GSK3 is a negative regulator of the thermogenic program in brown adipocytes

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Brown adipose tissue is a promising therapeutic target in metabolic disorders due to its ability to dissipate energy and improve systemic insulin sensitivity and glucose homeostasis. β-Adrenergic stimulation of brown adipocytes leads to an increase in oxygen consumption and induction of a thermogenic gene program that includes uncoupling protein 1 (Ucp1) and fibroblast growth factor 21 (Fgf21). In kinase inhibitor screens, we have identified glycogen synthase kinase 3 (GSK3) as a negative regulator of basal and β-adrenergically stimulated Fgf21 expression in cultured brown adipocytes. In addition, inhibition of GSK3 also caused increased Ucp1 expression and oxygen consumption. β-Adrenergic stimulation triggered an inhibitory phosphorylation of GSK3 in a protein kinase A (PKA)-dependent manner. Mechanistically, inhibition of GSK3 activated the mitogen activated protein kinase (MAPK) kinase 3/6-p38 MAPK-activating transcription factor 2 signaling module. In summary, our data describe GSK3 as a novel negative regulator of β-adrenergic signaling in brown adipocytes.

The discovery of active brown adipose tissue (BAT) in healthy adults has revitalized the concept of combating metabolic dysfunction via recruitment and activation of brown adipocytes1–3. BAT can dissipate energy by uncoupled respiration, a process called adaptive thermogenesis1–3. BAT is classically activated by cold, which through sympathetic nervous system-mediated release of norepinephrine at the surface of brown adipocytes activates β-adrenergic receptors. This activation results in augmented lipolysis, mitochondrial uncoupling, oxygen consumption and thermogenesis. In mice, activated BAT promotes glucose and triacylglycerol clearance, improves insulin sensitivity and glucose tolerance, and counteracts obesity1–3. Most of these effects of brown adipocytes depend on their high mitochondrial density, the unique presence of uncoupling protein 1 (UCP1) in the inner mitochondrial membrane and a high oxidative capacity1–3. In humans, BAT activity correlates with cold-induced energy expenditure and BAT activity is recruited after regular cold exposures4,5. In addition, brown-like (also called beige, brite or inducible brown) adipocytes appear in certain white adipose tissues in response to prolonged cold exposure or treatment with β-adrenergic agonists1–3.

Recent data suggest that BAT has beneficial metabolic functions beyond thermogenesis which might involve an endocrine role6,7. Several signaling molecules with hormonal properties have been found to be released by BAT, particularly under conditions of cold-induced BAT activation7. Additionally, the improved glucose tolerance, enhanced insulin sensitivity and decreased adiposity observed with BAT transplantation have also been associated with the endocrine properties of BAT6,9. Recent reports have established fibroblast growth factor 21 (FGF21) as a bona fide BAT-released factor, secreted by brown adipocytes following cold or β-adrenergic stimulation10–12. Fgf21 expression is controlled by activating transcription factor 2 (ATF2), which in turn is activated by β-adrenergic stimulation in a CAMP-protein kinase A (PKA)-mitogen activated protein kinase (MAPK) kinase 3/6 (MKK3/6)-p38 MAPK-dependent manner10. The same intracellular signaling pathway that controls Fgf21 expression in brown adipocytes is required for induction of a broader thermogenic gene expression program that also includes uncoupling protein 1 (Ucp1), type II iodothyronine deiodinase (Dio2) and peroxisome proliferator-activated receptor γ co-activator-1α (Ppargc1α)10,11,16. Pharmacological administration of FGF21 has been ascribed a number of beneficial metabolic effects, including lowering of adiposity and increased glucose tolerance, and some of these effects have been associated with a direct effect of FGF21 on adipocytes17–19. Recently, autocrine and/or endocrine actions of FGF21 were shown to induce BAT differentiation and WAT browning.

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in response to activation of G protein-coupled receptor 120. Glycogen synthase kinase 3 (GSK3) is a Ser/Thr kinase implicated in the insulin signaling pathway to control glycogen metabolism, but is now also recognized as a multifunctional kinase regulating an array of additional cellular functions. GSK3 exists as two paralogs: GSK3α and GSK3β. Small-molecule inhibitors of GSK3 have favourable metabolic effects in rodents, including prevention of diet-induced obesity and improved glucose tolerance. Thus, GSK3 inhibitors exert some of the same metabolic effects as FGF21 administration.

In a search for novel kinases regulating the thermogenic program of brown adipocytes, we carried out screens with a kinase inhibitor library. We identified GSK3 to be a novel negative regulator of Fgf21 and thermogenic gene expression in brown adipocytes. Following thermogenic activation, GSK3 becomes inactivated by phosphorylation in a PKA-dependent manner, which in turn leads to increased activity of the MKK3/6-p38 MAPK-ATF2 signaling module. Thus, inhibition of GSK3 unleashes thermogenic signaling in brown adipocytes, an observation pointing to GSK3 as a potentially interesting target in metabolic diseases.

Results

FGF21 is under β-adrenergic control in adipose tissue and cultured brown adipocytes. Unbiased kinase inhibitor screens have been applied to successfully identify novel roles for kinases in regulating the formation and function of thermogenic adipocytes. Here we aimed to identify novel kinase functions involved in the β-adrenergically induced thermogenic gene program in brown adipocytes. To this end we decided to use Fgf21 mRNA levels as a read-out due to its reported high inducibility in BAT upon cold exposure and brown adipocytes in response to β-adrenergic stimulation. Before searching for the involvement of novel kinases, we wanted to confirm Fgf21 as a meaningful read-out in our screens. Gene expression was measured by reverse transcription-quantitative PCR (RT-qPCR).

First, we exposed mice to cold or thermoneutrality for 4 days. Cold exposure led to a significant induction of Ucp1 in interscapular and axillary BAT (iBAT and aBAT, respectively) and in the browning-prone inguinal WAT (iWAT) (Fig. 1a). Ucp1 was barely expressed in epididymal WAT (eWAT) and liver, irrespective of housing temperature. In line with previous reports, we found that cold exposure strongly increased Fgf21 expression in thermogenesis-capable adipose tissue depots (BAT and iWAT) and only to a smaller extent in eWAT (Fig. 1b). Fgf21 expression was high in the liver but did not change with cold exposure. Beside transcriptional changes, cold exposure was associated with a decreased average body weight gain (Fig. 1c), a decreased iWAT mass (Fig. 1d) and an increased iBAT mass (Fig. 1e). However, we did not find the plasma concentration of FGF21 to be significantly altered by the cold exposure (Fig. 1f) despite the enhanced expression in adipose tissues.
Next, we determined Fgf21 expression in cultured immortalized and primary brown adipocytes in response to β-adrenergic stimulation. In both cell models, Fgf21 mRNA levels increased after 6 h of stimulation with the pan-β-adrenergic receptor agonist isoproterenol (ISO) (Fig. 1g), which also resulted in a significant increase in medium FGF21 (Fig. 1h).

Thus, in line with previous reports, we show that brown adipocytes induce Fgf21 expression and secretion following β-adrenergic stimulation in a cell-autonomous manner.

Kinase inhibitor screen identifies GSK3 as an inhibitor of Fgf21 expression and secretion in brown adipocytes. To identify novel kinases involved in the β-adrenergically stimulated expression of Fgf21 in brown adipocytes we carried out a screen with 90 different kinase inhibitors. Mature immortalized brown adipocytes were pre-treated with kinase inhibitor and stimulated with ISO. All samples have been normalized to the ISO-stimulated control cells to depict changes in ISO-induced Fgf21 mRNA levels (Fig. 2a). The unstimulated control (without ISO; blue column) showed that ISO stimulation increased Fgf21 expression by ~40 fold. Of notice, the majority of the kinase inhibitors potentiated the effect of ISO. Two broad-spectrum kinase inhibitors (red columns) served as positive controls and were found to largely block ISO-induced Fgf21 induction. Two GSK3 inhibitors, SB415286 and SB216763, increased the β-adrenergically induced Fgf21 mRNA expression ~3 fold, placing them among the 10 inhibitors with the strongest potentiating effect. A third GSK3 inhibitor, BIO, elicited a less pronounced potentiating effect on Fgf21 expression in the screen (~1.4-fold above ISO-stimulation alone). In dedicated GSK3 inhibitor experiments all three inhibitors were found to elevate both basal and ISO-induced expression of Fgf21 by ~2 fold (Fig. 2b).

In these experiments, BIO was as potent as SB415286 and SB216763. Treatment with SB216763 also increased both basal and ISO-induced FGF21 secretion into the cell culture medium (Fig. 2c). In addition to BAT, other key metabolic tissues such as liver, WAT and skeletal muscle also express Fgf21. Interestingly, SB216763 only increased basal expression of Fgf21 in mouse and human white adipocyte cell models (3T3-L1 and hMADS), but not in different hepatocyte cell models or muscle cell lines (Supplementary Fig. 1), suggesting that GSK3 might be an adipocyte-selective regulator of FGF21.

To confirm the kinase inhibitor results by genetic means, retroviral vectors encoding constitutively active (S9A) or kinase dead (K85A) HA-tagged human GSK3β were expressed in immortalized brown pre-adipocytes (Fig. 2d). Overexpression of GSK3β mutants did not appear to affect differentiation as indicated by normal expression of adipocyte-specific markers fatty acid binding protein 4 (Fabp4), adiponectin (AdipoQ) and CCAAT/enhancer binding protein α (Cebpα) (Fig. 2e). Constitutively active GSK3β decreased both basal and ISO-induced Fgf21 expression. The kinase dead mutant had a modest non-significant effect on the basal expression, but increased ISO-induced Fgf21 expression ~2–3 fold (Fig. 2f). Taken together, we have identified GSK3 as an adipocyte-selective negative regulator of FGF21.

GSK3 is a negative regulator of the thermogenic gene program. Next, we wanted to investigate if the effect of GSK3 was specific to the Fgf21 gene, or if GSK3 might regulate a broader thermogenic gene program in brown adipocytes. Therefore, we measured the effect of SB216763 on expression of the β-adrenergically induced thermogenic genes Ucp1, Dio2 and Ppargc1α, in addition to Fgf21, in primary brown adipocytes. In these cells, SB216763 augmented ISO-induced expression of Fgf21, Ucp1, Dio2 and Ppargc1α (Fig. 3a). Basal expression was also increased by SB216763 for Fgf21, Dio2 and Ppargc1α, but not for Ucp1. The increased Ucp1 mRNA levels translated into an increase in ISO-induced UCP1 protein (Fig. 3b). Consistent with the effects of SB216763, simultaneous siRNA-mediated knockdown of both GSK3 paralogs in mature primary brown adipocytes significantly induced the expression of Dio2 and Ppargc1α at both the basal and β-adrenergically induced level (Fig. 3c). Knockdown of GSK3 caused an increased expression of Fgf21 in response to ISO stimulation, but did not influence basal Fgf21 expression. There was no effect of GSK3 knockdown on expression of Ucp1 (Fig. 3c) even though GSK3α and GSK3β protein levels were both substantially reduced by the reverse GSK3 siRNA transfection (Fig. 3d).

The effect on the broader thermogenic gene program prompted us to measure the impact of GSK3 on oxygen consumption. Knockdown of both GSK3 paralogs in mature primary brown adipocytes caused a ~30% higher ISO-induced oxygen consumption rate compared with control cells (Fig. 3e,g), while no significant effect was observed under basal conditions (Fig. 3f).

Overall, this indicates that reduction of GSK3 activity in primary brown adipocytes activates the thermogenic gene program and oxygen consumption in response to β-adrenergic stimulation, consistent with GSK3 being a negative regulator of brown adipocyte thermogenic function.

GSK3 is inactivated after cold exposure and β-adrenergic stimulation in a PKA-dependent manner. GSK3 is believed to be ubiquitously expressed.39 Probing various metabolically active tissues such as BAT, WAT, liver, skeletal muscle and heart confirmed expression of both paralogs in all tissues, albeit at varying levels (Fig. 4a). GSK3 is thought to be constitutively active in most tissues under normal physiological conditions, and its activity is mainly regulated by post-translational phosphorylation of an inhibitory amino acid residue: Ser21 in GSK3α and Ser9 in GSK3β.31 Interestingly, iBAT from mice exposed to cold for 24 h showed a marked increase in the inhibitory phosphorylation of both GSK3α and GSK3β compared to mice housed at ambient temperature (Fig. 4b). This observation was mimicked in immortalized brown adipocytes, as ISO stimulation increased the inhibitory phosphorylation of both GSK3 paralogs (Fig. 4c). Thus, GSK3 becomes inactivated by phosphorylation in brown adipocytes in response to β-adrenergic stimulation.

The major transducer of the β-adrenergic signal in brown adipocytes is PKA and the amino acid sequence around Ser21 in GSK3α and Ser9 in GSK3β is a high probability PKA consensus phosphorylation sequence (Fig. 5a). In addition, GSK3 activity can be inhibited by PKA through phosphorylation of these serine residues.
in HEK293 and NIH3T3 cells. Pre-treatment of immortalized brown adipocytes with the PKA inhibitor H89 before β-adrenergic stimulation blunted the ISO-induced phosphorylation of both GSKα and GSK3β (Fig. 5b). Stimulation with the PKA-specific activator 6-MB-cAMP mimicked the effect of ISO on Ser9-phosphorylation of GSK3β, and to a smaller extent on Ser21-phosphorylation of GSK3α. iBAT from mice expressing a constitutively mutant allele of the PKA catalytic subunit specifically in adipose tissue (AdipoQ-caPKA mice) revealed a hyperphosphorylated pattern of the PKA substrate hormone sensitive lipase (HSL) as well as of GSK3α and GSK3β compared with control mice (Fig. 5c). Treatment of immortalized brown adipocytes with SB216763...
Figure 3. GSK3 restricts the thermogenic program in brown adipocytes. (a) Expression of thermogenic genes (Fgf21, Ucp1, Dio2 and Ppargc1α) in primary brown adipocytes pre-treated with 10μM SB216763 for 1 h before being stimulated with 0.1μM ISO for an additional 6 h. (b) Immunoblot analysis of UCP1 in primary brown adipocytes pre-treated with 10μM SB216763 for 1 h before being stimulated with 0.1μM ISO for an additional 24 h. TFIIB serves as loading control. (c) Expression of thermogenic genes (Fgf21, Ucp1, Dio2 and Ppargc1α) in siRNA-transfected primary brown adipocytes stimulated with 0.1μM ISO for 6 h. (d) Immunoblot analysis of GSK3α and GSK3β in siRNA-transfected primary brown adipocytes stimulated with 0.1μM ISO for 24 h. TFIIB serves as loading control. (e) Representative normalized Seahorse run of oxygen consumption rates (OCR) in siRNA-transfected primary brown adipocytes. Quantification of basal (f) and ISO (1μM)-induced (g) OCR. RT-qPCR data are presented as mean of means (+SEM) (n = 4). Statistical significance was determined by two-way ANOVA with repeated measures and Tukey's post hoc test for multiple comparisons. *p < 0.05 versus H2O. #p < 0.05 versus vehicle/scramble. Seahorse data are presented as mean (±SEM) of one representative experiment (e) or 3 independent experiments (f,g) and significance was determined by paired t-test (*p < 0.05).

For panels b and d, full-length blots/gels are presented in Supplementary Fig. 2.
did not result in increased basal or ISO-induced lipolysis (Fig. 5d) or altered phosphorylation status of major PKA target proteins (HSL, cAMP responsive element-binding protein (CREB) and PKA substrates detected with an anti-PKA substrate antibody) (Fig. 5e), suggesting that GSK3 does not interfere with PKA activity, but rather acts downstream of PKA in the β-adrenergic signaling pathway.

In summary, these data suggest that the inactivating phosphorylation of GSK3 in response to β-adrenergic stimulation is dependent on PKA activity in brown adipocytes.

Inhibition of GSK3 promotes activation of the MKK3/6-p38 MAPK-ATF2 signaling module.

Activation of the thermogenic gene program has been shown to depend on the MKK3/6-p38 MAPK-ATF2 signaling module that is activated downstream of PKA10,13–15. Treatment with SB216763 led to increased activating phosphorylations of MKK3/6, p38 MAPK and ATF2 in response to ISO stimulation (Fig. 6a). Moreover, SB216763 augmented the phosphorylation of p38 MAPK and ATF2 even in the absence of β-adrenergic stimulation. These observations suggest that GSK3 activity normally inhibits this signaling module (Fig. 6a). Forced expression of a kinase dead version of GSK3β caused increased phosphorylation p38 MAPK in response to ISO, whereas a constitutively active GSK3β mutant blunted the normal ISO-induced p38 MAPK phosphorylation (Fig. 6b). Furthermore, the effect of SB216763 on both basal and β-adrenergically induced expression of Fgf21 was abolished in cells pre-treated with the p38 MAPK inhibitor SB202190 (Fig. 6c). As expected, inhibition of p38 MAPK alone attenuated ISO-induced Fgf21 expression. Thus, these data demonstrate that GSK3 restricts p38 MAPK signaling in brown adipocytes and that the effect of GSK3 inhibition depends on p38 MAPK activity. A schematic model of the proposed novel regulatory circuit of GSK3 inhibition of the MKK3/6-p38 MAPK-ATF2 module in brown adipocytes is illustrated in Fig. 6d.

Discussion

In energy metabolism research, much effort is put into elucidating how to increase catabolic pathways, such as β-adrenergically induced thermogenesis in brown adipocytes. Here we show that under basal conditions, the expression of thermogenic genes in brown adipocytes is repressed through a GSK3-dependent inhibitory effect on the MKK3/6-p38 MAPK-ATF2 signaling module. Upon β-adrenergic stimulation, brown adipocytes partly release this repression on the thermogenic gene program through a PKA-dependent inactivation of GSK3 to expand the thermogenic transcriptional machinery, thereby forming a novel regulatory circuit.

GSK3 is known to be involved in glycogen storage and insulin signaling, but is now regarded as a multifunctional kinase regulating an array of cellular functions21. In kinase inhibitor screens, we identified GSK3 as a negative
regulator of the β-adrenergically stimulated increase in Fgf21 expression in mouse brown adipocytes (Fig. 2a). We confirmed the involvement of GSK3 by various approaches: chemical inhibitors, retroviral overexpression of mutant proteins, and siRNA-mediated knockdown. Inhibition of GSK3 by chemical inhibitors or depletion of GSK3 by siRNA was carried out in the mature adipocyte state, whereas retroviral transduction was performed in preadipocytes. Even though GSK3 activity has been associated with adipogenesis in different adipocyte cell models (3T3-L1...
and hMADS)\(^34-36\), we observed no effect of the GSK3\(\beta\) mutants on adipocyte differentiation (Fig. 2e). This might be due to redundant effects of the two paralogs, where GSK3\(\alpha\) was possibly able to sustain normal differentiation. Since forced expression of GSK3\(\beta\) mutants was sufficient to cause effects on \(\text{Fgf21}\) expression, it is possible that the GSK3 paralogs do not exert redundant effects on controlling \(\text{Fgf21}\) levels. Non-redundant effects of the paralogs have been described, e.g. the GSK3\(\beta\)-regulated control of mitochondrial elongation induced by PKA signaling\(^37\). The catalytic domains of GSK3\(\alpha\) and GSK3\(\beta\) are very similar but they differ in their C- and N-terminal regions\(^21\). However, from our results, we are unable to conclude on the relative contribution of each GSK3 paralog.

When analysing other thermogenic genes, we found that GSK3 was not just a regulator of \(\text{Fgf21}\) expression, but had a broader effect on \(\beta\)-adrenergically stimulated gene transcription (Fig. 6a,b). Similar to \(\text{Fgf21}\), \(\text{Dio2}\) and \(\text{Ppargc1}\alpha\) expression was increased with GSK3 inhibition or knockdown, both basally and following stimulation. \(\text{Ucp1}\) expression was augmented following \(\beta\)-adrenergic stimulation in GSK3 inhibitor-treated cells, which also correlated with increased protein levels, however basal levels were not affected. We achieved an efficient siRNA-mediated GSK3 knockdown (Fig. 6d), and even though the effects of SB216763 on \(\text{Fgf21}\), \(\text{Dio2}\) and \(\text{Ppargc1}\alpha\) were mimicked by the knockdown, the effect on \(\text{Ucp1}\) was not. In addition, we observed that knockdown of GSK3 led to increased oxygen consumption following \(\beta\)-adrenergic stimulation (Fig. 3e,g), which might be a consequence of increased levels of \(\text{Ppargc1a}\) and \(\text{Dio2}\) (Fig. 3c) and thereby mitochondrial biogenesis and intracellular conversion of \(\text{T}^{4}\) to \(\text{T}^{3}\).

Because of its effect on a range of thermogenic genes, we hypothesised that GSK3 activity would impact a common regulatory pathway such as the MKK3/6-p38 MAPK-ATF2 module, which is well described as controlling \(\text{Fgf21}\), \(\text{Ucp1}\) and \(\text{Ppargc1}\alpha\) expression in brown adipocytes\(^10,13-15\). We found that inhibition of GSK3 promoted activation of MKK3/6, p38 MAPK and ATF2, and that functional p38 MAPK signaling was necessary for the observed effects of a GSK3 inhibitor (Fig. 6). Therefore, our data suggest that GSK3 suppresses the signal upstream of MKK3/6. MKK3/6 is activated by members of the MAPK kinase kinase (MAP3K) family\(^38\). MAP3K involvement in controlling brown adipocyte gene expression is less well understood, however a few of the family members have been linked to specific functions in brown adipocytes. Recently, it was discovered that MAP3K5 regulates brown and beige adipocyte function\(^40\) and a second MAP3K, MAP3K4, has previously been reported to enhance thermogenic gene expression in adipocytes through activated growth arrest and DNA-damage-inducible 45-\(\gamma\) in a PKA-dependent manner\(^40\). Interestingly, MAP3K4 contains several GSK3 consensus phosphorylation
sites and GSK3 has been identified as a negative regulator of MAPK4 through binding to its kinase domain in COS cells and primary embryonic fibroblasts. It is thus possible that GSK3 impacts the MKK3/6-p38 MAPK-ATF2 module by negatively interfering with the activity of a specific MAPK3, such as MAPK4, thereby restricting the entire signaling module.

Interestingly, only few negative regulators of the adipocyte thermogenic gene program have been identified, and most of these work by targeting PGC-1α stability or thermogenic genes directly by binding to promoter regions. Here we have identified GSK3 as a novel negative regulator of the brown adipocyte thermogenic gene program, through active suppression of the PKA-dependent MKK3/6-p38 MAPK-ATF2 signaling module (Fig. 6d). For the upstream regulation of GSK3 activity, we show that following cold and β-adrenergic stimulation, the thermogenic gene program in brown adipocytes is de-repressed through induction of inhibitory phosphorylations of GSK3 (Fig. 4b,c). Recently, similar findings on GSK3 phosphorylation were made in 3T3-L1 cells after stimulation with ISO. Moreover, two independent in vitro studies and one in vivo study, all in cardiomyocytes, have shown that GSK3α and GSK3β become inactivated when treated with ISO. Since PKA is the master regulator of brown adipocyte activity and thermogenic gene expression following cold or β-adrenergic stimulation, we speculated that GSK3 inactivation might be downstream of PKA. Indeed, GSK3 inactivation (Fig. 6d). For the upstream regulation of GSK3 activity, we show that following cold and β-adrenergic stimulation, the thermogenic gene program in brown adipocytes is de-repressed through induction of inhibitory phosphorylations of GSK3 (Fig. 4b,c). Recently, similar findings on GSK3 phosphorylation were made in 3T3-L1 cells after stimulation with ISO. Moreover, two independent in vitro studies and one in vivo study, all in cardiomyocytes, have shown that GSK3α and GSK3β become inactivated when treated with ISO. Since PKA is the master regulator of brown adipocyte activity and thermogenic gene expression following cold or β-adrenergic stimulation, we speculated that GSK3 inactivation might be downstream of PKA. Indeed, GSK3 inactivation (Fig. 6d).

Small-molecule inhibitors of GSK3 are already being considered for the treatment of Alzheimer's disease, bipolar disorder, certain cancers and type 2 diabetes. Interestingly, studies with rodent models of metabolic disease have demonstrated that inhibiting GSK3 has beneficial effects on systemic metabolism, but also impact whole-body non-specific metabolic actions. GSK3 inhibitors were found to ameliorate diet-induced obesity, decrease adiposity and hepatic steatosis and improve glucose tolerance and lipid profiles. It is possible that GSK3β following cold and β-adrenergic stimulation could also be inhibited by p38 MAPK activity, downstream of PKA, adding a second layer of regulation to GSK3 activity in brown adipocytes.

To harvest the metabolic benefits of BAT, finding negative regulators of thermogenic activation is of importance, since these regulators might be druggable targets. Here, we propose that GSK3 is such a druggable target that has the potential to allow a release of the brake on BAT activity to impact whole-body energy metabolism.

Methods

Mice.

Mice used in Figs 1 and 4 were single-caged 10 weeks old male C57BL/6J BomTac mice (Taconic), housed at room temperature and fed standard chow diet. For cold experiments, mice were kept at 4 °C or 30 °C (thermoneutrality) for 4 days. Animals were killed by cervical dislocation, and iBAT, aBAT, iWAT, eWAT, heart, skeletal muscle and liver were rapidly excised and snap frozen in liquid nitrogen and stored at −80 °C. Blood was collected by sub-mandibular puncture and serum was prepared. Mouse experiments were preapproved and conducted in accordance with the legislation of Danish authorities. The AdipoQ-caPKA mice used in Fig. 5 have been described, and were maintained according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Cell culture.

Brown pre-adipocytes immortalized with SV40 large T antigen were kindly provided by Dr. C. Ronald Kahn. The cells were propagated in DMEM (Life Technologies) containing 10% fetal bovine serum (FBS) (Life Technologies). Two days post-confluence (designated day 0) differentiation was induced by addition of propagation medium supplemented with 1 μM dexamethasone (Sigma-Aldrich), 0.5 mM isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 5 μg/ml insulin (Roche) and 0.5 μM rosiglitazone (Cayman Chemical). At day 2, the medium was changed to medium containing 5 μg/ml insulin and 0.5 μM rosiglitazone. From day 4, cells were cultured in propagation medium and the cells were considered mature at day 8. The cells were kept at 37 °C in a humidified atmosphere with 5% CO₂. 3T3-L1, C2C12 and hMADS cells were kindly provided by Dr. Karsten Kristiansen (3T3-L1, C2C12) and Dr. Christian Dani (hMADS) 33. 3T3-L1 and hMADS cells were propagated and differentiated as described. C2C12, HepG2 (ATCC) and Hepa1-6 (ATCC) cells were propagated in the same medium as the immortalized brown pre-adipocytes. Differentiation of C2C12 cells was induced by replacing the propagation medium with DMEM containing 2% horse serum from two days post-confluence (designated day 0 and considered as myoblasts). The cells were considered mature myocytes at day 2. HepG2 and Hepa1-6 cells were harvested at 80% confluence. Primary CD-1 mouse hepatocytes were obtained from Life Technologies (MSCP10) and were propagated according to the manufacturer’s instructions. All media contained 62.5 μg/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich).
**Isolation and culture of primary brown adipocytes.** Isolation and culture of primary brown pre-adipocytes from male 3–4 weeks old NMRI mice (Taconic) were done as described\(^1\), except that the cell culture medium was changed at days 1, 4, 6 and 8 after isolation.

**Gene expression.** Isolation of total RNA, reverse transcription and RT-qPCR was done as described\(^1\), except that the SensiFAST SYBR Lo-ROX Kit was used. Primers used were: Tbp, fwd-ACCTTTACCAAAATGCTCTATT, rev-ATGATCCGTGCAAAATCCG; Ucp1, fwd-AGCCGGGCTTAATGACTCTGAG, rev-TCTG TAGGGCTGCAATGAGG; Dio2, fwd-AGATGAGCCTCTCTATGGTCG, rev-AGGGCTCGACTGTTACACAT; Fabp4, fwd-TGGAAAGCTTGTCCACTGTA, rev-AATCCCTATTTAGCTGATG; AdipoQ, fwd-AACCTTGCAAGTTGGA TGCC, rev-CTCTTCTCCTCTTCCAGGA; Cebpa, fwd-TGGACAAAGAAGCACGAG; rev-TCAGTGCTGACTCCAGC; Dio2, fwd-CAGTGTGCTGACGTTCCACATC, rev-TCGAACAAAGTTGACCCACAG; Pparγ1a, fwd-AGCCGGTGCAGTCAACAGGAG, rev-GCTGCAAGTGGTACGTAAG.

**FGF21 secretion measurements.** FGF21 levels in plasma and cell culture medium were determined with the Mouse/Rat FGF-21 Quantikine ELISA Kit (R&D Systems) according to the instructions of the manufacturer.

**Kinase inhibitors and activators.** The Tocriscreen Kinase Inhibitor Toolbox containing 80 kinase inhibitors was supplemented with 10 additional inhibitors (Tocris Bioscience)\(^2\). SB415286, SB216763, BIO, H89 and SB202190 were obtained from Tocris Bioscience. 6-MB-CAMP was from Biolog. All compounds were dissolved in DMSO, except for 6-MB-CAMP which was dissolved in water.

**Immunoblotting.** Preparation of whole-cell extracts and immunoblotting were carried out essentially as described\(^3\). Briefly, protein lysates were separated using NuPage 4–12% Bis-Tris gradient gels (Life Technologies) and transferred by semi-dry blotting onto polyvinylidene difluoride membrane (GE Healthcare). Equal loading was confirmed by Amido Black staining (Sigma-Aldrich). All washing and incubation steps were carried out with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk or BSA. Primary antibodies used were: CREB (#9192), p-CREB (Ser133) (#9198), GSK3\(^{\alpha}\) (#9316), GSK3\(^{\beta}\) (#12456), p-GSK3\(^{\alpha}\) (Ser9) (#5558), p38 MAPK (#9212), p-p38 MAPK (Thr180/Tyr182) (#9211), MKK6 (#8550), p-MKK3/6 (Ser189/Ser207) (#12260), ATF2 (#9226), p-ATF2 (Thr71) (#5112), HSL (#4107), p-HSL (Ser660) (#4126), Phospho-(Ser/Thr) PKA substrate (#9621) (all from Cell Signaling Technology); TFIIB (#sc-225) (Santa Cruz Biotechnology); Vinculin (#V9264) (Sigma-Aldrich); UCP1 (#10983) (Abcam) and HA (#11583816001) and p-HA (#12456) (all from Cell Signaling Technology). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse (DAKO). Chemiluminescence was confirmed by Amido Black staining (Sigma-Aldrich). All washing and incubation steps were carried out with PBS containing 0.1% Tween-20 and 5% non-fat dry milk or BSA. Primary antibodies used were: CREB (#9192), p-CREB (Ser133) (#9198), GSK3\(^{\alpha}\) (#9316), GSK3\(^{\beta}\) (#12456), p-GSK3\(^{\alpha}\) (Ser9) (#5558), p38 MAPK (#9212), p-p38 MAPK (Thr180/Tyr182) (#9211), MKK6 (#8550), p-MKK3/6 (Ser189/Ser207) (#12260), ATF2 (#9226), p-ATF2 (Thr71) (#5112), HSL (#4107), p-HSL (Ser660) (#4126), Phospho-(Ser/Thr) PKA substrate (#9621) (all from Cell Signaling Technology); TFIIB (#sc-225) (Santa Cruz Biotechnology); Vinculin (#V9264) (Sigma-Aldrich); UCP1 (#10983) (Abcam) and HA (#11583816001) and p-HA (#12456) (all from Cell Signaling Technology). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse (DAKO). EZ-ECL Enhanced Chemiluminescence Detection Kit for HRP (Biological Industries) was used for detection.

**Retroviral overexpression.** Human pcDNA3-HA-GSK3\(^{\beta}\)-S9A and human pcDNA3-HA-GSK3\(^{\beta}\)-K85A were a gift from Jim Woodgett (Addgene plasmid number #14755 and #14754). GSK3\(^{\beta}\)\(-S9A\) and GSK3\(^{\beta}\)-K85A were expressed in Phoenix-Eco cells. 50–60% confluent Phoenix-Eco cells were transfected with retroviral vectors at 50–60% confluence using Fugene HD Transfection Reagent (Promega). 48 and 72h after transfection, the virus-containing supernatant was harvested and filtered. Subconfluent immortalized brown adipocytes were transduced with the virus-containing supernatant diluted with DMEM containing 10% FBS and supplemented with 4.5 μg/ml polybrene (Sigma-Aldrich). Transduced cells were selected with 400 μg/ml G418 (Sigma-Aldrich).

**Reverse siRNA transfection.** Reverse siRNA transfections were performed as described\(^3\). siRNAs used were GSK3\(^{\alpha}\) (SASI_Mm01_00126759) and GSK3\(^{\beta}\) (SASI_Mm01_00141911) (Sigma-Aldrich). The MISSION\(^{\circledR}\) siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001) was used as control siRNA.

**Oxygen consumption measurements.** Real-time measurements of oxygen consumption rate (OCR) were performed using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies). Mature primary brown adipocytes were re-plated from 6-well plates at day 8 after isolation and reverse transfected in 96-well XF Cell Culture Microplates (Agilent Technologies) as previously described\(^4\). Cells were kept in growth medium until the day of the experiment, i.e. 4 days after transfection. The cell culture medium was changed 1 h before the first measurement to DMEM (without serum) supplemented with 5 mM glucose and adjusted to pH 7.4. OCR was measured under basal conditions and following isoproterenol injection.

**Glycerol release.** Glycerol release into the cell culture medium was measured with the Adipolysis Assay Kit (Cayman Chemical) following the instructions of the manufacturer.

**Statistics.** All statistical tests of gene expression were performed on log-transformed data. Unpaired two-tailed Student’s t-test was used for single comparisons. Two-way ANOVA with repeated measures and Tukey’s post hoc test was used for multiple comparisons.

**References**

1. Sidossis, L. & Kajimura, S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. *J Clin Invest* **125**, 478–486, https://doi.org/10.1172/JCI78362 (2015).
2. Betz, M. J. & Enerback, S. Human Brown Adipose Tissue: What We Have Learned So Far. *Diabetes* **64**, 2352–2360, https://doi.org/10.2337/db15-0146 (2015).
3. Schrauwen, P. & van Marken Lichtenbelt, W. D. Combatting type 2 diabetes by turning up the heat. *Diabetologia* **59**, 2269–2279, https://doi.org/10.1007/s00125-015-3572-4 (2016).
4. van der Lans, A. A. et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* **123**, 3395–3403, https://doi.org/10.1172/JCI68993 (2013).
40. Gantner, M. L., Hazen, B. C., Conkright, J. & Kralli, A. GADD45gamma regulates the thermogenic capacity of brown adipose tissue. *Nat Rev Endocrinol*, https://doi.org/10.1038/nrendo.2016.136 (2016).

41. Liu, X. et al. Brown adipose tissue transplantation improves whole-body energy metabolism. *Cell Res* 23, 851–854, https://doi.org/10.1038/cr.2013.64 (2013).

5. Yoneshiro, T. et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest* 123, 3404–3408, https://doi.org/10.1172/JCI67803 (2013).

6. Wang, G. X., Zhao, X. Y. & Lin, J. D. The brown fat secretome: metabolic functions beyond thermogenesis. *Trends Endocrinol Metab* 26, 231–237, https://doi.org/10.1016/j.tem.2015.03.002 (2015).

7. Villarroya, F., Cerejo, R., Villarroya, J. & Giralt, M. Brown adipose tissue as a secretory organ. *Nat Rev Endocrinol*, https://doi.org/10.1038/nrendo.2016.136 (2016).

8. Liu, X. et al. Activation of mTORC1 is essential for beta-adrenergic stimulation of adipose browning. *J Clin Invest* 126, 1704–1716, https://doi.org/10.1172/JCI83532 (2016).
44. Condorelli, G. et al. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. Proc Natl Acad Sci USA 99, 12333–12338, https://doi.org/10.1073/pnas.172376399 (2002).
45. Okumura, S. et al. Disruption of type 5 adenylyl cyclase enhances desensitization of cyclic adenosine monophosphate signal and increases Akt signal with chronic catecholamine stress. Circulation 116, 1776–1783, https://doi.org/10.1161/CIRCULATIONAHA.107.698662 (2007).
46. Webb, I. G., Sicard, P., Clark, J. E., Redwood, S. & Marber, M. S. Myocardial stress remodelling after regional infarction is independent of glycogen synthase kinase-3 inactivation. J Mol Cell Cardiol 49, 897–900, https://doi.org/10.1016/j.yjmcc.2010.07.021 (2010).
47. Dema, A. et al. The A-Kinase Anchoring Protein (AKAP) Glycogen Synthase Kinase 3beta Interaction Protein (GSKIP) Regulates beta-Catenin through Its Interactions with Both Protein Kinase A (PKA) and GSK3beta. J Biol Chem 291, 19618–19630, https://doi.org/10.1074/jbc.M116.738047 (2016).
48. Thornton, T. M. et al. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science 320, 667–670, https://doi.org/10.1126/science.1156037 (2008).
49. Cohen, P. & Goedert, M. GSK3 inhibitors: development and therapeutic potential. Nat Rev Drug Discov 3, 479–487, https://doi.org/10.1038/nrd1415 (2004).
50. Takahashi-Yanaga, F. Activator or inhibitor? GSK-3 as a new drug target. Biochem Pharmacol 86, 191–199, https://doi.org/10.1016/j.bcp.2013.04.022 (2013).
51. Fasshauer, M. et al. Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. J Biol Chem 275, 25494–25501, https://doi.org/10.1074/jbc.M004046200 (2000).
52. Rodriguez, A. M. et al. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J Exp Med 201, 1397–1405, https://doi.org/10.1084/jem.20042224 (2005).
53. Isidor, M. S. et al. An siRNA-based method for efficient silencing of gene expression in mature brown adipocytes. Adipocyte 5, 175–185, https://doi.org/10.1080/21623945.2015.1111972 (2016).
54. Murholm, M. et al. Dynamic regulation of genes involved in mitochondrial DNA replication and transcription during mouse brown fat cell differentiation and recruitment. PLoS One 4, e8458, https://doi.org/10.1371/journal.pone.0008458 (2009).
55. Hansen, J. B. et al. Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. J Biol Chem 274, 2386–2393 (1999).

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Author Contributions
All authors conceived and designed the experiments. L.K.M., S.W. and B.W. performed the experiments. L.K.M., S.W. and J.B.H. analyzed and interpreted the data. L.K.M. and S.W. prepared the figures. L.K.M., S.W. and J.B.H. wrote the paper. All authors read and approved the final manuscript.

Additional Information
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