000 ultrafiltration tube; Merck Millipore). Titer was determined by quantitative PCR. Male C57Bl6/N mice (Charles River) and/or GPR180 global knockouts and their wild-type littermates were injected i.v. with CTHRC1 AAV or stuffer (non- coding DNA), both at 5000–10 VG/mouse.

In high-fat diet cohorts (23.9% proteins, 4.9% fat, 5.0% ashes, Provimi Klbu SA) and FGF-2 was omitted after cells reached confluence (day 0) by adipogenic medium (DMEM/Ham F12 media (Lonza) containing 10 µg/ml Transferin, 10 ng/ml insulin and 0.2 µM triiodothyronine) supplemented with 1 µM dexamethasone and 300 µM isobutyl methylxanthine (IBMX). From day 2 to 9, cells were cultured in adipogenic medium. All compound names were obtained from Sigma–Aldrich except for resiglitazone (Adipogen). Cells were kept in culture until day 18 in absence of resiglitazone to obtain mature white adipocytes. To obtain

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beige adipocytes, cells were exposed to an additional rosiglitazone pulse (100 nM) between days 14 and 18. To investigate long-term effect of pharmacological agents on browning of white adipocytes, treatment was performed for three consecutive days in combination with rosiglitazone. Signalling studies were performed on day 18 in mature beige hMADS cells-derived adipocytes after 2 h fasting in adipogenic medium without supplements following acute stimulus with recombinant proteins up to 1 h. Recombinant CTHR1 was produced by human cells and purchased from 2 different vendors (Sino Biological and Creative Biomart). Treatment with both recombinant proteins yielded the same results. Recombinant FSTL1 (Abcam) was produced by CHO cells and IGBP7 (R&D Systems) was produced in mouse myeloma cells. TGFβ neutralizing antibody (1D11, ThermoFisher) was used to exclude contamination of TGFβ contaminant was estimated by calculating the ratio of CO2 production to O2 consumption. Animals were single-caged and acclimated to the metabolic cage for 48 h prior metabolic recording. Locomotor activity, food and water intake were monitored throughout the whole measurement.

**Surface temperature measurement.** Surface temperature was recorded with an infrared camera at room temperature (E60;FLIR) and analyzed with FLIR Tools software (FLIR).

**Tissue harvest.** Animals were euthanized singly in carbon dioxide atmosphere. All tissues were carefully dissected, weighed and either snap frozen in liquid nitrogen until further processing or fixed in 4% paraformaldehyde for tissue histology. Popliteal lymph nodes were carefully removed from iBAT for all gene and protein expression analyses. For RNA and protein isolation, whole adipose tissue depot was homogenized.

**Blood parameters.** Blood was collected from mice fasted for 6 h by cardiac puncture into EDTA coated tubes and plasma was obtained by centrifugation at 3000rpm for 20 min at 4°C. Plasma insulin was measured by Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem), ALT activity by kinetic colorimetric assay (Sigma–Aldrich), free fatty acids by NEFA-HR(2) assay (Wako Chemicals), cholesterol by LabAssay Cholesterol (Wako Chemicals) and triglycerides by Cobas TRIGB kit (Roche/Hitachi), all following manufacturer instructions. Absorbance was measured by SynergyMX Plate reader and data analyzed by Gen5 v3.08 software (BioTek). CTHR1 in human plasma was determined by sandwich ELISA following kit instructions (MMCRU)55 We have validated the antibody by overexpression of CTHR1 in HEK-293T cells, which do not express this protein, and determining CTHR1 levels in culture media. Importantly, we also spiked recombinant CTHR1 protein into plasma of patients with undetected circulating CTHR1.

**Liver histology and lipid accumulation.** Part of liver left lobe was fixed in 4% paraformaldehyde for tissue histology. After 24 h, samples were transferred into 70% ethanol prior tissue processing. Tissues were dehydrated through graded alcohols, cleared with xylene and infiltrated with paraffin by standard procedures. Tissues were sectioned at 4 µm cuts and stained with hematoxylin and eosin. Tissue sections were examined by light microscopy using AxiosPhot microscope equipped with AxiosCam MR (Zeiss). Another piece of left liver lobe was snap frozen in liquid nitrogen prior to lipid extraction. Total lipids were extracted using chloroform: methanol (2:1) mixture and normalized to tissue weight.

**Cellular respiration.** hMADS cells were differentiated on collagen-coated 96-well Seahorse microplates. On the day of experiment, adipogenic medium was replaced with XF Assay Medium (pH 7.4, Seahorse Bioscience) supplemented with glucose (1 g/L; Sigma–Aldrich), 2 mM sodium pyruvate (Invitrogen) and 2mM L-glutamine (Invitrogen). The oxygen consumption rate (OCR) was measured using the Extracellular flux analyzer XF96 and analyzed by Wave 2.6.0 (Agilent Seahorse). Test compounds were sequentially injected to obtain following concentrations: 0.5 µM mibuthyl cAMP (1 µM isoproterenol for murine iBAs), 1 µg/ml FCCP, 3 µM Rotenone with 2 µg/ml Antimycin A. All compounds were purchased from Sigma–Aldrich, except for Oligomycin (AdipoGen).

**Intraperitoneal glucose tolerance test.** To measure glucose tolerance, mice were fasted for 6 h by transfer to a clean cage without food and the test was performed at the end of the dark (active) phase. Mice were weighed and after fasting glucose levels were obtained from a tail vein using a standard glucometer (ACCÜ-CHEK Aviva, Roche), D-glucose (Sigma–Aldrich) was injected intraperitoneally at dose 1 mg/kg body weight. Blood glucose levels were measured 15, 30, 60 and 120 min after glucose injection using glucometer.

**Body composition measurement.** Live mice body composition was measured with a magnetic resonance imaging technique (EchoMRI 130, Echo Medical Systems). Mice were fasted for 6 h before measurement. Fat and lean mass was analyzed using EchoMRI 14 software.

**Indirect calorimetry.** Indirect calorimetry measurements were performed with the Phenomaster (TSE Systems) using TSE Phenomaster software v3.6.5 or ProMet (Sable Systems) and equipped with Oxigen, O2 and CO2 levels were measured for 60 s every 20 min continuously. In case of GPR180 global knockout mice, following basal measurement mice were injected i.p. with CL-316,243 (0.1 mg/kg/day) to activate non-shivering thermogenesis. In case of adipocyte-specific GPR180 knockout animals, recombination of floxed allele was induced by intraperitoneal injection of 2 mg/mouse tamoxifen on 2 consecutive days after basal measurement. Regarding experiment with Citrelin overexpression, AAV injection was performed prior to housing animals in metabolic cages. Energy expenditure was calculated according to the manufacturer’s guidelines. The heat produced by LabAssay Cholesterol (Wako Chemicals) and triglycerides by Cobas TRIGB kit (Roche/Hitachi), all following manufacturer instructions. Absorbance was measured by SynergyMX Plate reader and data analyzed by Gen5 v3.08 software (BioTek). CTHR1 in human plasma was determined by sandwich ELISA following kit instructions (MMCRU). We have validated the antibody by overexpression of CTHR1 in HEK-293T cells, which do not express this protein, and determining CTHR1 levels in culture media. Importantly, we also spiked recombinant CTHR1 protein into plasma of patients with undetected circulating CTHR1.

**Generation of stable HEK-293T cell line lacking GPR180**

**Lentiviral GPR180 overexpression**

**Indirect calorimetry.** Indirect calorimetry measurements were performed with the Phenomaster (TSE Systems) using TSE Phenomaster software v3.6.5 or ProMet (Sable Systems) and equipped with Oxigen, O2 and CO2 levels were measured for 60 s every 20 min continuously. In case of GPR180 global knockout mice, following basal measurement mice were injected i.p. with CL-316,243 (0.1 mg/kg/day) to activate non-shivering thermogenesis. In case of adipocyte-specific GPR180 knockout animals, recombination of floxed allele was induced by intraperitoneal injection of 2 mg/mouse tamoxifen on 2 consecutive days after basal measurement. Regarding experiment with Citrelin overexpression, AAV injection was performed prior to housing animals in metabolic cages. Energy expenditure was calculated according to the manufacturer’s guidelines. The heat produced by LabAssay Cholesterol (Wako Chemicals) and triglycerides by Cobas TRIGB kit (Roche/Hitachi), all following manufacturer instructions. Absorbance was measured by SynergyMX Plate reader and data analyzed by Gen5 v3.08 software (BioTek). CTHR1 in human plasma was determined by sandwich ELISA following kit instructions (MMCRU). We have validated the antibody by overexpression of CTHR1 in HEK-293T cells, which do not express this protein, and determining CTHR1 levels in culture media. Importantly, we also spiked recombinant CTHR1 protein into plasma of patients with undetected circulating CTHR1.
After washing, the cells were incubated in buffer (130 mM NaCl, 5 mM KCl, adipocytes were starved 2 h prior calcium measurement. Then the cells were loaded with PBS and subsequent blocking with 5% BSA in PBS under permeabilizing with 5% acetic acid in ethanol for 20 min at 4°C. After washing with PBS, cells were stained in parallel using Hoechst (Cell Signaling). After cells were washed 3 times with PBS, pictures were obtained using the automated Operetta imaging system (PerkinElmer). Cells overexpressing GFP were used to subtract background of unspecific antibody binding.

Assessment of SMAD3 shutting by immunofluorescence. To address SMAD3 shutting in response to CTHRC1, mature beige adipocytes at day 18 were fasted for 2 h and then acutely stimulated with recombinant CTHRC1 for 1 h. TGFβ was used as positive control. Then, cells were washed with PBS and fixed 20 m with 4% Formaldehyde at RT. After washing with PBS, cells were incubated with 5% acetic acid in ethanol for 20 min at −20°C to remove lipids. After washing with PBS and subsequent blocking with 5% BSA in PBS under permeabilizing (0.05% Triton X-100) conditions for 90 min, cells were stained overnight at 4°C with primary antibodies either anti-SMAD3 (Cell Signaling) or phospho-SMAD3 (Abcam) diluted 1:500 in blocking buffer and nuclei were stained in parallel with Hoechst (Cell Signaling). After cells were washed 3 times with PBS, pictures were obtained using the automated Operetta imaging system (PerkinElmer). The positive pSMAD3 cells were quantified based on standard image processing steps including thresholding, size filtering (for the nuclei) and by counting those cells whose nuclei overlap with the pSMAD3 staining. For the quantification of cytosolic/nucleus ratio, around each segmented nucleus at least 3 pixel wide ring was created by binary operations as accurate estimation of the individual cell borders were not possible. The ratio is determined by the average pSMAD3 signal intensity inside the nucleus relative to the average signal intensity within the ring. The quantified data has been filtered according to circularity of the nucleus (0.5<), estimated background (weak) signal for the SMAD3 staining, and nucleus/cytoplasmic ratio to hinder the artefactual influence of the segmentation (border estimation of the nuclei). The image processing is performed with Matlab 2019a.

Signalling studies. Phosphorylation of SMAD3 in beige adipocytes in response to TGFβ following knockdown of GPR180 was determined by SMAD3 (pSer423/ 
3425) ELISA kit (Abcam) while silencing of TGFBR2 was used as negative control. Involvement of G signaling following GPR180 knockdown or in response to CTHRC1 was assessed using fluorescent calcium indicator Fluo-4 (Thermo Fisher) or pIP1 ELISA (Cisbio) following manufacturer instructions. Briefly, mature beige adipocytes were starved 2 h prior calcium measurement. Then the cells were loaded with cell permeable Fluo-4 and probenecid mixture and incubated 45 min at 37°C. After were washed with PBS, cells were incubated with 5% acetic acid in ethanol for 20 min NaCl, 5, 10 mM HEPES, 2 mM CaCl2, 10 mM glucose, pH 7.4) for 20 min and fluorescence was measured with excitation at 494 nm and emission at 516 nm. After basal measurement, CTHRC1 or control were added and kinetic measurement continued. In the end of the assay, 2.5 mM ionophore A23187 was added as positive control to validate the assay. Fluorescence was normalized to nuclei number stained by Hoechst as described above. In case of IP1 measurement, 2 h of starvation of beige adipocytes was followed by CTHRC1 stimulus for 15 min in the presence of lithium chloride to suppress IP1 degradation. Endothelin (0.3 nM) was used as positive control. Absorbance was normalized to protein content. Levels of cAMP knockdown in or in response to CTHRC1 were determined by Direct cAMP ELISA kit (Enzo) in basal or forskolin (10 μM) pre-treated adipocytes for 15 min and normalized to protein content.

Binding studies. A HiBiT tag was cloned at the C terminus of human CTHRC1 coding sequence and inserted into pDNA3.1 expression vector (Invitrogen) to obtain a HiBiT-tagged CTHRC1. Three days after delivery of the overexpression construct into HEK-293T cells, standard culture medium (DMEM + 10% FBS + 1% Pen/Strep) was replaced by DMEM + 0.5% and collected after 8 h. Medium containing HiBiT-tagged CTHRC1 was centrifuged 300 x g for 5 min and sterile filtered. Wild-type and GPR180+/- HEK-293T cells were grown in 15 cm dishes until confluence, washed with PBS and serum starved in 15 ml DMEM for 2 h prior to the binding assay. One milliliter of medium containing HiBiT-tagged CTHRC1 protein was added to the starvation medium after 2 h and incubated for 10 min at 37°C. Cells were washed 5 times with ice-cold PBS to wash away unbound CTHRC1 and frozen at −80°C until protein extraction. Interaction of CTHRC1 with GPR180 was studied using the Nano-Glo® HiBiT Blotting System (Promega) according to manufacturer’s instructions.

Protein extraction and western blot. Adipose tissue samples and in vitro differentiated adipocytes were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 130 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented with protease (Complete, Roche) and phosphatase (Halt phosphatase inhibitor cocktail, ThermoFisher) inhibitor cocktails. Lysates were cleared by centrifugation at 12,000 × g for 15 min at 4°C. Protein concentration of the supernatants was determined by DC Protein Assay (Bio-Rad). Equal amount of proteins (5-20 μg) were separated on 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad) and blocked for 1 h (5% BSA in PBS). Phospho-SMAD3 (Ser423/425; 1:100, Abcam), phospho-SP1 (SN5; (Ser645/650; 1:1000, Abcam), SMAD3 (1:200, Abcam), CTHRC1 (1:750, Sigma–Aldrich), TGFβ1,2,3 (1:100, R and D Systems), TGFβ1F1,2F2 (1:2000, Abcam), TGFβ2F2 (1:1000, Abcam), RFP (1:2000, Evrogen), OXPHOS (1:500, Abcam), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204; 1:1000, Cell Signaling), p44/42 MAPK (ERK1/2) (1:1000, Cell Signaling), phospho-p38MAPK (Thr180/Tyr182; 1:1000, Cell Signaling), p38MAPK (1:1000, Cell Signaling), phospho-ERK (Ser218/Ser222; 1:1000, Cell Signaling), phospho-AKT (Thr495; 1:1000, Cell Signaling), phospho-AMPK (Thr172; 1:1000, Cell Signaling), phospho-CREB (Ser133; 1:1000, Cell Signaling), phospho-FAK (Tyr927; 1:1000, Cell Signaling), phospho-PI3K (Thr423; 1:1000, Cell Signaling), phospho-JNK (Thr183; 1:1000, Cell Signaling), phospho-AKT (Thr308; 1:1000, Cell Signaling), HSP90 (1:1000, Cell Signaling) and γ-tubulin (1:10000, Sigma–Aldrich). Signal of the HRP-conjugated secondary antibodies (1:10000, Merck) was visualized by the Image Quanti system (GE Healthcare Life Sciences). Quantification of western blots was done using ImageJ version 1.53e (NIH).
genes were filtered for membrane proteins and receptors. Furthermore, the genes with median expression of tpm < 2 were filtered out. Lastly, the resulting genes were filtered for log2 fold change significance. We processed the GPR180 KD and control RNA-seq data in the same manner described above using kallisto followed by DESeq2 and PCA analysis. In the DESeq2 workflow, first the count data was normalized by the median of ratios method. Next, the dispersion or biological variance was estimated. Thereafter, a generalized linear model was fitted for each gene to detect DE genes. We used the batch number as an additional covariate apart from condition in the generalized linear model of negative binomial distribution to account for the batch effect. The p-values obtained by Wald test were corrected by Benjamini–Hochberg multiple testing procedure. The DE genes were analyzed for KEGG pathway enrichment using GSEA pre-ranked method, which enables the analysis of up- and downregulated genes simultaneously. This approach significantly improved the sensitivity of the geneset enrichment analysis. The genes were sorted by their log2 fold change and analyzed by the GSEA pre-ranked algorithm implemented in GSEApp (https://github.com/zqfang/GSEApp). The data were processed by in house libraries lib called pyDESeq2 (python.org) with pandas library following standard DESeq2 and GSEA tutorials.

Proteomic analysis of cell-conditioned media. For identification of proteins secreted by hMADS cells, we collected 10 ml of cell-conditioned media. The medium was filtered (0.45 μm syringe filter) to remove cellular debris and concentrated 10 times using Amicon Ultra 15 centrifugal filter units with a 3 kDa cut-off (Millipore). For western blot, 30 μl of concentrated medium was loaded into SDS-PAGE gel to detect TGFβ isoforms or CTHRC1. For mass-spectrometry analysis, proteins and peptides present in the concentrated medium were precipitated using ice-cold 10% TCA. Protein pellets were washed twice with ice-cold acetone and re-solubilized in 90 μl of 10 mM Tris, 2 mM CaCl2 pH 8.2. Proteins were digested with 10 μl sequencing grade Trypsin (Promega; 100 ng/μl in 10 mM HCl) and 10 ul RapiGest (1% in water) for 16 h at 37°C. After tryptic digestion, samples were briefly spun; supernatants were dried, dissolved in 20 μl 0.1% formic acid and transferred to autosampler vials for LC/MS/MS. Database search was performed by using the Mascot (SwissProt). We filtered out the overlapping genes from MS and hMADS DE genes in brown over white adipocytes. We obtained a list of secreted proteins from Uniprot. The list of extracellular matrix components or proteins regulating immune response were obtained by GO and Reactome annotations and was used to narrow down the list of secreted proteins.

Quantification and statistical analysis. For in vivo studies, littermates were used for all experiments. Sample sizes were determined on the basis of previous culture experiments were performed with 2 mice. Two-tailed unpaired Student t-test was applied on comparisons of multiple groups. Paired Student t-test was used to analyse the differences in paired samples (e.g. BAT and WAT from the same animal). P-values were filtered for log2 fold change from the DE analysis. We applied on comparisons of multiple groups. Paired Student t-test was used to analyse the differences in paired samples (e.g. BAT and WAT from the same animal). P-values were filtered for log2 fold change from the DE analysis.

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Author contributions
L.B., H.N. and C.W. designed the study; L.B., M.B., B.H. and C.W. supervised the experiments; L.B., M.B., C.H., C.M., E.K., W.S., H.D., I.D. and V.E. performed the experiments; C.H. and A.H. performed image analysis; P.N., K.V. and T.N. performed the clinical transcriptome study; A.G. and U.G. performed all bioinformatics analyses; M.B., B.U., J.U. and Z.K. performed the clinical study and analyzed human samples; E.A. provided resources; P.P. generated global knockout; T.L. generated AAV; L.B., M.B. and C.W. wrote the paper; all authors reviewed and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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