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Adipose tissue mTORC2 regulates ChREBP-driven de novo lipogenesis and hepatic glucose metabolism

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Adipose tissue de novo lipogenesis (DNL) positively influences insulin sensitivity, is reduced in obesity, and predicts insulin resistance. Therefore, elucidating mechanisms controlling adipose tissue DNL could lead to therapies for type 2 diabetes. Here, we report that mechanistic target of rapamycin complex 2 (mTORC2) functions in white adipose tissue (WAT) to control expression of the lipogenic transcription factor ChREBPβ. Conditionally deleting the essential mTORC2 subunit Rictor in mature adipocytes decreases ChREBPβ expression, which reduces DNL in WAT, and impairs hepatic insulin sensitivity. Mechanistically, Rictor/mTORC2 promotes ChREBPβ expression in part by controlling glucose uptake, but without impairing pan-AKT signalling. High-fat diet also rapidly decreases adipose tissue ChREBPβ expression and insulin sensitivity in wild-type mice, and does not further exacerbate insulin resistance in adipose tissue Rictor knockout mice, implicating adipose tissue DNL as an early target in diet-induced insulin resistance. These data suggest mTORC2 functions in WAT as part of an extra-hepatic nutrient-sensing mechanism to control glucose homeostasis.
Insulin resistance is a comorbidity of obesity, a risk factor for type 2 diabetes (T2D), and a side effect of the immunosuppressant rapamycin; however, the exact mechanisms that can lead to insulin resistance remain poorly understood. A hallmark of T2D is failure of insulin to suppress hepatic glucose production (HGP) leading to hyperglycemia. In hepatocytes insulin suppresses HGP by stimulating AKT to inhibit FOXO1 (ref. 1). Insulin also regulates HGP in mice, in which hepatic insulin signalling is genetically ablated2–4 suggesting the existence of an extrahepatic insulin-sensing tissue that can indirectly control hepatic glucose output. Thus, understanding how organs communicate to control glucose homeostasis is critical to understanding T2D.

Traditionally adipose tissue de novo lipogenesis (DNL) was thought to function primarily to store excess energy from carbohydrates as more energy-dense lipid; however, unlike in the liver, in which increased DNL often correlates with insulin resistance, increased DNL in white adipose tissue (WAT) correlates with insulin sensitivity5,6. Moreover, reduced DNL in WAT is observed in obesity7,8 and can occur following prolonged rapamycin treatment9. Recent work in rodents shows that hepatic insulin resistance develops within a week of high-fat diet (HFD) feeding, coincident with reduced insulin-stimulated glucose uptake into adipose tissue, but without losing insulin-stimulated AKT signalling in fat, and before any detectable decrease in muscle glucose uptake, elevation of lipolysis or inflammation10. This supports a model in which HFD causes selective insulin resistance in fat and that adipose tissue glucose uptake and DNL is linked to an extra-hepatic insulin-sensitizing signal that may be targeted early in obesity. Deciphering the upstream mechanisms controlling adipose tissue DNL is therefore critical to understanding the pathogenesis of certain forms of insulin resistance.

DNL involves taking up glucose and converting glucose-derived citrate to acetyl-CoA (by ATP-citrate lyase/ACL), which is further converted to malonyl-CoA (by acetyl-CoA carboxylase/ACC), and eventually to palmitate (by fatty acid (FA) synthase/FASN). Palmitate is further modified by elongases and desaturases to produce diverse lipids. The transcriptional regulators sterol response element-binding protein 1c (SREBP1c) and carbohydrate response element-binding protein (ChREBP) control lipogenic gene expression11–13; however, their distinct regulation and functional roles in adipose tissues are still being worked out. Recently it was shown in adipose tissue that the ChREBPα isomeric functions in part by driving expression of the N-terminally truncated ChREBPβ isomeric from an alternative promoter5. ChREBPβ more potently induces DNL genes and its expression in human adipose tissue correlates with insulin sensitivity7,8, suggesting ChREBP activity in adipose tissue may be an important regulator of systemic glucose homeostasis.

The mechanistic target of rapamycin (mTOR) kinase is a master regulator of metabolism whose downstream functions are split between at least two distinct complexes. The best understood complex, mTOR complex 1 (mTORC1) is a well-known amino acid and growth factor sensor that promotes anabolic metabolism by driving protein and lipid biosynthesis and suppressing autophagy14. Its less understood sibling, mTORC2, is activated by growth factors and is best known as the AKT hydrophobic motif kinase (S473 in AKT1; S474 in AKT2)15. It is thought that mTORC2 activity is essential for maximal AKT signalling to its downstream substrates and whether AKT-independent mTORC2 pathways are critical in metabolism is not yet clear. The function of mTORC2 in adipose tissue was previously examined using the ap2-Cre driver to conditionally delete floxed alleles of Rictor, an essential subunit of the complex16,17. However, several recent studies indicate that adiponectin-Cre has greater efficiency and selectivity to mature adipocytes18–21. Thus, the in vivo role of mTORC2 in mature adipocytes remains unclear.

We recently reported that mTORC2 regulates expression of lipogenic genes in brown adipose tissue (BAT)22 leading us to hypothesize that mTORC2 might also regulate DNL in WAT to control insulin sensitivity. To test this we developed a new fat-specific mTORC2 knockout (KO) model by deleting Rictor with adiponectin-Cre. Here, we provide evidence supporting a model in which a primary function of adipocyte mTORC2 is to promote Chrebpβ expression and DNL. Moreover, we find that ablating Rictor/mTORC2 in WAT alters the lipid composition of fat and causes severe hepatic insulin resistance. Mechanistically, mTORC2 appears to promote Chrebpβ expression and DNL at least in part by controlling glucose uptake into adipocytes independently of the classic AKT-AS160 pathway. Adiponectin-Cre;Rictor conditional KO mice also exhibit reduced fat growth on a HFD suggesting mTORC2 additionally functions in mature adipocytes to control diet-induced adipose tissue expansion. These data provide a new framework for exploring the role of mTORC2 signalling in obesity and the pathogenesis of insulin resistance.

Results

Mice lacking adipocyte Rictor have normal body growth. To evaluate the role of mTORC2 specifically in adipose tissue, we generated Adiponectin-cre;Rictorβ/β mice (herein RictorAdipoq-cre mice). We previously confirmed that adiponectin-Cre targets mature adipocytes with high specificity and efficiency in our colony23. Note that significant differences exist between RictorAdipoq-cre and mice in which Rictor was targeted with aP2-cre24,25. We provide a detailed comparison in the Discussion and in Supplementary Table 1.

Deleting Rictor with adiponectin-Cre greatly reduces RICTOR, pAKT5473 and pAKT5473 (a growth factor insensitive mTORC2 target site) in the major visceral (that is, perigonadal or pgWAT), subcutaneous (that is, inguinal subcutaneous or sWAT), and brown fat (that is, interscapular BAT) depots (Fig. 1a and Supplementary Fig. 1a) and the residual signal is from stromal vascular fraction (SVF) cells because RICTOR and pAKT5473/pT5450 is undetectable in purified adipocytes (Fig. 1b). In contrast, pAKT5308 is intact (Fig. 1a,b), which maintains AKT’s ability to phosphorylate pFOXO124, pGSK3β59 and pPRAS40/7246 (Fig. 1a). SVF and hepatic RICTOR levels are normal (Fig. 1b and Supplementary Fig. 1b) confirming targeting specificity.

The body weight (Fig. 1c) and food intake (Fig. 1d) of RictorAdipoq-cre mice consuming a standard chow diet does not significantly differ from controls through 20 weeks. The mass of the major fat depots is also unaffected in both male and female mice (Fig. 1e and Supplementary Fig. 1c) and the RictorAdipoq-cre adipocytes appear normal by haematoxylin and eosin stain (Fig. 1f and Supplementary Fig. 1d). Liver mass increases in RictorAdipoq-cre mice by 18% and 13% in both males and females, respectively (Fig. 1g and Supplementary Fig. 1e). By haematoxylin and eosin stain the liver appears normal (Fig. 1h); however, despite no observable difference in Oil Red O staining in the liver between the control and RictorAdipoq-cre KO mice (Fig. 1h) there is a measurable increase in total hepatic TAG content (Fig. 1i) corresponding with a 16% decrease in the number of nuclei per field (Supplementary Fig. 1f) (indicative of increased cell size) that may in part explain the overall increase in liver mass. Heart mass also increases in RictorAdipoq-cre KO mice; however, kidney, spleen, lung, thymus and muscle mass is normal (Fig. 1g and Supplementary Fig. 1e). Total pancreas and pancreatic β-cell mass also does not significantly differ between KO and control...
**Figure 1 | Growth characteristics of mice lacking Rictor in adipose tissue.** (a) Western blots of the indicated total and phosphor-proteins in whole-fat tissue lysates of Rictorfl/fl (WT) and RictorAdipoq-Cre (KO) mice. (b) Western blots using lysates of purified adipocytes and the stromal vascular fraction (SVF) prepared from pgWAT and sWAT. (c) Body growth curves. n = 8. (d) Food consumption. n = 6. (e) Individual fat tissue mass analysis. n = 8-13. (f) Representative H&E images of pgWAT and sWAT. (g) Individual lean tissue mass analysis. n = 8-13. (h) Representative H&E images of liver and of liver oil Red O staining. (i) Liver TAG content. N = 6. (j) β-cell mass. N = 5. Data were analysed by Student’s t-test. Values are expressed as mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bar, 100 μM. H&E, haematoxylin and eosin.

(Fig. 1j and Supplementary Fig. 1g,h). These observations are in contrast to those reported for Rictor^P2-cre^ mice, which have increased total body size because of a global increase in lean tissue mass (including heart, kidney, spleen and pancreas but not the liver) that is attributed to high circulating IGFI (ref. 16). We find no difference in circulating IGFI levels between RictorAdipoq-cre mice and controls (Supplementary Fig. 1i) and conclude that in 20-week-old mice living under standard conditions, losing adipose tissue Rictor does not affect overall fat mass or increase whole-body lean tissue growth in this model.

**Adipocyte mTORC2 controls hepatic glucose production.** Blood glucose concentrations in fasting and fed RictorAdipoq-cre mice do not differ from controls; however, this requires approximately threefold higher plasma insulin suggesting insulin resistance (Fig. 2a,b). Insulin intolerance of RictorAdipoq-cre mice was supported by insulin tolerance tests (ITTs), showing an 89% increase in AUC (Fig. 2c and Supplementary Fig. 2a); glucose tolerance is normal (Fig. 2d). Acutely inhibiting mTORC2 by treating RictorAdipoq-creERT2 mice with tamoxifen also ablates RICTOR and pAKT^S473 within 3-week of treatment (Supplementary Fig. 2b), and this induces insulin intolerance similar to the congenital KOs without affecting glucose tolerance (Supplementary Fig. 2c-e) indicating insulin resistance occurs rapidly with Rictor loss.

To better assess insulin action we performed hyperinsulinenemic-euglycemic clamps in conscious mice. The steady-state glucose infusion rate required by RictorAdipoq-cre mice to maintain euglycemia is 46% lower than controls indicating severe insulin resistance (Fig. 2e). Insulin stimulated whole-body glucose uptake and glycolysis is diminished by 31 and 32%, respectively (Fig. 2f,g), and there is no difference in glycogen plus lipid synthesis (Fig. 2h). Interestingly, insulin stimulated glucose uptake into skeletal muscle—the main site of glucose synthesis (Fig. 2i) and they show less tolerance to a pyruvate bolus stimulation. However, basal HGP is unaltered between both cohorts; however, insulin fails to suppress HGP in RictorAdipoq-cre mice (Fig. 2j). Basal HGP is unaltered between both cohorts; however, insulin fails to suppress HGP in RictorAdipoq-cre mice (Fig. 2k). The RictorAdipoq-cre mice also express 2.2-fold more hepatic glucose 6-phosphatase (G6P) (Fig. 2l) and they show less tolerance to a pyruvate bolus (Supplementary Fig. 2f) consistent with increased hepatic gluconeogenesis. Hepatic phosphoenolpyruvate carboxylase (Pepck) is not significantly elevated (Fig. 2l).

We also examined genes involved in hepatic lipid regulation. Although RictorAdipoq-cre livers have elevated TAG content, most lipogenesis genes including hepatic Srebs (Srebp1a, Srebp1c and Srebp2), Chrebpz, and Lxrα express normally in the RictorAdipoq-cre KO livers (Supplementary Fig. 2g). We do detect increased Chrebpβ expression; however, expression of the ChREBP targets Acly, Acc and Fasn does not significantly differ.
Intracellular insulin action. As expected, insulin fails to stimulate AKT1 phosphorylation in RictorAdipoq-cre mice (Fig. 4a) consistent with hepatic insulin-resistance. Curiously, in RictorAdipoq-cre mice muscle IR phosphorylation is slightly higher in the unstimulated state, while insulin-stimulated pAKT1 is blunted (Fig. 4b) suggesting some muscle signalling might also be altered in the mutant mice. However, AKT1 phosphorylation is stimulated normally in RictorAdipoq-cre muscle (Fig. 4b), which is consistent with the clamp data showing normal insulin-stimulated muscle glucose clearance (Fig. 2i). These data support a model in which Rictor loss in fat most negatively affects hepatic function. Interestingly, primary hepatocytes isolated from RictorAdipoq-cre mice maintain insulin resistance even when cultured ex vivo (Fig. 4c). Thus, the
Figure 3 | Insulin action in adipose tissues. (a,b) Western blots of the indicated total and phospho-proteins in whole-tissue lysates prepared from pgWAT (a) and sWAT (b). Male Rictorfl/fl (WT) and RictorAdipoq-Cre (KO) mice (8–10 weeks old) were fasted for 6 h and then injected with insulin (0.75 U kg$^{-1}$) for 15 min before collecting samples. Quantifications are shown below. $n = 3$ or 4. Values are expressed as mean + s.e.m. (c) Western blot of indicated total and phospho-proteins in mature adipocytes isolated from the pgWAT of Rictorfl/fl (WT) and RictorAdipoq-Cre (KO) with or without insulin stimulation.

Figure 4 | Insulin action in liver and muscle. (a,b) Western blots of the indicated total and phospho-proteins in whole tissue lysates prepared from liver (a) and skeletal muscle (b). Male Rictorfl/fl (WT) and RictorAdipoq-Cre (KO) mice (8–10 weeks old) were fasted for 6 h and then injected with insulin (0.75 U kg$^{-1}$) for 15 min before collecting samples. (c) Western blots of the indicated total and phospho-proteins in isolated hepatocytes. In each panel, the Western blot quantifications are shown to the right. $n = 3$ or 4. Values are expressed as mean + s.e.m. *$p<0.05$; **$p<0.01$; ***$p<0.001$. 

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Altered lipid metabolism and composition in Rictor-deficient fat. Consistent with the model that DNL in WAT regulates insulin sensitivity, we find that adipose tissue Rictor loss dramatically reduces Acly, Acc and Fasn mRNA and protein expression (Figs 1a and 5a,b). The expression of Chrebp and Srebflc expression is unchanged in the mutant fat; however, Chrebpβ induction is almost completely blocked in both pgWAT and sWAT (Fig. 5c,d). This is consistent with Chrebpβ expression driving DNL in adipocytes. Indeed, overexpressing recombinant ChrEBPβ, constitutively active ChrEBPβ (ChrEBP-CA), and to a lesser extent ChrEBPα rescues expression of ACLY, ACC and FASN in Rictor-deficient adipocytes supporting this notion (Fig. 6). These data implicate adipocyte mTORC2 as a key upstream regulator of ChrEBPβ-driven DNL.

Although under standard dietary conditions the adipose tissues predominantly obtain free FAs (FFAs) from the liver and diet

Figure 5 | Deleting Rictor in adipose tissue reduces DNL and alters the lipid composition of fat and liver. (a-d) Relative mRNA expression of indicated genes in pgWAT (a,c) and sWAT (b,d); Rictorfl/fl (WT) and RictorAdipoq-Cre (KO) mice. n = 8. (e) The ratio of C16/C18:2n6 in pgWAT, sWAT and liver. n = 8. (f) Lipid profiles of pgWAT (f) and sWAT (g). n = 8. (h,i) The ratio of C18/C16, C18:1n7/C16:1n6 and C18:1n9/C18 in pgWAT and sWAT. n = 8. (j,k) Relative mRNA expression of indicated genes in pgWAT (j) and sWAT (k). (l) Lipids profiles of liver. n = 8. (m) The ratio of C18/C16, C18:1n7/C16:1n6 and C18:1n9/C18 in liver. n = 8. (n) Relative mRNA expression of the indicated genes in liver. n = 8. Data were analysed by Student’s t-test. Values are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.
rather than DNL, we wondered whether losing Rictor in WAT alters FA composition. The ratio of C16:0 (palmitate) to the essential FA C18:2n6 (linoleate)—an index of DNL—is slightly but significantly decreased in Rictor\textsuperscript{Adipoq-cre} sWAT (Fig. 5e) suggesting that despite the normal size of mutant WATs (though trending smaller) (Fig. 1e) DNL is decreased. Moreover, C18:0 (stearate) levels decrease while C16:1n7 (palmitoleate) and C18:1n9 (oleate) levels increase in Rictor\textsuperscript{Adipoq-cre} WATs (Fig. 5f). These findings are noteworthy because the de novo synthesis of palmitoleate by adipose tissue has been linked to improved systemic insulin action. Diets rich in oleate are also reportedly metabolically healthy. Thus, it does not appear that a palmitoleate or oleate deficiency in fat is causing insulin resistance in Rictor\textsuperscript{Adipoq-cre} mice.

The altered abundance of very long-chain FAs suggested mTORC2 might additionally regulate FA elongation and/or desaturation (Supplementary Fig. 3a). Elongation of very long-chain FAs 6 (ELOVL6) elongates C16:0 FAs to C18:0 FAs, while steryl CoA desaturase (SCD1) desaturates C16:0 and C18:0 FAs to C16:1n7 and C18:1n9, respectively. We calculated the ELOVL6 and SCD1 activity ratios by dividing their products by their substrates (Fig. 5h), which indicates a decrease and increase respectively in ELOVL6 and SCD1 activity supporting this hypothesis. A broad survey of elongase and desaturase gene expression further indicates that Elov6 expression in the WAT of chow-fed mice requires Rictor explaining the reduced C18:0/C16:0 ratio (Fig. 5k and Supplementary Fig. 3b). In contrast, Scd1 expression is unchanged (Fig. 5j,k). Elovl6 and Scd1 are reportedly co-regulated ChREBP targets in the liver. Thus, in adipose tissue Elovl6 and Scd1 are not necessarily co-regulated genes and the altered very long-chain FA profile in the WAT of Rictor\textsuperscript{Adipoq-cre} mice likely reflects defective elongation.

Deletion of Rictor in adipose tissue mirrors the effects of HFD. We also examined whether deleting adipose tissue Rictor affects hepatic lipid composition. Interestingly, Rictor\textsuperscript{Adipoq-cre} livers have a lipid profile and ELOVL6/SCD1 ratios that parallel the WATs but without changes in hepatic Elovl6 or Scd1 expression (Fig. 5l–n). The livers from Rictor\textsuperscript{Adipoq-cre} mice also have high levels of C14:0, C16:0, C18:1n9, C20:1n9 and C20:2 FAs with corresponding decreases in C18:2n6 and C20:4n6 FAs (Fig. 5l).

A lipogenic diet improves the insulin sensitivity of knockout mice. We next placed Rictor\textsuperscript{Adipoq-cre} mice on a high carbohydrate/zero-fat diet (ZFD) for 12 weeks to maximize effects caused by a DNL deficiency. Rictor\textsuperscript{Adipoq-cre} mice consuming a ZFD maintain a body weight similar to controls and consume the same amount of food (Fig. 7j,k). However, the adipose tissues are smaller (Fig. 7l) indicating that Rictor is also more critical for fat growth with increasing carbohydrate load. Liver mass also increases in ZFD-fed mutants (Fig. 7m) explaining why total body mass is unchanged relative to controls despite the decrease in fat mass.

Interestingly, consuming a ZFD restores insulin tolerance in Rictor\textsuperscript{Adipoq-cre} mice back to the level observed in the benchmark chow-fed controls (Fig. 7g,n). This correlates with restoration of most lipogenic genes (for example, Chrebp\textsuperscript{b}, Acly, Acc and Elovl6) back to the benchmark expression level (that is, not significantly different from chow-fed controls) (Fig. 7o). ZFD similarly restores sWAT lipogenic gene expression, and for Chrebp\textsuperscript{b}, Acly...
and Elovl6, to levels even higher than in the sWAT of chow-fed controls (Fig. 7p). These increases are mirrored by increases in ACLY, ACC and FASN protein expression (Supplementary Fig. 5a,b). Notably, while ZFD increases lipogenic gene expression in Rictor<sup>Adipoq-cre</sup> mice to chow-fed levels, lipogenic gene expression still remains lower relative to the ZFD-fed control group (Fig. 7o,p). In fact, lipogenic gene expression responds robustly in the ZFD-fed control group exemplified by ~11- and 28-fold Chrebp<sub>b</sub> induction in pgWAT and sWAT, respectively, over chow-fed controls (Fig. 7o,p). Thus, even though a ZFD improves insulin sensitivity in Rictor<sup>Adipoq-cre</sup> mice (presumably by ‘forcing’ more glucose into adipocytes), the lipogenic genes do not respond at full capacity. Notably, ZFD also increases hepatic lipogenic gene expression and steatosis in both the control and
Reduced glucose uptake and DNL in Rictor-deficient adipocytes.

To better define the mechanism by which adipose tissue Rictor loss alters adipocyte function, we generated primary white preadipocytes harboring a Rictor (UBC-CREERT2) inducible-KO (Rictor-iKO) system to examine the acute effects of inhibiting mTORC2 on differentiation and function. When compared with their isogenic controls, primary Rictor-iKO preadipocytes differentiate normally based on PPARγ, ChREBPα, C/ebpα, C/ebpβ, Ap2 and Adiponectin expression (Fig. 9a and Supplementary Fig. 6a). In high-glucose culture medium, DNL is the primary driver of lipid droplet formation and under these conditions Rictor-iKO cells have smaller lipid droplets (Fig. 9b). Rictor-iKO cells also fail to upregulate C/ebp mRNA and protein (Fig. 9a,c), consistent with a DNL deficiency. Moreover, both basal and insulin-stimulated glucose uptake as well as glucose incorporation into FFAs and TAGs are blunted in Rictor-iKO cells (Fig. 9d,e,f). As expected, RICTOR is completely ablated in the Rictor-iKO cells by day 6 of differentiation at which point AKT S473 phosphorylation is also undetectable (Fig. 9a). In contrast, phosphorylation of AKT T308, AS160 T642, and S6K 2399 (a measure of mTORC1 activity) is unchanged relative to the isogenic control (Fig. 9a). Thus, decreased glucose uptake and DNL is a primary consequence of Rictor loss.

GLUT4 is the major glucose transporter for insulin-stimulated glucose uptake into adipocytes. Therefore, we next examined whether GLUT4 regulation is defective in Rictor-deficient adipocytes. Indeed, in primary Rictor-iKO cells, Glut4 mRNA and protein fail to induce normally during differentiation (Fig. 9g and Supplementary Fig. 6b). Similarly, in mice consuming the lipogenic ZFD, Glut4 is also reduced in both the sWAT and pgWAT of RictorAdipoq-cre mice (Fig. 9h and Supplementary Fig. 6c). Thus, in highly lipogenic conditions such as in culture medium and a high-carbohydrate diet, Rictor is required for maximal Glut4 gene expression. In mice consuming normal chow however, Glut4 expression does not significantly differ in either depot from controls (Fig. 9h and Supplementary Fig. 6d,e). Moreover, in HFD-fed mice Glut4 expression only decreases in the mutant sWAT and not pgWAT (Fig. 9h and Supplementary Fig. 6c). Thus, additional mechanism(s) of glucose regulation by Rictor/mTORC2 in adipocytes likely exist.

We also considered other proposed modulators of insulin resistance, such as increased lipolysis. Primary Rictor-iKO adipocytes show no difference in basal or isoproterenol-stimulated glycerol release (Fig. 9i) arguing against increased lipolysis as a primary target of Rictor loss. However, pgWAT depots resected from RictorAdipoq-cre mice show a modest increase in basal glycerol release ex vivo; isoproterenol-stimulated glycerol release is normal (Fig. 9j). When administered at a low concentration, insulin is also ineffective at suppressing circulating FFA levels in vivo in fasted RictorAdipoq-cre mice (Supplementary Fig. 6e), which is consistent with insulin resistance but could reflect liver dysfunction. High cholesterol is detected in the mutants, however, circulating FFA and TAG levels are not significantly different between fasted control and RictorAdipoq-cre mice possibly due to the high basal insulin levels (Supplementary Fig. 6f–h). Moreover, RictorAdipoq-cre mice have normal hormone sensitive lipase (HSL) levels in both depots and increased HSL phosphorylation is not detected (Fig. 1a). Higher levels of adipose triglyceride lipase (ATGL) associate with increased lipolysis; however, ATGL levels are also normal in pgWAT and lower in sWAT (Fig. 1a). Thus, defective lipolysis may contribute to insulin resistance in the prolonged absence of Rictor/mTORC2 in fat but it does not appear to be a primary effect.

TNF-α is undetectable in RictorAdipoq-cre mice and leptin, resistin and PAI-1 levels are normal (Supplementary Fig. 6i–k). We do detect reduced plasma adiponectin by ~32% (Supplementary Fig. 6l), which could contribute to insulin resistance. However, reducing adiponectin levels reportedly has no or only a mild defect in insulin sensitivity in chow-fed mice31,32 suggesting this alone likely does not explain the severe insulin resistance of RictorAdipoq-cre mice. Thus, based on the collective in vivo and in vitro primary cell data, we propose that a primary function of mTORC2 in adipocytes is to control ChREBP activity by regulating glucose flux, which promotes DNL and the production of a signal(s) that promote insulin sensitivity and
possibly adipogenesis (Fig. 10). However, prolonged Rictor/ mTORC2 loss may lead to secondary metabolic changes that exacerbate the phenotype.

**Discussion**

We describe a novel model of mTORC2 in adipose tissue based on conditional deletion of Rictor with adiponectin-Cre. There are several phenotypic differences compared with mice in which Rictor was targeted with aP2-Cre. A detailed comparison can be found in Supplementary Table 1. These differences likely reflect the higher efficiency and specificity of adiponectin-Cre. For example, aP2-Cre incompletely targets adipocytes and additionally targets adipose tissue endothelial cells. We find that RictorAdipoq-Cre mice exhibit more severe insulin resistance. In addition, RictorAdipoq-Cre gain less weight than controls on HFD while in contrast RictorA2Cre mice gain more weight on HFD. The reduced weight of RictorAdipoq-Cre mice on HFD is largely due to a decrease in fat mass, but whether resistance to obesity reflects the deficiency in Chrebpβ expression or DNL is not yet clear. Interestingly, it was suggested recently that ChREBP may promote PPARγ activity by controlling the synthesis of FAs that function as PPARγ ligands. In addition, mice lacking Fasn in adipose tissue are also resistant to HFD and this is attributed to defective synthesis of a PPARγ ligand. Such a ligand could act in a paracrine manner to stimulate new adipogenesis. This requires further investigation.

Based on our results we hypothesize that adipose tissue mTORC2 functions as part of an extra-hepatic nutrient-sensing mechanism that relays the organism’s nutritional state to the liver to control insulin sensitivity and glucose homeostasis (Fig. 10). Mechanistically, our data suggest that adipocyte mTORC2 controls ChREBP activity and DNL at least in part by regulating glucose flux. Interestingly however, this mTORC2 function mechanism that relays the organism’s nutritional state to the liver to control insulin sensitivity and glucose homeostasis (Fig. 10). Mechanistically, our data suggest that adipocyte mTORC2 controls ChREBP activity and DNL at least in part by regulating glucose flux.
substrates the dependency for mTORC2 cannot be overcome. An alternative possibility is that only some AKT substrates require mTORC2-dependent hydrophobic motif phosphorylation (S473 in AKT1; S474 in AKT2) while PDK1 phosphorylates AKT in the kinase domain (T308 in AKT1; T309 in AKT2), which promotes maximal AKT activity. This includes stimulating GLUT4 translocation to the plasma membrane by inhibiting AS160. (2) By deleting Rictor in mature adipocytes, we provide evidence that Rictor/mTORC2 is not essential for AKT signalling to AS160 and other classic substrates, but it is required for normal glucose uptake and ChREBP activity. This suggests mTORC2 might only be essential for a specific AKT-target other than AS160 that promotes glucose uptake; or alternatively, mTORC2 may regulate glucose uptake by an AKT-independent mechanism (also see Discussion). (3) Furthermore, mTORC2-dependent glucose uptake drives ChREBP-dependent DNL and the production of a signal(s) that (4) promotes hepatic insulin sensitivity and possibly (5) HFD-induced adipogenesis. (6) Prolonged Rictor loss may lead to additional defects such as increased lipolysis.

Figure 10 | A model of RICTOR/mTORC2 function in adipocytes. (1) In adipocytes, mTORC2 phosphorylates AKT in the hydrophobic motif (S473 in AKT1; S474 in AKT2) while PDK1 phosphorylates AKT in the kinase domain (T308 in AKT1; T309 in AKT2), which promotes maximal AKT activity. This includes stimulating GLUT4 translocation to the plasma membrane by inhibiting AS160. (2) By deleting Rictor in mature adipocytes, we provide evidence that Rictor/mTORC2 is not essential for AKT signalling to AS160 and other classic substrates, but it is required for normal glucose uptake and ChREBP activity. This suggests mTORC2 might only be essential for a specific AKT-target other than AS160 that promotes glucose uptake; or alternatively, mTORC2 may regulate glucose uptake by an AKT-independent mechanism (also see Discussion). (3) Furthermore, mTORC2-dependent glucose uptake drives ChREBP-dependent DNL and the production of a signal(s) that (4) promotes hepatic insulin sensitivity and possibly (5) HFD-induced adipogenesis. (6) Prolonged Rictor loss may lead to additional defects such as increased lipolysis.

A complication after organ transplantation is a syndrome called new onset diabetes after transplantation. Immunosuppressants such as rapamycin associate with new onset diabetes after transplantation, and in rodent models, rapamycin causes glucose intolerance and insulin resistance; however, the mechanism of rapamycin-induced metabolic disease needs further investigation. One pathway suggested by our data is that mTORC2 might control glucose uptake by controlling Glut4 transcription. This function of mTORC2 appears to be most essential when the glucose load is high (for example, culture medium or when mice are consuming ZFD/high-carbohydrate diet). However, another mechanism(s) must exist because Glut4 expression is unchanged in the fat of Rictor Adipoq-Cre mice consuming normal chow. One possibility based on work in glioblastoma cells is that mTORC2 may regulate expression of glycolytic enzymes independently of AKT; however, the mechanism is not clear. Nevertheless, our findings suggest that selective mTORC2 activators may be useful anti-diabetic drugs.
Methods

**Mice.** Rictor-flxed mice were described previously and backcrossed with C57BL/6 for 10 generations. Floxed mice were crossed with mice expressing either adiponectin-Cre or adiponectin-CreERT2, or with Ubc-CreERT2 (JAX #007001) mice to generate conditional or inducible KO models. Floxed Cre-negative mice were used as wild-type controls. Mice were kept on a daily 12 h light/dark cycle and fed a normal chow diet (Prolabo Isopro RMH 3000) from Lab Diet ad libitum at 22 °C. All animal experiments were approved by the University of Massachusetts Medical school animal care and use committee. For the inducible CreER models, Rictorfl/fl (WT) and Adiponectin-CreERT2;Rictorfl/fl (KO) male mice at 8 weeks old were treated with 3 mg Tamoxifen per day (i.p.) for 6 constitutive days. The age of the mice used for all studies were 8–20 weeks old. For 2 weeks HFD experiment, mice were randomly placed into cages with chow or HFD. No other randomization was used while conducting experiments. No animals were excluded from any experiments, unless they displayed obvious wounds from fighting as determined by our veterinarians.

All animal studies were designed to minimize and control for confounding variables such as mouse gender and age. Based on our previous studies, we use a minimum of six animals per treatment group to achieve statistical power to detect significant differences when measuring RNA, tissue mass, body weight and blood metabolites. Researchers were not blinded to the genotype.

**Antibodies and reagents.** AS160 (07–741) was purchased from Cell Signaling Technologies. 4-hydroxy-tamoxifen (4-OHT) was obtained from Toronto Research Laboratories. Antibodies and reagents were collected by centrifugation (at 300 g) and incorporated into the appropriate tissues. The incorporation was then normalized with protein content measured by BCA protein assay kit (Bio-Rad).

**Tissue harvest and histology.** Adipose tissue depots were carefully dissected to avoid contamination from surrounding tissue. Samples for RNA or protein were immediately frozen in liquid nitrogen and then stored at −80 °C for further analysis. For histology, tissue pieces were fixed by 10% formalin. Embedding, sectioning, and Hematoxylin & Eosin (HE) and PAS staining was done by the UMass Medical School Morphology Core. For Oil Red O staining, liver samples were embedded in OCT before sectioning and staining. For cell size measurements, 12–14 images were taken from each mouse (n = 3 wild-type and 3 conditional KOs). Image J was used to measure cell size and the distribution of cell size as percentage of total counted cells was analysed.

**Western blots.** Insulin stimulated signalling in each tissue was determined in mice that were fasting for 6 h before injection with 0.75 unit kg⁻¹ of body weight of insulin for 15 min. Each tissue was collected and frozen down immediately in liquid nitrogen and then stored at −80 °C for subsequent lysis and western blots analysis with the indicated antibodies. For liver and epididymal fat pads (Efps), Hepes, pH 7.4, 40 mM NaCl, 2 mM EDTA, 1.5 mM NaVO₄, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate and 1% Triton X-100 typically 16 h after the cells were replenished with fresh culture medium. Tissues were homogenized using a TissueLyser (Qagen) in the same lysis buffer, where additionally supplemented with 0.1% SDS, 1% sodium deoxycholate. An equal amount of total protein was loaded into acrylamide/bis-acrylamide gels and transferred to polyvinylidene fluoride membranes for detection with the indicated antibodies. Briefly, membranes were incubated with primary antibodies (1:1,000 dilution) in 5% milk/ PBST or 5% BSA/PBST overnight. Horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution) were added for 1 h followed by washing 3 times with PBST and visualizing with enhanced chemiluminescence (PierInElmer) and detected by X-ray films.

**Primary cell isolation and in vitro differentiation.** SVF cells were isolated by digesting the sWAT of Ucbictror mice in digestion buffer (NaCl (123 mM), KCl (5 mM), CaCl₂ (1.3 mM), glucose (5 mM), HEPES (100 mM), P/S (1%), BSA (4%), pH 7.4) containing collagenase A at 1.5 mg ml⁻¹. After 45 min of digestion, the digested tissue was filtered through 100 μm cell strainers (BD Falcon). Cells were collected by centrifugation (300 g, 5 min) and cultured in DMEM (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin at 37 °C. To induce Rictor deletion, Ucbictror SVF cells were treated with 1 μM 4-OHT or vehicle for 2 days when start to differentiate by adding differentiation medium containing 2 μg ml⁻¹ dexamethasone, 100 nM insulin, 1 μM rosiglitazone and 0.5 mM IBMX for 2 days, and then induced with 100 nM insulin for another 2 days before changing back to regular medium. At different time points, the differentiated adipocytes were collected for protein, mRNA or Oil-Red-O staining analysis. For Oil Red O staining, the differentiated cells were washed three times with PBS and fixed with 10% buffered formalin at 4 °C overnight. Cells were then stained for 10 min at 37 °C with a filtered Oil Red O solution (0.5% Oil Red O in isopropyl alcohol), washed three times with distilled water, and visualized.

**Lipogenesis assay.** Isolated SVF from Ucbictror mouse were cultured and differentiated into adipocytes for 7 days. The cells were then incubated in KRH buffer supplemented with 2.5% BSA and 2 μg ml⁻¹ of [U-14C]-glucose (PerkinElmer) for 4.5 h with or without the presence of 150 nM insulin. The medium was then transferred to mice expressing either Ubc/rictor fl/fl or 1 μM 4-OHT (vvv: = 4:1:1), and newly synthesized lipids were extracted with hexane. About 1/3 of hexane phase was left for extraction of triglycerides (TAGs), and the remaining 2/3 was used for extraction of free fatty acids (FFAs). An equal 1/3 of total protein was loaded into acrylamide/bis-acrylamide gels and transferred to polyvinylidene fluoride membranes for detection with the indicated antibodies. Briefly, membranes were incubated with primary antibodies (1:1,000 dilution) in 5% milk/PBST or 5% BSA/PBST overnight. Horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution) were added for 1 h followed by washing 3 times with PBST and visualizing with enhanced chemiluminescence (PierInElmer) and detected by X-ray films.

**2-DOG glucose uptake.** Isolated SVF from Ucbictror mouse were cultured and differentiated into adipocytes for 7 days. The cells were then incubated in KRH buffer supplemented with 0.3% BSA and glucose sodium 10 μM 1H2O4 in the presence of 150 nM insulin stimulation for 15 min. [1, 2-3H]-2-deoxy-D-glucose (PerkinElmer) was added to the samples and incubated at 37 °C for 10 min, and the assays were
3. O-Sullivan, I. et al. NATURE COMMUNICATIONS | 7:11365 | DOI: 10.1038/ncomms11365 | www.nature.com/naturecommunications

dissolved in scintillation buffer and uptaken $^3$H was determined by counting CPM terminated by three-time KRH wash. The cells were lysed with 1% Triton, NATURE COMMUNICATIONS | DOI: 10.1038/ncomms11365 ARTICLE

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Author contributions

Y.T. and D.A.G. designed experiments. Y.T performed the experiments with the adipose tissue Rictor knockout mice. P.L.L. performed the experiments with lipolysis. J.S.-G. helped with HFD experiments using wild-type mice. W.-Y.H. performed glucose uptake and GLUT4 translocation assays. M.W. and C.M.M. performed lipid analysis and analysed the lipid profiling data. S.V. helped perform experiments with purified hepatocytes. H.L. assisted with animal maintenance. Y.T and D.A.G. analysed and interpreted the data and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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