Enhanced β-adrenergic signalling underlies an age-dependent beneficial metabolic effect of PI3K p110α inactivation in adipose tissue

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The insulin/IGF-1 signalling pathway is a key regulator of metabolism and the rate of ageing. We previously documented that systemic inactivation of phosphoinositide 3-kinase (PI3K) p110α, the principal PI3K isoform that positively regulates insulin signalling, results in a beneficial metabolic effect in aged mice. Here we demonstrate that deletion of p110α specifically in the adipose tissue leads to less fat accumulation over a significant part of adult life and allows the maintenance of normal glucose tolerance despite insulin resistance. This effect of p110α inactivation is due to a potentiating effect on β-adrenergic signalling, which leads to increased catecholamine-induced energy expenditure in the adipose tissue. Our findings provide a paradigm of how partial inactivation of an essential component of the insulin signalling pathway can have an overall beneficial metabolic effect and suggest that PI3K inhibition could potentiate the effect of β-adrenergic agonists in the treatment of obesity and its associated comorbidities.
Interventions to increase the lifespan and healthspan of organisms have been the subject of intense research efforts. Loss-of-function mutations in components of the insulin/IGF-1 signalling (IIS) pathway have long been known to have a life- and healthspan extending effect in diverse model organisms. The fact that inactivating mutations in a pathway essential for regulation of metabolic physiology extend lifespan is seemingly paradoxical and has been referred to as the insulin paradox. Although evolutionary theories have been evoked to explain the phenomenon of lifespan extension through loss-of-function mutations in essential genetic pathways such as the IIS, mechanistic insights as to how this is achieved at the molecular level have only recently started to emerge.

A number of genetic and other interventions that extend lifespan and metabolism have also beneficial effects on metabolic physiology, highlighting the interconnectedness of metabolism and ageing. Prime examples of gene mutations that extend lifespan and improve glucose and lipid homoeostasis in old mice are mutations in the growth hormone/IGF-1 signalling axis in hypopituitary dwarf long-lived mutant mice. Furthermore, calorie restriction, the best known intervention to extend lifespan in a number of species, has also profound beneficial metabolic effects, manifested as improved insulin sensitivity and glucose homoeostasis in calorie restricted mice.

In the context of interventions shown to affect both lifespan and metabolism, the adipose tissue has a prominent role. The adipose tissue is an important organ in the regulation of energy homoeostasis. Moreover, the adipose tissue has been shown to play an important role in the lifespan-extending effects of insulin pathway downregulation. A seminal finding in this regard has been the demonstration that adipose tissue-specific insulin receptor knockout mice (FIRKO) display extended longevity and they are also protected against age-related metabolic pathologies.

We and others have documented that p110α is the key PI3K isoform in the insulin signalling pathway. We recently reported that global partial inactivation of p110α has an age-dependent beneficial effect on metabolism and causes a modest increase in lifespan. To gain further insights into the mechanism underlying these effects, we assessed the role of the adipose tissue in the beneficial metabolic impact seen upon global p110α inactivation. To this end, we inactivated p110α specifically in the mouse adipose tissue by Cre/loxP approaches and characterised the metabolic profiles of these mice over the course of ageing, which is one of the most physiologically relevant stresses an experimental model can be subjected to. We report here that partial inactivation of p110α potentiates β-adrenergic signalling in the adipose tissue. This results in enhanced catecholamine-induced energy expenditure (EE), which counteracts the effects of insulin resistance thus preserving normal glucose homoeostasis over the course of ageing. These findings have clear implications for the development of therapeutic interventions for age-related obesity and associated comorbidities.

Results

p110α deletion in adipose tissue causes insulin resistance. We applied Cre/loxP-mediated conditional gene deletion to inactivate p110α in the adipose tissue of mice by crossing conditionally targeted p110α mice (p110αFLOX) with a transgenic line expressing Cre under control of the adiponectin promoter (Adipoq–Cre)14, which is more specific for deletion in the adipose tissue than the earlier reported aP2–Cre line15. We isolated metabolic tissues from mice expressing the Adipoq–Cre transgene (p110αDEL) or not (p110αFLOX), and assessed p110α protein expression by immunoblot analysis. Significant reduction of p110α protein expression was only seen in the white and brown adipose tissues (WAT and BAT) of p110αDEL mice, with a reduction of an average of 70% (WAT) and 90% (BAT), compared to the expression levels in p110αFLOX littermates (Fig. 1a, b and Supplementary Fig. 1). The expression of p110β, the other broadly expressed PI3K isoform, as well as that of the p85 regulatory subunit was not affected under these conditions in p110αDEL mice.

We next tested insulin sensitivity in metabolic tissues of approximately 1-year-old mice by injecting insulin and assessing PI3K activity and Akt phosphorylation as readouts of insulin pathway activation. Consistent with the previously demonstrated role of p110α in insulin receptor signalling, the PI3K activity associated with IRS-1 immunoprecipitates was significantly reduced in visceral (epididymal) WAT (eWAT) of p110αDEL mice (Fig. 1c). In line with this, insulin-induced Akt phosphorylation was also significantly impaired in eWAT, but only marginally affected in iWAT and BAT isolated from p110αDEL mice (Fig. 1d). Akt phosphorylation was impaired to a lower extent also in the liver and skeletal muscle of p110αDEL mice (Supplementary Fig. 2), indicating the development of systemic insulin resistance. This was further corroborated by hyperinsulinemic–euglycemic clamp experiments in 20-week-old mice that revealed profound systemic insulin resistance at this age point, as evidenced by an impaired rate of glucose disappearance, increased hepatic glucose production and elevated free fatty acids in the plasma in p110αDEL mice (Fig. 1e) under clamp conditions. Notably, the rate of glucose disappearance at basal level was slightly elevated in p110αDEL mice, which suggests that p110αDEL mice may have a higher basal metabolic rate under clamp conditions.

p110αDEL mice maintain normal glucose tolerance over ageing. We assessed the metabolic profile of p110αDEL mice by performing glucose tolerance and insulin tolerance tests over a number of time points ranging from 3 to 24 months of age. In line with the impaired insulin signalling in metabolic tissues and hyperinsulinemic–euglycemic clamp studies (Fig. 1d, e and Supplementary Fig. 2), p110αDEL mice displayed systemic insulin resistance at all of the time points tested (Fig. 2a and Supplementary Fig. 3). However, glucose tolerance was essentially unaffected throughout the life course. The lack of any effect seen in mice expressing only Adipoq–Cre, and lacking targeted p110α alleles, under the same experimental conditions, attests that these phenotypes can confidently be attributed to the deletion of p110α alleles (Supplementary Fig. 4). A number of hormones and cytokines and lipid profiles tested in the plasma of fasted one year old mice did not show any prominent differences, with the exception of substantially reduced insulin and leptin and slightly reduced adiponectin, and increased total cholesterol in the plasma of male p110αDEL mice (Fig. 2b–d). Notably, β-AR agonists have previously been reported to reduce production and secretion of adiponectin from the adipose tissue. As both leptin and adiponectin are known to influence insulin sensitivity of metabolic tissues, reduced levels of leptin and adiponectin might play a role in the development of insulin resistance in liver and skeletal muscle, as seen in p110αDEL mice (Supplementary Fig. 2). Also, the differential impact of p110α inactivation on fasting insulin levels between male and female mice (Fig. 2b) suggests a sexually dimorphic phenotypic effect. Sex differences in the function of the adipose tissue are well-known in both mice and humans. Such effects could account for a differential impact of p110α inactivation on insulin sensitivity and thus on fasting insulin levels between two sexes at the age point of this measurement. ITTs were also in line with female p110αDEL mice being substantially more insulin resistant than their control littermates, in
contrast to male mice, at this age point of 12 months (Supplementary Fig. 3).

When mice were subjected to high-fat feeding (45% calories derived from fat), weight gain was identical between the two genotypes (Fig. 3a). However, high-fat fed p110αDEL mice were significantly more glucose tolerant despite being as insulin sensitive as p110αFLOX littermates (Fig. 3b, c). To gain further insight into this unexpected effect of p110α inactivation, mice were subjected to a tissue-specific glucose uptake assay over a glucose tolerance test. We found that the BAT of p110αDEL mice cleared approximately twice as much glucose from the circulation as p110αFLOX littermates (Fig. 3d). No significant differences were detected in other organs, with the exception of visceral WAT, which in p110αDEL mice cleared half as much glucose as control littermates. High-fat feeding is known to activate BAT and increase expression of UCP1, consistent with higher BAT activity can counter the effects of insulin resistance on glucose homoeostasis of p110αDEL mice.

p110α inactivation potentiates β-adrenergic/cAMP signalling. Since it is well-established that catecholamine stimulation of adrenergic signalling regulates glucose and lipid metabolism in BAT, the enhanced glucose clearance in the face of insulin...
Fig. 2 Insulin resistant p110αDEL mice maintain normal glucose tolerance over ageing. a Cohorts of p110αDEL and p110αFLOX male mice were subjected to intraperitoneal glucose and insulin tolerance tests at various time points over ageing. For glucose tolerance test a bolus of glucose (2 g per kg of body weight) was injected intraperitoneally. Insulin doses were adjusted according to the age of the mice (0.75 U kg−1 for 3-month-old and 2 U kg−1 for 18- and 24-month-old). n = 8 per genotype at 3 months, 16 per genotype at 18 months, 14 per genotype at 24 months. AUC, area under curve. Additional age points and data for females are shown in Supplementary Fig. 3. b Fasting plasma levels of metabolic hormones in 1-year-old p110αDEL and p110αFLOX mice. n (p110αFLOX/p110αDEL), Insulin: Female, 9/8; Male, 4/5. Leptin: Female, 8/8; Male, 7/10. Adiponectin: Female, 8/8; Male, 10/10. c Fasting plasma levels of pro-inflammatory cytokines (IL6 and TNFα) in 1-year-old p110αDEL and p110αFLOX mice. n (p110αFLOX/p110αDEL), IL-6: Female, 6/7; Male, 6/9. TNFα: Female, 8/8; Male, 7/7. d Fasting plasma levels of lipids (total cholesterol, free fatty acids and triglycerides) in 1-year-old p110αDEL and p110αFLOX mice. n (p110αFLOX/p110αDEL), Cholesterol: Female, 8/7; Male, 3/4. FFAs: Female, 10/9; Male, 4/5. Triglycerides: Female, 8/9; Male, 10/9. n (p110αFLOX/ p110αDEL), Cholesterol: Female, 8/7; Male, 3/4. FFAs: Female, 10/9; Male, 4/5. Triglycerides: Female, 8/9; Male, 10/9. Data are presented as mean ± SEM. Statistical analyses: Unpaired two-tailed t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001
resistance prompted us to investigate the impact of p110α inactivation on the activity of adrenergic pathways in response to catecholamine stimulation. Furthermore, adrenergic pathways control fat metabolism by modulating lipolysis in adipose tissues. β-Adrenergic receptors (β-ARs) activate adenylyl cyclase through coupling to Gs subunits of heterotrimeric G proteins, resulting in the elevation of intracellular levels of cAMP and activation of its effector molecules, notably of protein kinase A (PKA).

Explants from subcutaneous (inguinal) and visceral adipose tissue were stimulated with the synthetic β-AR agonist isoproterenol or the natural catecholamine norepinephrine. As readouts of β-AR/cAMP pathway activation, we assessed phosphorylation of hormone sensitive lipase (HSL), perilipin and cAMP response element binding (CREB) protein, all of which are targets of PKA in the adrenergic pathway in adipocytes. Tissue explants isolated from subcutaneous adipose tissue of p110αDEL mice, as well as those isolated from p110αFLOX mice and treated with the p110α selective inhibitor A66, displayed enhanced sensitivity to β-AR stimulation by both isoproterenol (Fig. 4a, b) and norepinephrine (Supplementary Fig. 6a, b). However, a similar effect was not the case in explants isolated from visceral adipose tissue.

Consistent with the observed increase in the phosphorylation of lipolysis regulatory molecules upon p110α inactivation, release of glycerol, which provides an index of the rate of lipolysis (Fig. 4c and Supplementary Fig. 6c), and intracellular cAMP levels (Fig. 4d and Supplementary Fig. 6d) were found to be increased in the subcutaneous adipose tissue of both p110αDEL mice and that of p110αFLOX mice treated with A66.
Since differences in the expression of various adrenergic receptor classes and subtypes have been reported between mouse and human adipose tissue, we tested whether inhibition of p110α has a similar potentiating effect on adipocytes differentiated from human multipotent adipose-derived stem (hMADS) cells. As with mouse adipocytes, treatment of hMADS cell-derived adipocytes with A66 resulted in potentiation of signalling induced by a number of β-AR agonists (Fig. 4e).

Mechanism of adrenergic signalling enhancement. We sought to gain further insights in the molecular mechanism underlying...
the potentiation of adrenergic signalling by p110α inhibition. To this end, we probed the levels of β-AR subtype expression in adipose tissues of p110αFLOX and p110αDEL mice. We found no significant differences in the expression of the various β-AR subtypes among the different depots tested (Supplementary Fig. 7). At the level of PI3K isoform expression, we observed similar levels of expression of p110α between iWAT and vWAT, but a tendency for higher level of p110β in iWAT compared to vWAT (Supplementary Fig. 7). This is consistent with the milder effect of p110α deletion on insulin sensitivity in iWAT compared to vWAT (Fig. 1), as higher levels of p110β could partially compensate for p110α deficiency in iWAT.

Insulin exerts its anti-lipolytic action mainly, though not exclusively, through PI3K/Akt-mediated phosphorylation and activation of the cAMP degrading enzyme phosphodiesterase 3B (PDE3B)25,26. We tested whether β-adrenergic stimulation can also activate Akt phosphorylation in iWAT and vWAT. We found that isoproterenol stimulation induced a substantial increase in Akt phosphorylation in both iWAT and vWAT explants (Fig. 5a). We also tested immortalised brown adipocytes and BAT explants derived from p110αFLOX mice. Similar to iWAT, inhibition of p110α in brown adipocytes (Fig. 5b) and in BAT explants (Fig. 5c) potentiated NE-stimulated (immortalised brown adipocytes) or ISO-stimulated (BAT explants) adrenergic signalling, as assessed by phosphorylation of HSL and perilipin. p110α inhibition also potentiated phosphorylation of p38 MAPK, which has previously been shown to be an important mediator of cAMP-dependent induction of UCP1 expression in brown adipocytes27. Similar to WAT, NE-stimulated Akt phosphorylation in brown adipocytes in a PI3K dependent manner (Fig. 5d). Notably, the effect of p110α inhibitor A66 on Akt phosphorylation in brown adipocytes was dependent on the level of phosphorylated PI3K (Fig. 5d). Inactivation of p110α potentiates adrenergic signalling in vivo and increases recruitment of beige adipocytes upon adrenergic stimulation (Fig. 5f). Consistent with this, inhibition of p110α with A66 fails to increase adrenergic signalling above the level attained through treatment with the general phosphodiesterase inhibitor isobutyl-methylxanthine (Supplementary Fig. 9).

Increased NE-stimulated EE in p110αDEL mice. Adrenergic stimulation of adipose tissue results in the recruitment of brown-like (beige or brite) adipocytes in WAT31. In order to further investigate the effect of p110α inhibition in the adipose tissue in vivo, we administered CL316,243, a β3-AR-specific agonist, to 18-week-old mice daily for a period of 5 days. As shown in Fig. 6a, administration of CL316,243 increased the mRNA expression of Ucp1 and other thermogenic genes, markers of brown and beige adipocytes, specifically in the inguinal WAT of p110αFLOX and p110αDEL mice. Increased expression of Ucp1 mRNA was accompanied by increased protein levels as determined by immunoblot analysis (Fig. 6b, c and Supplementary Fig. 10) and immunohistochemistry (Fig. 6d). In line with the above documented increase in adrenergic signalling in the subcutaneous WAT of p110αDEL mice, a higher expression of all these markers was observed in the inguinal WAT of p110αDEL compared to p110αFLOX mice. This suggests that inactivation of p110α potentiates adrenergic signalling in vivo and increases recruitment of beige adipocytes upon adrenergic stimulation of subcutaneous adipose tissue.

To complete the physiological phenotypic analysis of p110αDEL mice, we performed indirect calorimetry to determine EE under free-living conditions, as well as in response to catecholamine stimulation. As shown in Fig. 6e, f, EE and respiratory exchange ratio were similar between approximately 1-year-old p110αFLOX and p110αDEL mice under free-living conditions. These experiments were performed in weight-matched groups of mice to avoid the confounding effects of different metabolic mass. No differences in water and food consumption or in loss of body weight over the time course of the experiment were observed between the genotypes (Fig. 6g). However, injection of norepinephrine into conscious mice measured at thermoneutrality (30°C) showed a significantly increased EE in p110αDEL mice compared to p110αFLOX littermates (Fig. 6h, i). p110αDEL mice also tended to display a slightly higher basal EE, which was consistent with their higher basal glucose turnover during the
clamp studies (Fig. 1f), but this did not reach statistical significance. The weights of all the mice were very similar (Fig. 6j), whereas basal EE was 10% larger in p110αDEL mice, suggesting that changes in EE were not simply a product of changes in body weight and therefore metabolic mass. These findings are consistent with the enhanced β-adrenergic signalling in isolated adipose tissue explants from p110αDEL mice (Fig. 4 and Supplementary Fig. 6).
Reduced age-dependent fat accumulation in p110αDEL mice. We generated cohorts of p110αFLOX and p110αDEL littermates and monitored their weight gain over time. As shown in Fig. 7a, b, p110αDEL mice were born with a body weight similar to that of p110αFLOX littermates. However, their growth curves started to diverge at around 8 months of age and p110αDEL mice did not accumulate as much weight as p110αFLOX littermates with age. More specifically, the average weight of p110αDEL mice for the period between 8–26 months of age was 7% lower for male and female mice, respectively, compared to their p110αFLOX littersmates. Gonadal WAT and interscapular BAT weights were also reduced in p110αDEL mice at 1.5 years of age, whereas BAT masses normalised to body weight were similar in 2-year-old mice, indicating a slower degeneration rate in BAT of p110αDEL mice (Fig. 7c). Assessment of food intake in 1-year-old mice showed no significant differences in food intake between p110αDEL and p110αFLOX littermates (Fig. 7d, e), except for the tendency of p110αDEL male mice to consume slightly more food (Fig. 7e), demonstrating that the weight difference between the two genotypes was not due to different levels of food consumption. Therefore, the increased catecholamine-stimulated EE documented in our physiological studies described above could be reducing fat accumulation over the life course in p110αDEL mice.

Discussion
Metabolism is an essential process for all organisms and, like many other biological processes, is adversely affected by ageing. However, this relationship is also valid in the inverse direction in that metabolic deterioration accelerates the rate of ageing in a vicious cycle. In fact, glucose and lipid homeostasis are emerging as key determinants of the rate of ageing in a diverse range of animal organisms. Consistent with this, genetic and pharmacological interventions affecting signalling pathways that regulate metabolism, notably the IIS, mTOR and AMPK pathways, have been shown to affect the lifespan of various organisms. The fact that downregulation of essential signalling pathways, such as IIS and mTOR, exerts beneficial effects is seemingly paradoxical. In the context of metabolism, the gross phenotypic effects of inactivation of these pathways are in many cases initially adverse, consistent with the importance of these pathways, and beneficial effects emerge only later in life. It is therefore essential that longitudinal studies are conducted in model organisms in order to assess the long-term effects of such interventions and to decipher the molecular mechanisms underlying these beneficial effects. These insights will help to reconcile the paradox of beneficial effects ensuing from the downregulation of essential metabolic pathways.

Here, we report the findings of a longitudinal metabolic study in mice that characterised the effects of adipose tissue-specific deletion of PI3K p110α, the partial global inactivation of which has been shown to confer a beneficial effect later in life and to moderately increase lifespan in mice. We found that adipose tissue-specific inactivation of p110α potentiated adrenergic signalling, specifically in subcutaneous white adipose tissue and in brown adipose tissue. The molecular mechanism underlying this effect appears to involve engagement of p110α in catecholamine-stimulated Akt phosphorylation, which in turns activates phosphodiesterase and degradation of cAMP. Deletion of p110α reduced catecholamine-induced PDE activation thus enhancing cAMP-mediated signalling. The significance of cAMP-mediated signalling in thermogenic conversion of white adipose tissue has recently been demonstrated by knockout of PDE3B shown to promote browning of epididymal white adipose tissue.

The potentiating effect on adrenergic signalling observed upon p110α inactivation was modest and most likely for this reason insufficient to confer protection from weight gain during high-fat feeding. However, glucose uptake by BAT was higher in p110αDEL mice fed a high-fat diet, despite their similar insulin sensitivity with p110αFLOX mice. This increased glucose uptake from BAT likely explains the normal glucose tolerance in the face of systemic insulin resistance documented over ageing of p110αDEL mice. Moreover, p110αDEL mice did not accumulate as much fat as p110αFLOX littersmates upon ageing, which is arguably a more physiological type of metabolic stress than high-fat feeding, demonstrating that the modest effect of p110α deletion on adrenergic signalling is sufficient to confer a beneficial effect under physiological conditions. Indeed, there is substantial evidence demonstrating that ageing diminishes the sensitivity of adrenergic stimulation to reduce EE in humans as well as in rodents. In this regard, p110α inactivation could be counteracting the negative impact of ageing via regulating adrenergic signalling in the adipose tissue and in particular in BAT where it increased levels of UCP1.

Recently, another study reported a phenotype of adipose tissue-specific p110α deletion using the aP2–Cre line. The reported phenotype is fundamentally different to the phenotype described here and includes a decrease in thermogenic capacity of the adipose tissue, as a result of downregulation of UCP1 and consequent development of obesity and liver steatosis. The reason for this striking discrepancy could be related to the different
conditional $\alpha$ alleles or the different Cre lines (aP2-Cre versus adiponectin-Cre) used in these two studies. Also, potential differences in the level of $\alpha$ deletion could critically determine the nature of the ensuing phenotypes. In the present study, we documented a reduction in $\alpha$ protein levels of approximately 70–90%, depending on the tissue. However, it is likely that higher levels of $\alpha$ deletion could result in more severe insulin resistance, of which the negative impact would outweigh the potential benefits. Nevertheless, the phenotype described in the present study is consistent with the beneficial metabolic effects of deletion of the IR in the adipose tissue of FIRKO mice as well as with the recent demonstration that administration of a dual $\alpha/\delta$ PI3K inhibitor protects mice and monkeys from diet-induced obesity and metabolic syndrome, an effect more recently shown to be largely recapitulated by administration of a $\alpha$ selective inhibitor. In the FIRKO mice, an increase in oxidative metabolism in the adipose tissue was reported, and in the latter model, PI3K inhibition was shown to increase the levels of UCP1 expression and fuel oxidation in BAT, in line with our findings.

The physiological importance of adrenergic signalling in the development of age-related obesity has recently been highlighted.
Fig. 6  p110α^DEL mice display increased catecholamine-stimulated energy expenditure.  

- mRNA-expression levels of brown adipocyte markers in adipose tissues isolated from 18-week-old p110α^DEL and p110α^FLOX littermate male mice (n = 4 per genotype) following intraperitoneal administration of the β3-AR selective agonist CL316,243 (1 mg per kg per day) over five consecutive days. RNA was extracted from inguinal and epididymal adipose tissues and mRNA expression of Ucp1, Cidea, Ppca, Deiodinase 2 (Dio2) and Elovi3 was analysed by quantitative PCR. 

b UCPI protein expression detected by immunoblot analysis (blot shown in Supplementary Fig. 10) in iWAT from the same mice as in (a) (n = 4 per genotype).

c Immunoblot analysis (c) and immunohistochemical detection (d) of UCPI protein expression in iWAT of 6-week-old male mice injected with CL316,243 as in (a) (ns non-specific band).

Scale bar: 50 μm. e Raw energy expenditure of approximately 1-year-old p110α^FLOX and p110α^DEL female mice measured for 48 h at room temperature.

f Respiratory exchange ratio (RER) for the 48 h caloriometry run.

g Water intake (WI), food intake (FI), change in body weight (dBW) and average body weight (BW) for the 48 h of the caloriometry run.

h j Norepinephrine (NE)-stimulated energy expenditure (EE). k Energy expenditure measured in unconscious mice housed at 30°C prior to (basal) and after injection with 0.5 mg kg⁻¹ NE. Bars are averages of three stable readings for baseline prior to injection. NE-stimulated is the average of the three largest readings recorded.

l Graph showing the effects of NE injection on energy expenditure with time.

m Body weights of the mice used at the time they were analysed.

n-c The effects of cross-activation of other types of adrenergic output by degrading catecholamines in the adipose tissue could be a potentiation of adrenergic signalling in adipose tissue could be a paradox at the mechanistic level.

Finally, the present work provides an example of how an intervention that negatively affects an essential signalling pathway, such as the IIS pathway, can at the same time act bene

Methods

Mouse strains and maintenance. To generate mouse lines with adipose tissue-specific deletion of PI3K p110α, mice with conditionally targeted p110α alleles recently shown efficacy in activation of human BAT. Also, as mentioned above, administration of a PI3K inhibitor reversed diet-induced obesity in mice and monkeys, providing proof-of-principle of the utility of PI3K inhibitors in the context of metabolic disease treatment. Moreover, a number of p110α inhibitors are undergoing clinical trials in oncology. Taken together, these suggest that testing combinations of p110α inhibitors and β3-adrenergic agonists in the treatment of obesity is a realistic possibility.

Finally, the present work provides an example of how an intervention that negatively affects an essential signalling pathway, such as the IIS pathway, can at the same time act beneficially to mitigate the adverse effects, thus reconciling the insulin paradox at the mechanistic level.
Maintenance and differentiation of hMADS cells. hMADS-3 cells isolated form the pubic fat pad of a 4-month-old male were maintained in DMEM supplemented with 10% foetal bovine serum and 2.5 ng ml−1 human fibroblast growth factors. Adipocyte differentiation was induced by replacing maintenance medium with differentiation induction medium consisting of DMEM/F12 (1:1) supplemented with 5 μg ml−1 human insulin, 10 μg ml−1 human transferrin, 0.2 mM 3,3',5'-Triiodo-l-thyronine (T3), 1 μg/ml rosiglitazone, 100 μM isobutylmethylxanthine (IBMX) and 1 μM dexamethasone. Three days later, the medium was replaced with differentiation induction medium without IBMX and dexamethasone. Adipocytes were used in experiments upon completion of differentiation, typically 5–7 days after induction.

Isolation, immortalisation and differentiation of brown pre-adipocytes. Interscapular BAT was isolated from three p110αFLOX and three p110αDEL 5-week-old male mice, minced with fine scissors and digested with 1 mg ml−1 collagenase for 1 h at 37°C. Mature adipocytes were separated from the stromal vascular fraction (SVF) by centrifugation at 250 g for 5 min. Pellets containing the SVF were seeded in 6-well cell culture dishes in DMEM supplemented with 10% foetal bovine serum and transduced with retroviruses carrying a plasmid encoding SV40 Large T antigen (pBabe-puro-SV40LT, Addgene plasmid no 14088). Transduced cells were seeded in 6-well cell culture dishes in DMEM supplemented with 10% foetal bovine serum and 2.5 ng ml−1 human insulin, 100 μg/ml isobutylmethylxanthine (IBMX) and 1 μM dexamethasone. Three days later, the medium was replaced with fresh DMEM supplemented with 10% foetal bovine serum. Cell monolayers were used in experiments upon completion of differentiation, typically 1 week after induction.

Body weight and food intake analysis. Body weight was measured monthly from 4 weeks of age onwards. For food intake assays, mice were housed in small groups of 2–3, with food restricted to ad libitum level for 3 days before the experiment. Pellet and mouse body weights were measured at the beginning and end of a 3-day measurement. Results are expressed as cumulative food intake (grams of chow per kg of body weight per 3 days).

Blood metabolic biomarkers. Blood samples from fed or fasted (16 h) mice were collected from the tail vein with heparinized capillaries and then spun in a benchtop microcentrifuge. Totally, 0.6 ml of the supernatant was used for each measurement. Blood glucose concentrations were measured in tail vein blood samples using a Contour XT glucometer (Bayer). Glucose and insulin turnover tests. Results are expressed as cumulative food intake (grams of chow per kg of body weight per 3 days).

Glucose and insulin tolerance tests. GTTs and ITTs were performed on mice at different ages, fasted for 16 or 5 h, respectively. Blood glucose levels were determined at several time points (up to 120 min) after intraperitoneal injection of a glucose solution (2 g per kg of body weight) or human insulin (Actrapid, Novo Nordisk) (0.75–2 U per kg of body weight). Blood glucose concentrations were measured in tail vein blood samples using a Contour XT glucometer (Bayer).

Hyperinsulinemic–euglycemic clamp. Hyperinsulinemic–euglycemic clamp were performed in anaesthetised animals on thermally controlled pads to maintain their core body temperature. Animals were anaesthetised by intraperitoneal injection of a glucose solution (2 g per kg of body weight) or human insulin (Actrapid, Novo Nordisk) (0.75–2 U per kg of body weight). Blood glucose concentrations were measured in tail vein blood samples using a Contour XT glucometer (Bayer).
The mixture was vortexed and the phases were separated by centrifugation at 10,000 x g for 2 min. The lower organic phase was spotted onto thin layer silica (TLC) gel-60 plates (Merck), which had been treated with 1% oxalic acid, 1 mM EDTA in water:methanol (6:4). TLC plates were developed in a solvent consisting of propanol:1:2 M acetic acid (13:7). Images of radiolabelled lipid products were captured using a Fuji FLA-2000 phosphorimaginer and analysed using ImageJ software.

**UpI immunohistochemistry.** Adipose tissues were fixed in formalin for 24 h and embedded in paraffin sections were deparaffinised, subjected to antigen unmasking (boiling in 10 mM sodium citrate pH 6 in a pressure cooker for 30 min), blocked with 5% bovine serum albumin (BSA) and incubated with UCPI antibody (Abcam, cat. no. 10983) diluted 1:1000 for 16 h at 4 °C. Sections were then incubated with SignalStain® Boost IHC Detection Reagent (Cell Signalling Technology, cat. no. 8114P) and immune complexes were visualised with SignalStain® DAB reagent (Cell Signalling Technology, cat. no. 8059).

**RNA extraction and qPCR.** RNA was extracted from adipose tissue samples using TriZol (Thermo Fisher) and reverse transcribed to cDNA using Qiagen Omniscript RT kit according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using an ABI7900HT Fast Real Time PCR detection system and Power SYBR-Green PCR Master Mix (ABI). Relative gene expression was quantified using the 2−ΔΔCt method. Mouse ribosomal protein S18 (rps18) was used as a loading control. A list of primers used in the present study is given in Supplementary Table 1.

**Lipolysis and cAMP levels in adipose tissue explants.** Adipose tissue explants were adjusted to approximately 100 mg of weight and then minced with fine scissors. Explants were incubated in serum-free DMEM supplemented with 2% (w/v) fatty acid-free BSA for 2 h at 37 °C in 5% CO2 incubator then transferred to Kreb’s-Ringer’s buffer (30 mM Hepes pH 7.4, 10 mM NaHCO3, 120 mM NaCl, 4 mM KH2PO4, 1 mM MgSO4, 0.75 mM CaCl2) supplemented with 0.2% fatty acid-free BSA and 10 mM glucose and incubated for an initial period. Inhibitors were added for the last 15 min of the incubation. Lipolysis was stimulated by addition of 1 μM Isoproterenol or Norepinephrine-i-bitartate (Sigma). Totally, 50 μl of medium was removed 30 min following stimulation and glycerol content was determined using Glycerol Reagent and Glycerol Standard (Sigma). For determination of intracellular cAMP levels tissue explants were processed, incubated and stimulated as above. Explants were collected 20 min following adrenergic stimulation, frozen in dry ice and kept in −80 °C until further processing. Samples were homogenised in 0.5 mL of 0.1 N HCl using a mechanical homogeniser. Homogenates were clarified by centrifugation for 5 min in a benchtop refrigerated microcentrifuge. Supernatants were diluted 1 in 4 with cAMP ELISA buffer and the cAMP content was determined with a cyclic AMP Select ELISA kit (Cayman, cat. no. 501040).

**Phosphodiesterase activity assay.** BAT-derived adipocytes were lysed in a buffer containing 20 mM Hepes-NaOH (pH 7.4), 0.5 mM EDTA, 2 mM MgCl2, 0.1% Triton X-100 in 0.5 mg/mL EGTA, 1 mM microsatin-LR (Calbiochem) protein inhibitors. Lysates were briefly sonicated using a probe sonicator and were desalted using Zea Spin desalting columns, 7 kD molecular weight cut-off (Thermo Scientific, cat. no. 89882), equilibrated in PDE assay buffer. Desalted lysates (15 μg protein) were assayed for phosphodiesterase activity using a kit from Abcam (cat. no. ab139460) according to the manufacturer's instructions.

**Indirect calorimetry.** EE and norepinephrine-induced thermogenesis were measured using a Metabolic Sense gas analyser (Creative Scientific, UK). The gas analyser uses a paramagnetic oxygen analyzer and an infra-red CO2 sensor to measure oxygen and carbon dioxide concentrations. The analyser operates in push mode with airflow to each chamber controlled by a mass flow controller set to flow 400 ml per min. Cages were multiplexed to the analyser, with four cages being read by each analyser. For free-living EE cages were sampled for 30 s every 11 min, with a 90 s washout period between each chamber. EE was calculated from the VO2 and CO2 according to the modified Weir equation: EE (J min−1) = 15.818 x VO2 (ml min−1) + 5.176 x VCO2 (ml min−1). For NE-induced thermogenesis, mice were anesthetized with sodium pentobarbital (90 mg kg−1) and placed in a 30 °C chamber. Mice were injected with 0.5 mg NE per kg. The dose of NE gave an average increase in EE of ~15% over the basal EE, while normal maximal thermogenic capacity experiments were for mice acclimated to a similar temperature a 150% increase would be expected. Therefore, the applied NE dose fulfilled the requirement of being a sub-maximal dose and could potentially allow detection of changes in sensitivity to NE, not just changes in brown adipose tissue thermogenic capacity.

**Statistical analysis.** Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 8. Specific tests are detailed in the figure legends.
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**Author contributions**

L.C.F. conceived the study and drafted the paper; C.A., A.Y., L.B., I.S., S.V., A.V.P. and L.F. conducted the research; C.A., A.Y., L.B., I.S., S.V., A.K.M. and L.C.F. acquired and analyzed data; C.A., A.Y., L.B., I.S., S.V., C.D., A.V.P. and L.C.F. interpreted the data and edited the paper.

**Additional information**

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