Ablation of adipocyte creatine transport impairs thermogenesis and causes diet-induced obesity

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Depleting creatine levels in thermogenic adipocytes by inhibiting creatine biosynthesis reduces thermogenesis and causes obesity. However, whether creatine import from the circulation affects adipocyte thermogenesis is unknown. Here we show that deletion of the cell-surface creatine transporter (CrT) selectively in fat (AdCrTKO) substantially reduces adipocyte creatine and phosphocreatine levels, and reduces whole-body energy expenditure in mice. AdCrTKO mice are cold intolerant and become more obese than wild-type animals when fed a high-fat diet. Loss of adipocyte creatine transport blunts diet- and β3-adrenergic-induced thermogenesis, whereas creatine supplementation during high-fat feeding increases whole-body energy expenditure in response to β3-adrenergic agonism. In humans, CrT expression in purified subcutaneous adipocytes correlates with lower body mass index and increased insulin sensitivity. Our data indicate that adipocyte creatine abundance depends on creatine sequestration from the circulation. Given that it affects whole-body energy expenditure, enhancing creatine uptake into adipocytes may offer an opportunity to combat obesity and obesity-associated metabolic dysfunction.

The escalation in prevalence of obesity worldwide has led to a surge in type 2 diabetes, cardiovascular disease and many cancers 1–12. Excess fat storage occurs when caloric intake persists exceeding caloric expenditure 1. The capacity for thermogenic adipose tissues (brown and beige) to dissipate chemical energy offers great potential to combat obesity. Some individuals who are predisposed to obesity exhibit decreased adipose thermogenic capacity 4, thus suggesting that this impairment is relevant in the context of human weight gain. Several pathways in adipose and non-adipose tissues have emerged in recent years and have been found to play critical roles in adaptive thermogenesis 5–11.

Creatine supports thermogenic respiration by stimulating mitochondrial ATP turnover in vitro 12,13. Importantly, pharmacological reduction of endogenous creatine levels in adipocytes blunts adrenergically-stimulated thermogenesis and core body temperature in vivo, and reduces oxidative metabolism in beige fat and brown adipose tissue (BAT) 13–15. Selective inactivation of creatine synthesis in fat (Adipo-Gatm knockout mice) causes a reduction in creatine levels in BAT, suppresses whole-body energy expenditure and results in obesity. Critically, creatine supplementation of Adipo-Gatm knockout animals prolongs the ability to sustain adrenergic thermogenesis, thus suggesting that creatine transport can be limited under certain physiological conditions in vivo and that adipocyte creatine levels are at least partly regulated by sequestration from the circulation 16.

Results

Fat-selective deletion of the creatine transporter depletes adipocyte creatine abundance. Creatine can be taken up into cells by a creatine transporter, CrT (also known as Slc6a8) 17, and the rate of sequestration into BAT from the circulation is substantial, as it can match that of skeletal muscle 18. To begin to examine the contribution of creatine transport to adipocyte creatine abundance, we purified the BAT stromal vascular fraction from mice containing a floxed allele of CrT 16, which we refer to as CrTflo/y mice. CrTflo/y primary brown adipocytes were differentiated in vitro and infected with adenovirus encoding Cre recombinase or green fluorescent protein (GFP). CrT transcript abundance was significantly reduced (~60%) 6 d after expression of CRE compared with expression of GFP (Fig. 1a). There was no defect in adipocyte differentiation of the cells on the basis of messenger-RNA expression levels of AdipoQ, Fabp4 and Pparγ2 (Fig. 1a). Similarly, transcript levels of mitochondrial creatine kinases (Ckm1 and Ckm2) and Gatm were not changed, but Gatm levels were marginally elevated. We also observed an increase in Ucp1 mRNA abundance on CrT deletion (Fig. 1a). This potentially compensatory relationship between Ucp1 and genes of creatine metabolism is consistently observed in various mouse models 19,20,21 and is suggestive of parallel thermogenic pathways.

Using liquid chromatography coupled to mass spectrometry (LC–MS), we detected a ~50% reduction in steady-state creatine and phosphocreatine (PCr) levels in CRE-infected CrTflo/y primary brown adipocytes compared with GFP-infected cells (Fig. 1b). In contrast, while CRE infection of Gatmlos/y brown adipocytes reduced Gatm transcript abundance substantially, by 60% (Supplementary Fig. 1a), these adipocytes exhibited no change in creatine or PCr levels in vitro (Supplementary Fig. 1b). Thus, while the creatine pool in BAT is partly regulated by GATM-dependent creatine synthesis in vivo 19, creatine abundance is primarily regulated by cellular transport by CRT in cultured brown adipocytes.
in vitro. In agreement with a thermogenic role for creatine, CRE-infected CrT\textsuperscript{lox/lox} primary brown adipocytes exhibited decreased norepinephrine-dependent thermogenic respiration, as compared with GFP-infected cells (Fig. 1c).

We next explored the role of creatine transport-related metabolism in vivo. We reduced the adipocyte creatine abundance through fat-selective inactivation of CrT by crossing Adiponectin-Cre transgenic animals\textsuperscript{12} with CrT\textsuperscript{lox/lox} mice (Fig. 1d). We hereafter refer to this model as adipose-specific CrT knockout (AdCrTKO) mice. The selective deletion of CrT in adipocytes was evaluated by reverse transcription quantitative PCR (RT–qPCR) analysis of BAT, subcutaneous adipose tissue (SQ), perigonadal white adipose tissue (PgWAT), kidney and skeletal muscle. Significant depletion of CrT mRNA abundance was detected in bulk BAT and SQ of AdCrTKO animals relative to CrT\textsuperscript{lox/lox} controls (BAT: CrT\textsuperscript{lox/lox} 1.00 ± 0.06, AdCrTKO 0.39 ± 0.04; SQ: CrT\textsuperscript{lox/lox} 0.39 ± 0.06, AdCrTKO 0.21 ± 0.04) but not in PgWAT, kidney or skeletal muscle (Fig. 1e). Moreover, these data demonstrate that, of the adipose depots analysed, BAT displayed the highest CrT expression levels (approximately threefold higher than SQ and PgWAT).

We next determined whether CrT deletion perturbs creatine abundance in vivo. Indeed, creatine and PCr levels were significantly reduced in the BAT of AdCrTKO mice compared with CrT\textsuperscript{lox/lox} controls, as demonstrated by NMR (creatine: ~60% reduced; PCr: ~40% reduced) (Fig. 1f). Serum creatine levels were unaltered between AdCrTKO and CrT\textsuperscript{lox/lox} mice (Supplementary Fig. 1c), thus indicating that loss of adipocyte creatine does not alter systemic abundance of this metabolite. Our previous work with Adipo-Gatm knockout animals has shown that creatine is depleted by 30–40% in BAT\textsuperscript{10}. Therefore, in terms of relative contributions of the different pathways for creatine accumulation, adipocyte creatine abundance is regulated by both intracellular synthesis and cellular transport in vivo.

**AdCrTKO mice have impaired energy expenditure.** Mice with adipocyte-specific loss of creatine synthesis are unable to maintain thermal homeostasis in response to acute cold (4°C) exposure\textsuperscript{41}. Thus, we explored body-temperature defence in AdCrTKO animals. On acute challenge to 4°C, the body temperature of AdCrTKO mice dropped at a significantly faster rate than that of CrT\textsuperscript{lox/lox} control mice (CrT\textsuperscript{lox/lox} 35.1°C ± 0.69°C, AdCrTKO 33.6°C ± 0.57°C after 3 h) (Fig. 2a). These data indicate that loss of adipocyte creatine transport may impair thermogenic capacity in vivo. Thermogenic and non-thermogenic factors contribute to the maintenance of body temperature on exposure to decreased environmental temperature. Thus, we next studied whole-body energy expenditure by examining resting and adrenergic energy expenditure in animals relieved from thermal stress at thermonuclear (30°C) housing\textsuperscript{42}. Energy expenditure at rest during chow feeding was not different between AdCrTKO animals and littermate age-matched controls (CrT\textsuperscript{lox/lox} control mice) (Fig. 2b). Energy expenditure at rest during chow feeding was not different between AdCrTKO animals and littermate age-matched controls (Supplementary Fig. 2a), nor was there any difference between genotypes when accounting for any differences in body mass by analysis of covariance (ANCOVA) (Supplementary Fig. 2b). There was no difference in ambulatory movement between genotypes (Supplementary Fig. 2c). To examine the direct contribution of adipose tissue to whole-body metabolic rate, we used the relatively adi-
pose-selective β3-adrenergic receptor agonist CL 316,243 (ref. 24), which we refer to as CL. Because adrenergic responsiveness can be masked at subthermoneutral temperatures (when obligatory thermogenesis is not sufficient to maintain thermal homeostasis) we examined the change in energy expenditure of conscious free-moving mice housed at 30 °C after intraperitoneal administration of CL. Strikingly, the activation of CL-induced energy expenditure was significantly blunted (~10%) in AdCrTKO animals compared with CrTlox/lox controls (Fig. 2b), thus confirming that loss of creatine transport impairs adrenergic thermogenesis in fat. Next, we applied
regression analysis to investigate the effect of inactivation of CrT on the relationship between CL-induced thermogenesis and body mass. The average energy expenditure per unit change in body mass was proportionately and significantly lower in AdCrTKO animals than their age-matched littermate controls (Fig. 2c). Thus, ablation of creatine transport into fat reduces whole-body metabolic rate in response to adrenergic stimulation.

Several genes involved in creatine metabolism are regulated at the mRNA level by acute caloric excess. On the basis of this, we examined CRT protein abundance in purified brown adipocytes isolated from wild-type (C57BL/6/N) mice after acute high-fat feeding for 3 d, by using liquid chromatography–tandem mass spectrometry (LC–MS/MS) high-resolution quantitative proteomics. Strikingly, CRT protein levels were significantly elevated (20%) after acute high-fat feeding (Supplementary Fig. 2d), a finding consistent with the idea that creatine transport may be important in triggering an increase in energy expenditure in response to caloric excess, known as diet-induced thermogenesis. Thus, we began to explore the interaction of adipocyte creatine transport with diet-induced thermogenesis by examining resting metabolic rate after acute exposure (3 d) to high-fat feeding. In contrast to chow feeding, acute high-fat feeding revealed a significant difference in resting metabolic rate between AdCrTKO animals and littermate age-matched controls (Fig. 2d), and this difference was maintained when accounting for any differences in body mass by ANCOVA (Fig. 2e). There was no difference in ambulatory movement between genotypes (Supplementary Fig. 2e). Moreover, adrenergic stimulation of adipocyte thermogenesis was reduced (~20%) in AdCrTKO animals compared with CrTlox/y (control) mice; the average energy expenditure per unit body mass was proportionately and significantly lower in AdCrTKO animals than their age-matched littermate controls (Fig. 2g). Next, we monitored the resting metabolic rate of mice at 30 °C to determine the change in energy expenditure mediated by high-fat feeding itself. Strikingly, AdCrTKO mice had a blunted (~40%) ability to activate resting metabolic rate in response to high-fat feeding, as compared with CrTlox/y littermate controls (Fig. 2h and Supplementary Fig. 2f). Therefore, loss of adipocyte creatine transport impairs diet-induced thermogenesis.

We next explored the effect of CR T-alimentation on metabolic rate at standard temperature (22 °C) housing. The energy expenditure of chow-fed AdCrTKO mice was similar to that of CrTlox/y littermates at rest (Supplementary Fig. 2g), and when the variation in body mass of individual mice was accounted for by ANCOVA (Supplementary Fig. 2h). Moreover, the reduced energy expenditure on CL administration that we detected at 30 °C with chow-diet feeding was not present in chow-fed animals at 22 °C (Supplementary Fig. 2i). We hypothesized that the cold stress of 22 °C housing might mask adrenergically mediated creatine-dependent thermogenesis, such that an additional (temperature-independent) thermogenic stimulus might be required to reveal the defect in whole-body energy expenditure of AdCrTKO animals. In agreement with this idea, AdCrTKO animals began to exhibit a trend toward reduced energy expenditure at rest, as compared with control mice, when fed a high-fat diet acutely (Supplementary Fig. 2k,l). However, this trend was not statistically significant. Strikingly, the activation of CL-induced energy expenditure was significantly blunted (~15%) in acute high-fat-diet-fed AdCrTKO animals compared with CrTlox/y controls (Fig. 2i). Regression analysis demonstrated that the average energy expenditure per unit change in body mass was proportionately lower in AdCrTKO animals than their age-matched littermate controls (Fig. 2j). Moreover, AdCrTKO mice exhibited reduced diet-induced thermogenesis, as compared with CrTlox/y animals at 22 °C (Fig. 2k and Supplementary Fig. 2m). Therefore, at 30 °C housing, decreased adrenergic thermogenesis is consistently observed in animals with depletion of adipocyte creatine stores, whether chow or high-fat fed. At 22 °C housing, the induction of diet-induced thermogenesis with acute high-fat feeding is required to reveal the impairment in adrenergic thermogenesis after depleting of BAT creatine.

**Creatine supplementation increases energy expenditure during high-fat feeding.** Since we hypothesized that creatine transport controls diet-induced thermogenesis, we explored the effect of dietary creatine supplementation on whole-body energy expenditure in wild-type mice. After two weeks of consuming creatine-supplemented diet (chow or chow followed by acute high fat), BAT creatine abundance was significantly higher than that in animals receiving no supplementation, as measured by LC–MS (Supplementary Fig. 2n). CL-induced activation of whole-body energy expenditure was not altered by creatine supplementation under Chow-fed conditions (Fig. 2j). In contrast, it was significantly increased in creatine-supplemented animals after acute transition to high-fat diet, as compared with controls (Fig. 2m). Notably, creatine supplementation with acute high-fat feeding resulted in significantly higher BAT creatine levels than did creatine supplementation with chow diet (Supplementary Fig. 2n). Together, these data demonstrate that adipocyte creatine transport is critical for supporting diet-induced thermogenesis in vivo. This is caused by (1) an interaction between adipocyte creatine with certain components of high-fat diet, (2) increased sequestration of creatine into BAT on high-fat feeding or (3) a combination of the two.

**AdCrTKO mice become more obese than control littermates on high-fat feeding.** The decreased energy expenditure of AdCrTKO animals, particularly under high-fat feeding conditions, prompted us to explore their propensity for diet-induced obesity. AdCrTKO mice and age-matched CrTlox/y littermate controls were challenged with ad libitum feeding of high-fat diet over 8 weeks at two environmental temperatures (30 °C and 22 °C). At 30 °C housing, cumulative food intake was indistinguishable between AdCrTKO mice and CrTlox/y animals (Fig. 3a), whereas the body mass of AdCrTKO mice increased over controls (Fig. 3b). Metabolic efficiency is a hallmark of obesity, representing the fraction of assimilated energy that is stored somatically. AdCrTKO mice exhibited higher metabolic efficiency than CrTlox/y control mice (CrTlox/y 3.51 ± 0.55, AdCrTKO 5.26 ± 0.54) (Fig. 3c), strongly suggesting that fat-specific deletion of CrT may increase the propensity towards diet-induced obesity. In agreement with this hypothesis, AdCrTKO animals accreted a significantly greater quantity of fat mass than CrTlox/y animals (Fig. 3d), whereas lean mass was indistinguishable between genotypes (Supplementary Fig. 3a). Most striking was the triglyceride abundance in individual fat depots. In agreement with CrT deficiency causing adipocyte thermogenic impairment, the BAT triglyceride content from AdCrTKO animals was significantly greater (~five-fold) than BAT triglyceride content from CrTlox/y mice, and the SQ triglyceride content of AdCrTKO animals was nearly double that of CrTlox/y mice (Fig. 3e). The obese phenotype exhibited by AdCrTKO animals was not observed when these animals were fed a chow diet (Supplementary Fig. 3b,c). Similarly, there was no difference in lean mass between genotypes (Supplementary Fig. 3d).

At 22 °C housing, the cumulative food intake was identical between AdCrTKO mice and CrTlox/y animals (Fig. 3f), while the body mass of AdCrTKO mice trended higher than controls (Fig. 3g). AdCrTKO mice exhibited significantly higher metabolic efficiency than CrTlox/y control mice (CrTlox/y 3.2 ± 0.48, AdCrTKO 4.39 ± 0.30), a result further consistent with the hypothesis that deficiency in creatine transport into fat causes obesity (Fig. 3h). Indeed, the fat mass of AdCrTKO animals expanded more than the fat mass from CrTlox/y animals after high-fat feeding (Fig. 3i), whereas the lean mass was similar between genotypes (Supplementary Fig. 3e). BAT triglyceride levels from AdCrTKO animals were significantly greater (~threefold) than BAT triglyceride content from CrTlox/y mice, and...
AdCrTKO mice exhibit increases in kininogen expression in BAT and SQ. Inactivation of creatine transport into adipose tissue did not alter Ucp1 mRNA levels in BAT or SQ between AdCrTKO and CrTlox/gy animals, regardless of housing temperature (Fig. 4a,b). Quantitative proteomics was next used to determine the global BAT expression profile of AdCrTKO animals compared with CrTlox/gy littermate controls, housed at 30 °C or 22 °C. We were primarily interested in defining a common signature resulting from CrT inactivation, irrespective of housing temperature (Fig. 4c). In agreement with the mRNA levels, we did not detect any difference in BAT or SQ UCP1 protein expression between AdCrTKO and CrTlox/gy animals, regardless of housing temperature (Fig. 4d,e), nor was there any significant difference in mitochondrial proteins (Supplementary Data 3), thus suggesting that the thermogenic impairment exhibited by AdCrTKO animals was not a result of reduced UCP1-dependent thermogenesis or general mitochondrial abundance. We identified 6,099 and 6,377 unique proteins from BAT of mice housed at 30 °C or 22 °C, respectively (Fig. 4c, Supplementary Data 1 and Supplementary Data 2). Intersection of these two datasets resulted in identification of 5,508 proteins (Fig. 4c and Supplementary Data 3). We next performed unbiased clustering of the samples by using proteins exhibiting the highest variance across all conditions and an empirical Bayes method to find proteins differentially expressed between AdCrTKO and CrTlox/gy BAT consistently at 30 °C and 22 °C (Fig. 4f). This analysis yielded a set of 15 differentially expressed proteins at a false discovery rate (FDR) of 0.05 (Fig. 4g and Supplementary Fig. 3i). Gene ontology analysis on the differentially expressed proteins indicated an enrichment of proteins with known functions in the control of blood pressure (Fig. 4h). Specifically, kinogen 1 and 2 (KNG1 and KNG2) along with several kallikreins (involved in proteolytic processing of kininogens) were elevated in AdCrTKO compared with CrTlox/gy BAT consistently at 30 °C and 22 °C (Fig. 4i). In agreement with elevated KNG1 expression in BAT, SQ from AdCrTKO animals also exhibited significantly elevated KNG1 protein expression at both 30 °C and 22 °C (Fig. 4i).

AdCrTKO mice incur adaptive increases in the cold-inducible high-molecular-weight isofrom of Kng1. Adipose tissue plays an important role in maintaining vascular homeostasis10,11, and BAT vasculature is critical for optimal thermogenic function. Obesity can impair BAT-mediated thermogenesis partly through capillary rarefaction, and obesity has been posited to be associated with impaired arterial distensibility12,13. In contrast, increasing BAT perfusion can increase glucose uptake into BAT, which may (indirectly) support thermogenic respiration12,13. However, Kng1 mRNA has been identified in several reports to encode a BAT-enriched

SQ triglyceride content of AdCrTKO animals was nearly double that of CrTlox/gy mice (Fig. 3). Obesity did not occur in AdCrTKO mice fed chow diet (Supplementary Fig. 3g), nor was there a change in lean mass (Supplementary Fig. 3h). Thus, the consistent findings of obesity at the whole-body level and triglyceride accumulation in specific depots (BAT and SQ) at 30 °C and 22 °C, along with prior work28, strongly indicate that adipocyte creatine depletion causes obesity, owing to increased metabolic efficiency and impaired diet-induced thermogenesis.

Fig. 3 | AdCrTKO mice become obese on a high-fat diet. a–d. Cumulative energy intake in megajoules (MJ) (a), body mass (b), metabolic efficiency (c) and fat mass (d) over 8 weeks of high-fat feeding at 30 °C (CrTlox/gy, n = 6; AdCrTKO, n = 5). e. Triglyceride abundance in BAT and SQ after 12 weeks of high-fat feeding at 30 °C (BAT and SQ: CrTlox/gy, n = 6; AdCrTKO, n = 6). f–j. Cumulative energy intake (MJ) (f), body mass (g), metabolic efficiency (h) and fat mass (i) over 8 weeks of high-fat feeding at 22 °C (CrTlox/gy, n = 7; AdCrTKO, n = 10). j. Triglyceride abundance in BAT and SQ after 8 weeks of high-fat feeding at 22 °C (BAT: CrTlox/gy, n = 7; AdCrTKO, n = 6; SQ: CrTlox/gy, n = 7; AdCrTKO, n = 7). Data are presented as mean ± s.e.m. of biologically independent samples. Two-tailed Student’s t-tests (c,h); multiple two-tailed Student’s t-tests (a,b,d–g,i,j).
secreted protein, and to be elevated in SQ fat on cold exposure\(^6,10\), thereby suggesting that KNG1 may have a thermogenic function. Thus, kinogen upregulation in AdCrTKO BAT and SQ suggested that this was an adaptive response that counters the impaired thermogenesis caused by creatine depletion. Alternative splicing leads to two Kng1 isoforms, yielding a high-molecular-weight kinogen and a low-molecular-weight kinogen. Using RT–qPCR, we compared the mRNA abundance of Kng1 and Kng2 between AdCrTKO and CrT\(^{lox/y}\) BAT. We detected a significant increase in Kng1 mRNA abundance in AdCrTKO compared with CrT\(^{lox/y}\), using primers detecting all Kng1 isoforms or primers specific to the high-molecular-weight isoform (Fig. 5a). In contrast, the low-molecular-weight isoform of Kng1, as well as Kng2, were not differentially expressed at the mRNA level (Fig. 5a). These data suggest that in AdCrTKO BAT, KNG1 and KNG2 protein upregulation (Fig. 4g) is at least partly regulated at the transcriptional and post-transcriptional level, respectively, and that the high-molecular-weight KNG1 isoform is the primary KNG1 isoform elevated in response to creative depletion in adipose tissue.

Next, we examined the expression of Kng1 and Kng2 mRNA between different fat depots, as well as their regulation following acute and chronic exposure to cold (4 °C), compared with 30 °C housing. Kng1 and Kng2 expression was highly enriched in BAT, compared with SQ and PgWAT (Fig. 5b). Furthermore, abundance of the high-molecular-weight isoform of Kng1 was significantly increased in BAT, SQ and PgWAT after 4 °C exposure (Fig. 5c).

Kng2 levels were significantly elevated in SQ and PgWAT (but not BAT) on exposure to environmental cold (Fig. 5c). Together, our findings and the findings of others\(^6,10\) suggest a potential role for kinogens (particularly KNG1) in adaptive thermogenesis. If true, elevated KNG1 and KNG2 abundance at the protein level in AdCrTKO BAT is consistent with an adaptive response resulting from impaired creatine-dependent thermogenesis.

**CRT levels in human adipocytes correlate with lower body mass index and increased insulin sensitivity.** Creative metabolism is selective for BAT compared with white fat in humans\(^36\). A recent analysis of ~2,850 \[^{18}F\]fluorodeoxyglucose positron-emission tomography/computed tomography scans from 1,644 human subjects has demonstrated that BAT creatine energetics may be a significant predictor of total activated human BAT\(^7\). Moreover, in a recent study of human BAT-mediated postprandial thermogenesis, 52 genes have been found to be significantly correlated with UCP1 expression, two of which are the mitochondrial creatine kinases (CKMT1A and CKMT2)\(^8\). We investigated the expression of genes that regulate the abundance of creatine in purified human subcutaneous adipocytes of 43 patients with a wide body mass index (BMI) range, who were undergoing elective plastic surgery. A diagnosis of diabetes was a criterion for exclusion, as the focus here was on proximal changes associated with obesity and insulin resistance, rather than downstream changes resulting from hyperglycaemia (and drug treatments). We compared the mRNA expression of genes

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**Fig. 4 | Increased kininogen expression from BAT and SQ of AdCrTKO mice.** a, RT–qPCR from BAT of mice housed at 30 °C (CrT\(^{lox/y}\), n = 5; AdCrTKO, n = 6), or 22 °C (CrT\(^{lox/y}\), n = 6; AdCrTKO, n = 6). b, RT–qPCR from SQ of mice housed at 30 °C (CrT\(^{lox/y}\), n = 4; AdCrTKO, n = 6), or 22 °C (CrT\(^{lox/y}\), n = 6; AdCrTKO, n = 6). c, Cartoon of BAT proteomics experiment. d, Relative UCP1 protein abundance in BAT from mice housed at 30 °C (CrT\(^{lox/y}\), n = 5 (light grey); AdCrTKO, n = 5 (red)) or 22 °C (CrT\(^{lox/y}\), n = 5 (black); AdCrTKO, n = 5 (blue)). e, Relative UCPI protein abundance in SQ from mice housed at 30 °C (CrT\(^{lox/y}\), n = 4 (light grey); AdCrTKO, n = 5 (red)) or 22 °C (CrT\(^{lox/y}\), n = 5 (black); AdCrTKO, n = 5 (blue)). f, Principal component analysis of proteomics results, using the 50 proteins with highest variance across all samples (n = 5 mice per group). g, Volcano plot showing expression fold changes (FC) (log_2) between AdCrTKO and CrT\(^{lox/y}\) controls from both 30 °C and 22 °C housing, compared with their associated P values. Red dots represent proteins with significant changes at 0.05 FDR (CrT\(^{lox/y}\), n = 10; AdCrTKO, n = 10). h, Gene ontology (GO) enrichment of differentially abundant proteins (CrT\(^{lox/y}\), n = 10; AdCrTKO, n = 10). i, Relative KNG1 protein abundance in SQ from mice housed at 30 °C (CrT\(^{lox/y}\), n = 4 (light grey); AdCrTKO, n = 5 (red)) or 22 °C (CrT\(^{lox/y}\), n = 5 (black); AdCrTKO, n = 5 (blue)). Box plots were generated in Graphpad Prism by using default parameters. Boxes stretch from the seventy-fifth percentile; black horizontal line within each box represents the median. Data are presented as mean ± s.e.m. of biologically independent samples. Two-tailed Student’s t-tests (a–d); moderated t-tests using the limma (v3.32.10) package in R\(^{51,52}\) as justified in ref. \(^{53}\) (g); hypergeometric test implemented in the GORilla tool (h); q values and FDR obtained by using the Benjamini–Hochberg approach (g, h).
that regulate creatine abundance (CRT, GATM, and GAMT) by RNA sequencing. CRT was the most abundant, followed by GATM and then GAMT (Fig. 6a). Strikingly, CRT abundance was inversely and significantly correlated with the BMI of patients (Fig. 6b), thus suggesting a possible causal relationship between decreased creatine transport and human obesity. Furthermore, when patients were stratified into insulin-sensitive and insulin-resistant groups, CRT expression was significantly lower in insulin-resistant patients than insulin-sensitive patients, whereas GATM and GAMT expression was not different between these groups (Fig. 6c). Finally, the insulin-sensitive group could be stratified into those with relatively low or high CRT expression. The subgroup with low CRT expression had a significantly greater BMI than the subgroup with high CRT expression (Fig. 6c,d). Thus, the strong inverse correlation of CRT transcript levels with BMI and insulin resistance suggests that creatine transport-mediated energetics plays an important role in human adipocyte metabolism. Importantly, the insulin-sensitive subgroup data (Fig. 6d) indicate that CRT expression can be uncoupled from insulin resistance, but not BMI, thus suggesting that creatine energetics is primarily associated with obesity and that insulin resistance may arise consequently, secondary to obesity, in the population with low CRT expression.

Discussion
Several properties of creatine transport suggest that it functions as a mediator for diet-induced thermogenesis: (1) it is required to support full thermogenic activation on pharmacological adrenergic stimulation, (2) its loss substantially reduces activation of whole-body energy expenditure in response to caloric excess, (3) selective modulation of energy expenditure by creatine occurs primarily during high-fat feeding, and (4) loss of creatine transport increases metabolic efficiency without effecting food intake, thus leading to obesity. It is important to note that creatine-dependent thermogenesis does not require Ucp1 inactivation to reveal its physiological relevance, and UCP1 protein levels are even modestly elevated in BAT of AdCrTKO animals compared with CrT+/− littersmates.

While we detected a statistically significant decrease (80%) in BAT CrT mRNA levels at both 30 °C and 22 °C, CrT abundance was only significantly reduced (85%) in SQ from animals housed at 30 °C (Fig. 4b). Interestingly, conditions in which CL-dependent energy expenditure is lower in AdCrTKO mice compared with controls (Fig. 2b,d,i and Supplementary Fig. 2i) correspond well with conditions in which CrT abundance is significantly reduced in both BAT and SQ (Fig. 4a,b). These data support the idea that BAT and SQ may both contribute to creatine-dependent thermogenesis.

It is still debated what rodent housing temperature most precisely mimics human thermal conditions. The results presented herein were derived from experiments conducted at 30 °C and 22 °C, and so the drive for heat production to combat heat loss was either eliminated or mildly present, respectively. Importantly, irrespective of housing temperature, adipocyte transport is critical for...
mitigating fat mass gain, thus indicating that it may have important clinical relevance to human obesity.

Our and others’ prior work, using in vitro and chemical approaches, has suggested a critical role for adipose tissue creatine metabolism in enhancing energy expenditure through stimulation of ATP turnover. While the phenotype of the currently available tissue-specific genetically engineered mouse models (Adipo-Gatm knockout and AdCrTKO) substantiate the initial mechanistic interpretations in isolated mitochondria, it is currently unknown whether the thermogenic role for creatine in vivo is more complex than the proposed mechanism of mitochondrial ATP turnover. This is the focus of our current research efforts. Nevertheless, evidence in support of creatine playing a key role in adipocyte thermogenesis has accumulated in recent years from work independent from ours. Global CrT knockout mice, which exhibit similar levels of creatine depletion to those of Adipo-Gatm knockout and AdCrTKO animals, have greater body fat stores than controls, despite a decrease in food intake. Creatine kinase U-type (Ckmt1) and brain-type creatine kinase (Ckb) double-knockout animals are cold sensitive and have an impaired capacity to activate thermogenic respiration in response to norepinephrine administration, thus providing additional supporting evidence for a role of creatine metabolism in thermoregulation in vivo. Future work in this area will require the generation of new animal models with fat-selective creatine kinase deletion. These models will undoubtedly be useful to further delineate the molecular mechanism of creatine-dependent thermogenesis and its role in obesity and metabolic disease. The current findings, in conjunction with previous work from our group and others, place adipocyte creatine energetic as a central regulator of whole-body energy expenditure and obesity.

Methods

Animals. Mice were housed at 22°C under a 12-h light/dark cycle and given free access to food and water until 8 weeks of age. CrTlox/lox animals were obtained from the Jackson Laboratory (B6.SJL-S6Gatm1.1Clar). Adipo-Gatm mice (B6FVB-Tg(Adipog cre)-1Evr/J, stock 020642), Adiponecin-Cre mice (B6FVB-Tg(Adipog cre)-1Evr/J, stock 028020), and on a C57BL/6J background, were bred to Crtlox/lox animals to generate experimental groups. All experiments used age-matched male littermates and were conducted at least 30°C or 22°C. Animals were housed in groups of three unless otherwise stated. Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

High-fat feeding. AdCrTKO mice and age-matched littermate controls (CrT+/-) were genotyped at 2 weeks of age. At 3 weeks of age, mice were weaned by genotype into groups of three per cage, where they were housed at 22°C and fed a chow diet. Mice of different genotypes were housed in cages side by side to limit variability in exposure to differential temperatures within the housing facility. At 8 weeks of age, mice (housed three per cage) were either transferred to incubators set at 30°C or maintained in the housing room at 22°C. The high-fat diet (initiated at 8 weeks of age) was a rodent diet (OpenSource Diets, D12492) with 60% kcal fat, 20% kcal carbohydrate and 20% kcal protein. C57BL/6N mice (Jackson Laboratory; stock 003304) were used as wild-type mice for examining CrT protein levels after acute high-fat feeding.

Calorimetric measurements. Indirect calorimetry studies were conducted at the Brigham and Women’s Hospital Metabolic Core facility. Animals were housed individually in metabolic chambers maintained at 30°C or 22°C under a 12-h light/dark cycle and given free access to food and water. The mice were acclimated to metabolic cages for 24 h before measurements. Whole-body metabolic rate was measured by using an Oxymax open-circuit indirect calorimeter, Comprehensive Lab Animal Monitoring System (Columbus Instruments), available to the Brigham and Women’s Hospital Metabolic Phenotyping Core.

Diet-induced thermogenesis. Animals were housed individually in metabolic chambers maintained at 30°C or 22°C under a 12-h light/dark cycle and given free access to food and water. The resting metabolic rate at each ambient temperature (30°C or 22°C) was calculated from the mean of three lowest energy expenditure (kcal) readings. Change in energy expenditure (kcal) in response to high-fat feeding was plotted as the absolute increase in resting metabolic rate from the last day of Chow feeding.

Metabolic efficiency. Metabolic efficiency was measured as body mass accumulation as a function of cumulative energy intake.

Body composition. Body composition was examined with Echo MRI (Echo Medical Systems) by using a 3-12 Echo MRI Composition Analyzer.

Adrenergic activation of metabolic rate. Animals were housed individually in metabolic chambers maintained at 30°C or 22°C under a 12-h light/dark cycle and given free access to food and water. Mice were injected with CL 316,243 (dissolved in saline) intraperitoneally at 1 mg kg⁻¹ at 6:00 hours. Mice were subsequently placed back in their metabolic cages, and oxygen consumption was monitored.

Metabolic analyses of primary brown adipocytes by mass spectrometry. Creatine and phosphocreatine were profiled with General Metabolomics, LLC, and extracted at the General Metabolomics laboratories according to previously published methodology. Briefly, 2×10⁶ adipocytes were washed three times with fresh prewarmed (37°C) 75 mM ammonium carbonate, pH 7.4. Metabolites were extracted with 700 µl of pre-heated (75°C) extraction solvent (70% (v/v) ethanol (absolute) in HPLC-grade water). Extraction solvent was kept on cells for 3 min. Samples were rapidly transferred to a dry ice/ethanol bath. Samples were centrifuged 16,000 g for 10 min at 4°C. An equal volume of extract supernatant was recovered and stored at −80°C until metabolite analysis. Analysis was performed on a platform consisting of an Agilent Series 1100 LC pump coupled to a Gerstel MPS2 autosampler and an Agilent 6520 Series Quadrupole time-of-flight mass spectrometer equipped with an electrospray source operating in positive mode. For online mass axis correction, 2-propanol (in the mobile phase), taurocholic acid and hexakis(1H,1H,3H-tetrafluoropropoxy)phosphaznine (HP-9921, Agilent Technologies) for negative mode and creatine, spermine and spermidine (in mobile phase), respectively, were added to the mobile phase. Mass spectra were recorded in profile mode from m/z 50 to 1,000, with a frequency of 1.4 spectra per second for 0.48 min, by using the highest resolving power (4 GHz). The source temperature was set to 25°C with 5 min¹⁻ dry gas and a nebulizer pressure of 30 psig. Fragmentor, skimmer and octopole voltages were set to 175, 65 and 750 V, respectively.

Metabolic analyses of BAT by mass spectrometry. Creatine was profiled at the Rosalind and Morris Goodman Cancer Research Centre Metabolomics Core Facility. Briefly, BAT (10 mg) was combined with 1.14 ml of methanol/HPLC-grade water (30:50 (v/v)), 0.66 ml of ice-cold acetoneitrile (ACN) (at ~20°C or colder) and four ceramic beads (2.8-mm diameter). The mixture was homogenized in a bead beater (Qiagen TissueLyser) for 2 min at 30Hz. Next, samples were transferred into a 5-ml tube containing 1.8 ml of ice-cold dichloromethane and 0.9 ml of ice-cold HPLC-grade water. The samples were vortexed for 1 min, incubated on ice for 10 min and centrifuged at 4,000 rpm, for 10 min at 1°C. Water-soluble metabolites in the upper polar phase were collected and dried by using a chilled ASciPrepVac set at 4°C. Samples were resuspended in 50 µl of HPLC-grade water before LC–MS analysis. For targeted metabolite analysis and semi-quantitative concentration determination of creatine, samples were injected onto an Agilent 6430 Triple Quadrupole (QQQ)–LC–MS/MS. Chromatography was achieved by using a 1290 Infinity ultra-performance LC system (Agilent Technologies) consisting of vacuum degasser, autosampler and a binary pump. The mass spectrometer was equipped with an electrospray ionization source, and samples were analysed in positive mode. Multiple reaction monitoring was optimized on authentic metabolite standards. The quantifying and qualifying ion transitions for creatine were 132.1 → 90.1 and 132.1 → 44.2, respectively. The source-gas temperature and flow were set at 350°C and 101 min⁻¹, respectively, the nebulizer pressure was set at 40 psi, and capillary voltage was set at 3,500 V. Chromatographic separation of creatine was achieved by using an Intrada Amino Acid column, 3 µm, 3.0×150 mm² (Intakt Corp.). The chromatographic gradient started at 100% mobile phase B (0.3% formic acid in ACN) with a 3-min gradient to 27% mobile phase A (100 mM ammonium formate in 20% ACN/80% water) followed with a 19.5-min gradient to 100% A at a flow rate of 0.6 ml min⁻¹. This was followed by a 5.5-min hold time at 100% mobile phase A and a subsequent re-equilibration time (7 min) before the next injection. For all LC–MS analyses, 5 µl of sample was injected. The column temperature was maintained at 10°C. Creatine eluted at 7.9 min. Relative concentrations were determined from external calibration curves. Data were analysed by using MassHunter Quant (Agilent Technologies). No additional corrections were made for ion suppression; thus, concentrations are relative, not absolute.

NMR spectroscopy. Brown fat tissue samples (~20 mg) were homogenized and extracted with a mixture of ice-cold solvents water/methanol/chloroform (H₂O/MeOH/CHCl₃, 1:1:1 (v/v/v)). The water/methanol phase was separated and dried in a 5-ml vial. The extract was resuspended in 550 µl of phosphate buffer, pH 7.4, and 50 µl of 1 mM of trimethylsilyl propanoic acid (TSP-d₃) in D₂O, vortexed for 20s and transferred to 5-mm NMR tubes. The
NMR spectra were acquired on a Bruker 600-MHz Avance III HD spectrometer equipped with a BBI room temperature probehead and SampleJet autosampler (Bruker Biospin). 1H NMR spectra were recorded by using a one-dimensional nuclear Overhauser enhancement spectroscopy pulse sequence with 90° pulse excitation (noesygppr1d), collecting 256 scans with calibrated 90° pulse (~11 μs). 4.5 s acquisition time and 4-s relaxation delay. Metabolites were identified and quantified by using Chenomx NMR suite 8.2 software, by fitting the spectral lines of library compounds into the recorded NMR spectrum of tissue extract. The quantification was based on the basis of peak area of TSP-δ signal. The metabolite concentrations are exported as micromolar values in NMR samples and normalized to wet tissue mass (nmol per mg of tissue).

Body temperature. A mouse rectal probe (World Precision Instruments) was used to examine body temperature. Mice were group-housed (three per cage) at 30°C for 5 d. Core temperature was measured in individually housed mice at 4°C after acclimation at 30°C for 5 d.

Triglyceride quantification. Triglyceride content in BAT and SQ tissues was measured by using a High Sensitive TG Assay Kit (Sigma-Aldrich, MAK264). Five standard solutions were prepared at 40, 80, 120, 160 and 200 pmol per well in 50μl of triglyceride buffer. BAT and SQ tissues (10 mg) were weighed and homogenized in 100 μl of cold triglyceride buffer. To ensure that the lipid layer was not separated from the aqueous phase, samples were boiled for 30–45 s. Insoluble materials were removed by centrifuging the samples at 12,000g for 5 min. Supernatant, including the entire fat layer, was transferred into clean tubes. Each sample was then diluted 5,000× with cold triglyceride buffer and transferred to a 96-well plate at a final volume of 50 μl. Lipase (2 μl) was added to each sample, which was subsequently incubated at 37°C for 20 min. After hydrolysis of triglycerides to glycerol and fatty acids, 50 μl of the master reaction mix was added to the wells, and the samples were incubated at 37°C for 30 min. Fluorescence intensity was measured with excitation λ535/λem590. The standard curve concentration is exported as micromolar values in NMR samples and normalized to wet tissue mass (nmol per mg of tissue).

Stromal-vascular-fraction isolation of mouse brown preadipocytes. Interscapular BAT stromal vascular fraction was obtained from 3-week-old animals after euthanasia. The protocol for the XFe24 was set to analyse cellular respirometry by using a mix:waıtmeasure ratio of 4.2:2.2. Norepinephrine (100 nM) was used to stimulate thermogenic respiration. A mix of rotenone and antimycin A (3 μM each) was used to inhibit mitochondrial respiration.

Collection of human adipose tissue samples. Subcutaneous adipose tissue was collected under IRB 2013000079 (approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigations) from subjects recruited from the plastic-surgeon operating-room schedule at Beth Israel Deaconess Medical Center in a consecutive fashion, as scheduling permitted, to process the sample. The inclusion criteria were healthy male and female subjects, ages 18–64 receiving abdominal surgery. The exclusion criteria were diagnosis of diabetes, any subjects taking insulin-sensitizing medications such as thiazolidinediones or metformin, chromatin-modifying enzymes such as valproic acid, and drugs known to induce insulin resistance such as TO-MOR inhibitors (for example, sirolimus or tacrolimus) or systemic steroid medications. Fasting serum was collected and tested for insulin, glucose, free fatty acids, and a lipid-panel was performed in a Clinical Laboratory Improvement Amendments-approved laboratory. BMI measures were derived from electronic medical records and confirmed by self-reporting, and measures of insulin resistance, the homeostasis model assessment-estimated insulin resistance index (HOMA-IR) and revised quantitative insulin sensitivity check index (QUICKI) were calculated39–41. Female subjects in the first and fourth quartiles for either HOMA-IR or QUICKI and matched for age and BMI were processed for RNA-seq.

RNA isolation from mature human adipocytes. Total RNA from ~400 µl of thawed adipocytes was isolated in TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RNA-seq library construction, mRNA was purified from 100 ng of total RNA by using a Ribo-Zero rRNA removal kit (Epicentre) to deplete ribosomal RNA and convert into double-stranded complementary DNA by using an NEBNext mRNA Second Strand Synthesis Module (E6111L). cDNA was subsequently tagmented and amplified for 12 cycles by using a Nextera XT DNA Library Preparation Kit (Illumina FC-131). Sequencing libraries were analysed by Qubit and Agilent Bioanalyzer, pooled at a final loading concentration of 1.8 pM and sequenced on a NextSeq500. Sequencing reads were demultiplexed by using bc12fastq and aligned to the mm10 mouse genome by using HISAT2 (ref. 39). PCR duplicates and low-quality reads were removed by Picard (https://broadinstitute.org/picard). Final reads were assigned to the annotated transcriptome and quantified by using featureCounts50.

Cellular respirometry. Primary brown preadipocytes were plated at 15,000 cells per well of a XF24 V7 cell culture microplate, and differentiation was induced 12 h later. On day 10 post-differentiation, adipocytes were washed once and maintained for ~45–50% DME supplemented with 1 mM pyruvate. The protocol for the XF24 was set to analyse cellular respirometry by using a mix:waıtmeasure ratio of 4.2:2.2. Norepinephrine (100 nM) was used to stimulate thermogenic respiration. A mix of rotenone and antimycin A (3 μM each) was used to inhibit mitochondrial respiration.

Purification of mature mouse brown adipocytes. Interscapular BAT was digested in isolation buffer HBSS (Sigma), 2 mg/ml collagenase B ( Worthington), 1 mg/ml soybean trypsin inhibitor (Worthington) and 4% BSA with continuous shaking at 37°C for 45 min. The tissue suspension was filtered through a 100-μm cell strainer and spun at 30 g for 5 min at room temperature. The infranatant was removed, and adipocytes were washed with 20 ml ice-cold PBS and centrifuged again. Infranatant was removed, and cells were snap-frozen in liquid nitrogen until downstream analysis.
Basic pH reversed-phase separation. TMT-labelled peptides were solubilized in 50 mM soluble in ACN/CH3CN (1:1) at pH 8.0 and separated by an Agilent 300 Extend C18 column (5-μm particle diameter and 220 mm in length). An Agilent 1100 binary pump coupled with a photodiode array detector (ThermoScientific) was used to separate the peptides. A 45-min linear gradient from 18–45% acetonitrile in 10 mM ammonium bicarbonate pH 8.0 (flow rate of 0.8 ml min⁻¹) eluted the peptide mixtures into a total of 96 fractions (36 samples). A total of 96 fractions were consolidated into 24 samples in a chequerboard fashion, acidified with 20 μl of 10 mM formic acid and vacuum dried to completion. Each sample was re-dissolved in 12 µl 5% formic acid/5% ACN, and desalted before LC–MS/MS analysis.

Data processing and spectra assignement. A comprehensive in-house developed software was used to convert mass spectrometric data (Raw file) to the mzXML format, as well as to correct monoisotopic m/z measurements and erroneous assignments of peptide charge state27. All experiments used the Mouse UniProt database (downloaded on 28 April 2017) where cleaved protein sequences and known contaminants such as human keratins were appended. SEQUEST searches were performed by using a 20-p.p.m. precursor-ion tolerance, while requiring each peptide's amino/carboxy (N/C) terminus to have trypsin protease specificity and allowing up to two missed cleavages. 10-plex TMT tags on peptide N termini and lysine residues (+229.162932 Da) and carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications, while methionine oxidation (+15.99492 Da) was set as variable modification. A MS/MS spectra assignment FDR of less than 1% was achieved by applying the target-decoy database search strategy28. Filtering was performed by using an in-house linear discrimination analysis method to create one combined filter parameter from the following peptide-level and MS/MS spectra metrics: SEQUEST parameters XCORr, ΔCN, peptide-ion mass accuracy and charge state, in-solution charge of peptide, peptide length and miscleavages. Linear discrimination scores were used to assign probabilities to each MS/MS spectrum for being assigned correctly and these probabilities were further used to filter the dataset with a MS/MS spectra assignment FDR of smaller than a 1% at the protein level.

Determination of TMT reporter-ion intensities and quantitative data analysis. For quantification, a 0.03 m/z window centred on the theoretical m/z value of each of the ten reporter ions and the intensity of the signal closest to the theoretical m/z value was recorded. Reporter-ion intensities were further de-normalized on the basis of their ion accumulation time for each MS/MS spectrum or for three MS/MS spectra and adjusted on the basis of the overlap of isotopic envelopes of all reporter ions (as determined by the manufacturer). The total signal intensity across all peptides quantified was summed for each TMT channel, and all intensity values were adjusted to account for potentially uneven TMT labelling and/or sample handling variance.

Differential protein expression. We determined proteins exhibiting differential expression between C57Bl/6 and AdCrTKO BAT at 30 °C (n = 10) and 22 °C (n = 10) conditions. Expression measurements were performed across all samples in TMT 5,508. The principal component analysis plot was produced in R using ggplot2 (v3.0.0) and ggfortify (v0.4.5) package.
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Author contributions

L.K. conceptualized the study, designed research, performed biochemical, cellular and in vivo research experiments, analysed data and wrote the paper. J.F.R., B.S., G.Z.L. and F.Y.D. performed in vivo experiments. M.L. performed proteomics experiments. M.L., J.C.R. and I.R.W. analysed proteomics data. S.Z. performed and analysed NMR experiments. E.T.C., P.D. and E.D.R. provided resources. L.T. recruited human subjects and isolated adipocytes, and D.T. performed RNA-seq experiments. L.K. and B.M.S. co-wrote the paper, with assistance from co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
  - A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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  - A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
  - For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
    - Give P values as exact values whenever suitable.
  - For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
  - For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
  - Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | NMR: Chenomx NMR suite 8.2  
QPCR: CFX Maestro 2017  
Seahorse: Wave 2.4 |
|-----------------|----------------------------------|
| Data analysis   | Peptide mass spectra were processed with an house SEQUEST-based software pipeline (Huttlin, E.L., et all. Cell. 143, 1174–1189 (2010). Details available upon reasonable request  
GraphPad Prism, 7  
Microsoft office Excel 2016  
limma (v3.32.10) package in R |

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The data that support the findings of this study are available from the corresponding author upon request.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample sizes were predetermined based on effect size, standard deviation, and significance level required to attain statistical significance of \( p<0.05 \) with a 90% probability on the basis of previous experiments using similar methodologies and were deemed sufficient to account for any biological/technical variability.

Data exclusions

No data were excluded.

Replication

All attempts of in vivo replication were successful. Proteomics was performed with five independent samples at two environmental temperatures. Only replicated proteomic data are discussed, effectively increasing independent sample number to 10 per genotype. Proteomic data were further followed up by alternative approaches, such as RT-qPCR.

Randomization

For in vivo studies, mice in each genotype were randomly assigned to treatment groups. For MS analyses, samples were processed in random order and experimenters were blinded to experimental conditions.

Blinding

For MS analyses, samples were processed in random order and experimenters were blinded to experimental conditions.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Antibodies            |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology         |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |
| ☒   | Clinical data         |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq              |
| ☒   | Flow cytometry        |
| ☒   | MRI-based neuroimaging |

Eukaryotic cell lines

| Policy information about | cell lines |
|--------------------------|------------|
| Cell line source(s)      | Primary brown adipocytes from CrT(lox/y) mice (strain: C57BL6/J, Stock#: 000664). Mice were bred and cells were isolated in-house. |
| Authentication            | N/A        |
| Mycoplasma contamination  | N/A        |
| Commonly misidentified lines | No commonly misidentified cell lines were used |
| (See ICLAC register)      |            |

Animals and other organisms

| Policy information about | studies involving animals. ARRIVE guidelines | recommended for reporting animal research |
|--------------------------|------------------------------------------------|-----------------------------------------|
| Laboratory animals       | Mice were housed at 22°C under a 12 hr light/dark cycle with free access to food and water until 8 weeks of age. CrT(lox/y) animals were obtained from the Jackson Laboratory [B6(SIL)-Sic8a8tm1.1Clar/J], Stock No: 020642]. Adiponectin-Cre mice (B6:FVB-Tg(Adipoq-cre)1Evdr/J, Stock No: 010803), maintained on a C57BL/6J background, were bred to CrT(lox/y) animals to generate experimental groups. All experiments used age-matched male littermates and were conducted at either 30°C or 22°C. Animal experiments were performed according to procedures approved by the Animal Resource Centre at McGill University and |
Comply with guidelines set by the Canadian Council of Animal Care, and experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the Beth Israel Deaconess Medical Center or the Harvard Center for Comparative Medicine. Unless otherwise stated, mice used were age-matched littermates (8–12 weeks of age), and housed in at (22 °C or 30 °C) on a 12 h light/dark cycle. AdCrTKO (CrT[lox/y]; AdipoQ-Cre) and age-matched littermates (CrT[lox/y]) were used. Brown adipocytes were cultured from male and female pups after genotyping (14 days old).

| Wild animals | N/A |
| Field-collected samples | N/A |
| Ethics oversight | Animal experiments were performed according to procedures approved by the Animal Resource Centre at McGill University and comply with guidelines set by the Canadian Council of Animal Care. Experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the Beth Israel Deaconess Medical Center. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Human research participants**

Policy information about studies involving human research participants

**Population characteristics**

Inclusion criteria included healthy male and female subjects, ages 18-64 receiving abdominal surgery. Exclusion criteria were diagnosis of diabetes, any subjects taking insulin-sensitizing medications like thiazolidinediones or metformin, chromatin modifying enzymes such as valproic acid, and drugs known to induce insulin resistance such as mTOR inhibitors (e.g. sirolimus, tacrolimus) or systemic steroid medications.

| Recruitment | Subjects recruited from plastic surgeon operating room schedule at Beth Israel Deaconess Medical Center in consecutive fashion as scheduling permitted to process the sample. |
| Ethics oversight | Subcutaneous adipose tissue was collected under IRB 2011P000079, and approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigations |

Note that full information on the approval of the study protocol must also be provided in the manuscript.